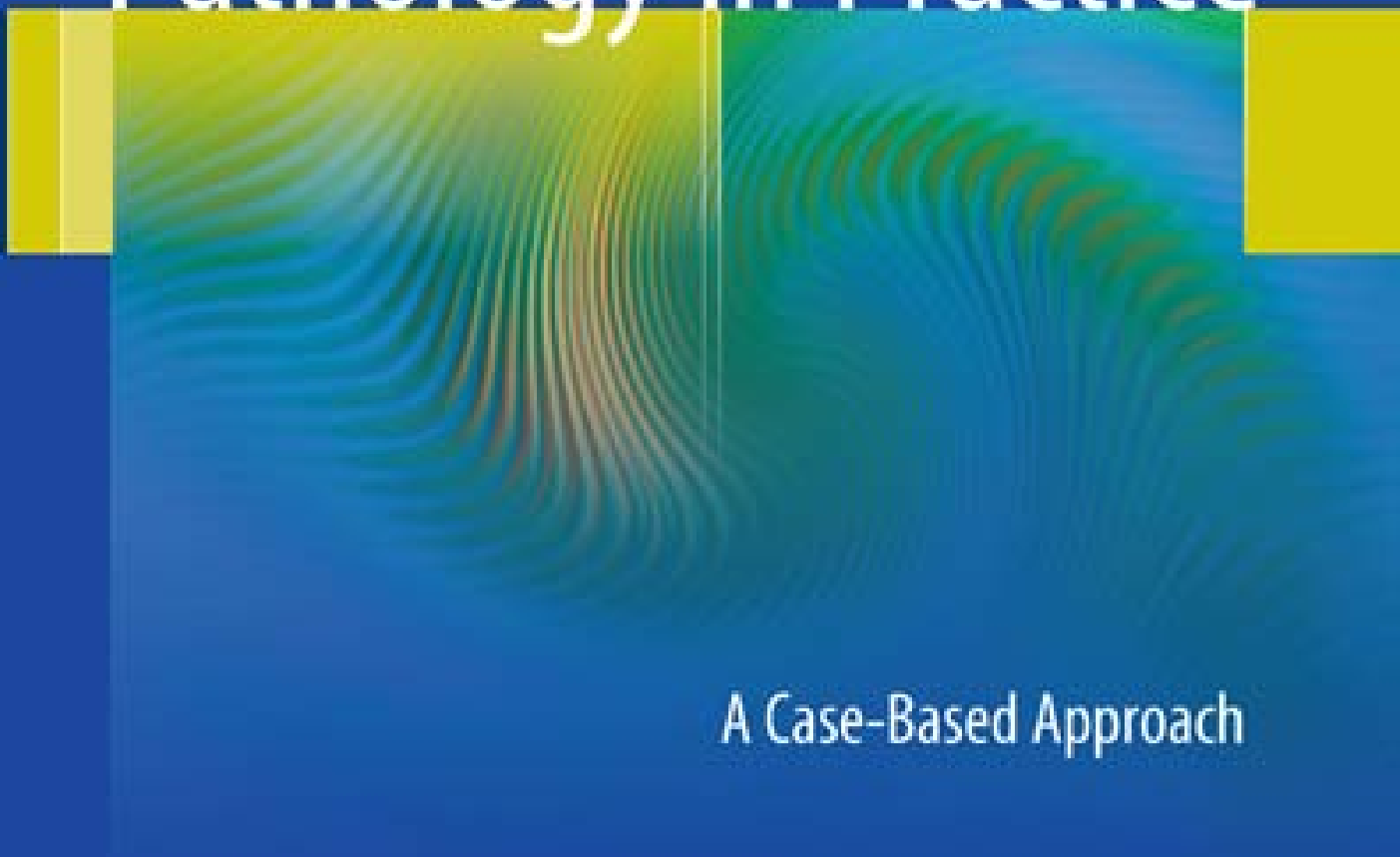
Iris Schrijver *Editor*

Diagnostic Molecular Pathology in Practice



A Case-Based Approach

Springer

Diagnostic Molecular Pathology in Practice

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Editor

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Foreword

It is my distinct pleasure to provide a few words of introduction to Dr. Iris Schrijver and her new book, *Diagnostic Molecular Pathology in Practice: A Case-Based Approach*. I have known Dr. Schrijver since she was a junior faculty member and just beginning her career in Molecular Pathology, and have followed with great pleasure her career development into one of the leaders in Molecular Pathology. I was very pleased when Dr. Schrijver talked with me about her idea for a new Molecular Pathology book. I was highly enthusiastic at the time because of the unique nature of the book she wanted to create. I also was pleased when the publisher of my most recent textbook agreed to work with Dr. Schrijver to translate her idea into reality.

This textbook is unique among Molecular Pathology textbooks. As a practicing molecular pathologist, I know that much of our experience as laboratory directors comes from the difficult cases and problems we face in the laboratory on a regular basis. This book opens up this world of unique and difficult cases to the reader. Whether used as a source of teaching cases by professors or for study in preparation for the practice of molecular pathology by students, the cases in this book illustrate real-world clinical laboratory problems in Molecular Pathology and provide novel insights into the practice of Molecular Pathology. Dr. Schrijver has involved many other leaders in Molecular Pathology as chapter contributors to the book. The reader will learn from experts in each of the book's topic areas. Included in the design of the book are questions about each situation, to allow the reader to assess their understanding of the information and issues presented.

As a more senior member of the Molecular Pathology community and an editor of two textbooks myself, I understand the passion that leads someone to want to share their knowledge through the writing of a textbook. I also understand the joy, mixed with a sigh of relief, that comes from seeing the fruits of your labors and passion. Dr. Schrijver clearly can be proud of this book, and I encourage readers to explore the topics of this book and gain from the knowledge shared by the experts.

New York, USA — Debra G.B. Leonard

Preface

The specialty of molecular genetic pathology (MGP) is rapidly growing and evolving. It focuses on the molecular identification of inherited genetic conditions, of acquired genetic diseases such as solid tumors and hematologic malignancies, and of infectious diseases. Specialty board examinations in MGP are available to physicians who are pathologists or medical geneticists and who have completed subspecialty training in an accredited MGP training program. Prior to the conception of MGP, specialty board examinations were already administered in clinical molecular genetics (CMG), which requires training by M.D. or Ph.D. post-doctoral trainees. CMG training programs focus specifically on inherited genetic conditions. The intended audience for this text comprises trainees in MGP and CMG, as well as residents and fellows in medical specialties to which molecular genetic pathology is pertinent. It is also relevant to the practicing pathologist who wants to learn more about the current practice of molecular diagnostics.

In the past few years, much needed reference textbooks have become available and provide a terrific knowledge foundation and resource. The book in your hands takes a complementary approach. It is a practical, completely case-based book with examples of molecular diagnostic cases (which are composites with fictitious patient names), as they can be encountered in molecular pathology laboratories. The cases are divided into the four main areas addressed in MGP: inherited conditions, hematopathology, solid tumors, and infectious diseases. Each section includes topics ranging from test selection, qualitative and quantitative laboratory techniques, test interpretation, and prognostic and therapeutic considerations, to ethical considerations, technical troubleshooting, and result reporting. This reflects a rich variety of teaching points associated with the diversity of cases in molecular laboratories and represents a cross section of current practical issues which are encountered in the day-to-day activities of a molecular genetic pathologist. The scenarios presented are not intended to indicate the preferred or only approach, but rather represent examples of current practice. Some of the cases in this book reflect common scenarios, whereas others are complex "puzzlers." All provide an opportunity to actively engage with the presented material and to independently develop approaches, solutions, and diagnostic interpretations. As such, it is a practice-based preparation for board examination, for the extensive range of clinical scenarios in the medical specialty of MGP, and, most importantly, for its successful practice.

Stanford, California, USA — Iris Schrijver

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Part I
Inherited Diseases

Cystic Fibrosis

1

Ruth A. Heim

Clinical Background

Mary Lombardi was 32 years old, of Italian descent, and pregnant for the first time. There was no history of cystic fibrosis (CF) in her family or in her husband's family. As recommended by the American College of Obstetricians and Gynecologists [1], her physician offered her CF carrier screening at her first prenatal visit. She tested negative for the mutations analyzed. The mutation panel ordered for Mary's carrier screen had a detection rate of 93% in Caucasians. After testing, Mary's risk to be a carrier of CF was reduced from 1/25 (4%) to 1/343 (0.3%), based on the negative result, her ethnicity, and the negative family history. Mary's husband, Martin Lombardi, was not screened for CF mutations, based on Mary's negative result and his negative family history. Although some physicians offer couples-based tested initially, a typical approach is maternal testing followed by assessment of need for paternal testing based on the maternal result. At 16 weeks gestation, prenatal ultrasound identified an echogenic bowel abnormality.

Question 1: What is your differential diagnosis?
Question 2: Mary tested negative for CF mutations; could the fetus have CF?

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Reason for Molecular Testing

Echogenic bowel can be associated with CF. CF is inherited as an autosomal recessive condition, therefore if both parents are carriers of a CF mutation there is a 25% risk that the fetus is affected. Mary may have carried a rare mutation not detected by a targeted mutation screening panel. It was possible that Martin was a carrier of CF. Since carrier status cannot be determined by physical examination, it would be clinically reasonable to request a molecular test for both parents to determine carrier status. Similarly, it would be reasonable to request a molecular test for the fetus, although this would ideally be done after parental testing. A diagnosis of CF cannot be made clinically in a fetus, but the presence of two CF mutations known to be clinically significant can be used prenatally to predict CF.

Test Ordered

There were several possibilities for CF testing in this family. Which tests are ordered first is typically based on cost of testing and on timing. CF sequence analysis could have been ordered for Mary to determine if she carried a rare mutation. Targeted mutation analysis could have been ordered for Martin, with a reflex to CF sequence analysis if targeted analysis were negative. If both parents were shown to be carriers, prenatal testing could have been ordered. Targeted mutation analysis costs less than sequence analysis; however, at 16 weeks of gestation and with the additional risk factor of the abnormal fetal ultrasound findings, the physician chose to test the fetus immediately.

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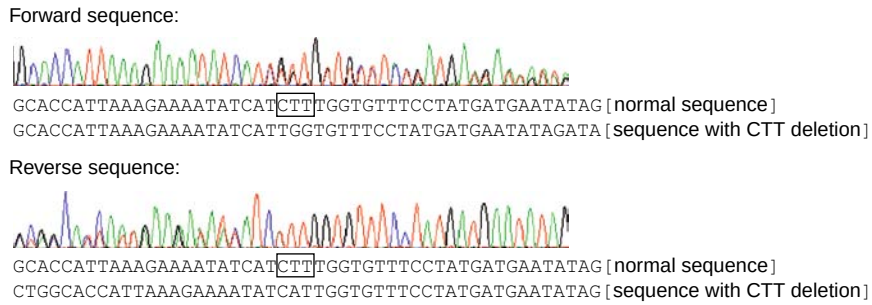

Fig. 1.1 Bi-directional sequence analysis showing the F508del (1653delCTT) [p.Phe508del (c.1521_1523delCTT)] mutation in the *CFTR* gene

An amniocentesis was performed and amniotic fluid was sent to the laboratory for CF sequence analysis. For all prenatal molecular testing the laboratory required a maternal sample for maternal cell contamination (MCC) studies; therefore a maternal peripheral blood specimen was sent for MCC analysis.

Question 3: Should the parents be tested as well as the fetus?
Question 4: What happens if there are not enough fetal cells in the amniotic fluid?
Questions 5: Is MCC analysis really necessary?

Laboratory Test Performed

Full Sequence Analysis of the Fetal Sample

DNA was extracted from amniocytes and amplified by the polymerase chain reaction (PCR). Multiple regions of the *CFTR* gene were analyzed by bi-directional DNA sequencing using capillary gel electrophoresis and fluorescence detection. The regions amplified included the 27 exons of the *CFTR* gene and their flanking intronic sequences (at least 15 bp upstream and 6 bp downstream of each exon), as well as the regions of introns 1, 2, 11, and 19 known to contain clinically significant mutations.

Question 6: What are the limitations of sequence analysis?

MCC Analysis

DNA from maternal and fetal samples was isolated and amplified by the polymerase chain reaction (PCR). Polymorphic markers were analyzed by capillary gel electrophoresis and fluorescence detection. Maternal and fetal markers were compared for evidence of MCC.

Question 7: What are the limitations of MCC analysis?

Results with Interpretation Guideline

Comparison of maternal and fetal DNA markers indicated that MCC was unlikely to have interfered with the fetal results. *CFTR* sequence analysis of the fetus identified four sequence changes. The four changes are listed below twice, first using historical nomenclature, and then using the Human Genome Variation Society (HGVS, http://www.hgvs.org/) nomenclature:

V232D (827T > A) [p.Val232Asp (c.695T > A)] (heterozygous) in exon 6a

M470V (1540A > G) [p.Met470Val (c.1408A > G)] (homozygous) in exon 10

F508del (1653delCTT) [p.Phe508del (c.1521_1523 delCTT)] (heterozygous) in exon 10

I1027T (3212T > C) [p.Ile1027Thr (c.3080T > C)] (heterozygous) in exon 17a

An example of bi-directional sequence showing the F508del three base-pair deletion is shown in Fig. 1.1. Both the normal nucleotide sequence and the sequence with the three base-pair deletion are provided below the data for reference.

Question 8: Why was Mary's first CF mutation screening result negative?
Question 9: Are these sequence changes pathogenic?

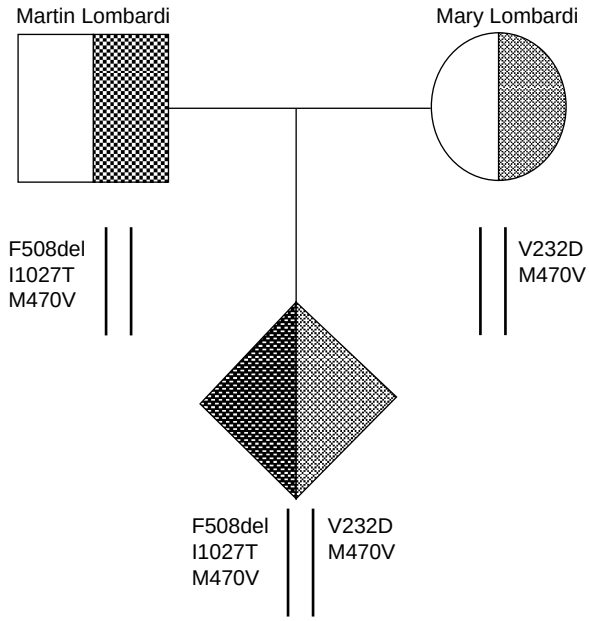

Fig. 1.2 Pedigree showing familial mutations

Result Interpretation

Of the sequence changes identified, one was known to be pathogenic (F508del); one was likely to be pathogenic (V232D); one had unknown clinical consequences (I1027T); and one was a benign variant (M470V). To interpret this information it was necessary to determine which sequence changes were inherited together, so that they could be phased in the fetus. The physician ordered partial sequence analysis of exons 6a, 10, and 17a, for both Mary and Martin.

The pedigree in Fig. 1.2 shows the results of parental testing. Mary was found to carry the clinically significant V232D mutation as well as the benign variant M470V on the allele inherited by the fetus. Martin was found to carry the clinically significant F508del mutation, the mutation of unknown significance, I1027T, and M470V, on the allele inherited by the fetus. The fetal chromosomes are depicted in Fig. 1.2 with the mutations phased based on the parental results. The final result interpretation was that the fetus was a compound heterozygote for two clinically significant CF mutations. The fetus was predicted to be affected with CF, a disorder with a wide range of clinical symptoms and a variable age of onset.

Question 10. Does this result explain the presence of echogenic bowel?

Other Considerations

Mary and Martin may have considered how to prepare for the birth of a child with CF, including identifying support systems, or they may have considered terminating the pregnancy. Consultation with a physician and/or genetic counselor was recommended to discuss the potential clinical and reproductive implications of this result, as well as to consider recommendations for testing other family members for their own information.

Background and Molecular Pathology

Cystic fibrosis (CF) is one of the most common autosomal recessive disorders. Approximately 1 in 2,500 live-born children in the United States has CF. Life expectancy has increased to the late 30s, but CF remains a serious and often lethal disorder. CF is a multi-system disorder in which defective chloride transport across membranes causes dehydrated secretions, resulting in tenacious mucus in the lungs, mucus plugs in the pancreas, and characteristically high sweat chloride levels. Nearly all males with CF are infertile. CF is most common among the Caucasian population, but also occurs in other ethnic groups [2].

CF is the result of mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. All types of mutations are distributed throughout the gene, including missense, frameshift, nonsense, splicing, and small and large in-frame deletions or insertions. Genotype and phenotype correlations have been studied, although this has only been done for a few mutations and these predictions may have limited use in clinical practice. The use of mutation analysis in clinical practice continues to evolve (e.g. [3, 4]).

Multiple Choice Questions

1. When ordering a CF screening test, is it important to obtain information about the ethnicity of the individual to be tested?
   A. No, a diagnosis of CF does not depend on knowledge of ethnicity
   B. No, interpretation of any molecular test is independent of ethnicity

C. Either yes or no, depending on the family history of CF
D. Yes, in some ethnic groups a positive CF carrier result is considered a false positive
E. Yes, this information is needed for accurate risk assessment of CF carrier status.

2. Which of the following is NOT important for interpreting a CF screening test result?
   A. Accurate sample tube labeling
   B. A clear indication for testing
   C. Knowledge of any family history of CF
   D. Pregnancy status
   E. The mutation detection rate of the panel used
3. Which of the following is NOT used when assessing the clinical significance of a *CFTR* sequence variant?
   A. Information about the sequence variant curated by the Consortium for CF genetic analysis [5]
   B. Laboratory knowledge of the structure and function of the CFTR protein
   C. The clinical status of the individual being tested
   D. The effect on the CFTR protein of the change in the amino acid sequence caused by the sequence variant
   E. The presence of the sequence variant in unrelated individuals with CF, as reported in the literature
4. Which of the following is NOT a limitation of sequence analysis?
   A. It may not be possible to interpret the clinical significance of a sequence variant
   B. Large deletions may prevent analysis of one allele
   C. Rare sequence variants are technically more difficult to sequence than common variants
   D. Some regions of a gene may not be analyzed, because of the size of the gene or technical constraints
   E. Variants may interfere with the sequencing primers
5. Is it necessary to determine whether maternal cell contamination is present in a fetal sample?
   A. Either yes or no, depending on the experience of the physician obtaining the sample
   B. Either yes or no, depending on whether the sample type is amniotic fluid or a chorionic villus sample (CVS)
   C. No, culturing cells from any fetal sample type will eliminate maternal cell contamination because the fetal cells will out-compete the maternal cells
   D. No, the laboratory is testing the fetal sample and maternal cell contamination, if any, will not interfere with the interpretation of the fetal result
   E. Yes, maternal cells can be present in any fetal sample, cultured or uncultured, and can interfere with the interpretation of the fetal result

Answers to Questions Embedded in the Text

Question 1. What is your differential diagnosis?

Echogenic bowel can be seen in normal fetuses, in fetuses with CF, or in fetuses with other conditions, including aneuploidy (particularly trisomy 21), intrauterine growth retardation, congenital viral infections, and thalassemia [6]. In Mary's case, fetal cytogenetic analysis and maternal testing for cytomegalovirus, parvovirus, and toxoplasmosis were ordered in addition to the CF testing. Results were negative for a chromosomal abnormality and negative for viral infection.

Question 2: Mary tested negative for CF mutations; could the fetus have CF?

Yes. After carrier screening, Mary's risk to be a carrier was reduced to 0.3%, but she was still at risk for carrying a rare mutation. More than 1,700 *CFTR* sequence variants have been identified, although it is unclear how many of these are pathogenic, and most of the variants are "private" (i.e., have been reported in only one family) [5]. Martin, who was also Italian, had a carrier risk of 1 in 25, which is equivalent to the general population risk for individuals of his ethnic background. If both parents were carriers, the risk for the fetus to be affected would be 1 in 4 (25%).

Question 3: Should the parents be tested as well as the fetus?

Possibly, depending on the laboratory requirements and the patient's needs. When there is a 25% risk that the fetus could be affected, both parents may be tested for internal laboratory QA, so that the fetal result can be interpreted accurately. For example, if one or both parental mutations cannot be identified using a specific laboratory test, then a negative fetal result obtained using the same test cannot predict the CF status of the fetus (carrier, affected, or unaffected). In this case, the

fetal risk was not known to be 25%. While it may have been useful to test both parents so that their results would be available to interpret the fetal results if needed, it was not required by the laboratory, and Martin was temporarily unavailable. Based on cost and logistics, the family decided to test the parents later if needed.

Question 4: What happens if there are not enough fetal cells in the amniotic fluid?

The amount of amniotic fluid available for testing is dependent on the technical and clinical realities of amniocentesis, including the location of the fetus and its gestational age. The amount of DNA extracted from amniotic fluid is not always sufficient for testing. It is important to maintain a backup of cultured cells to be available for testing if direct testing of the amniotic fluid is unsuccessful. If cultured cells were required by the laboratory then parental testing could be performed concurrently with culturing of fetal cells, which typically takes about two weeks.

Question 5: Is MCC analysis really necessary?

Yes. If MCC is present in a prenatal sample it poses a serious risk for prenatal misdiagnosis. The risk of MCC being a source of ambiguous results is increased when sensitive PCR-based methods are used. Therefore, MCC testing is performed to rule out the presence of contaminating maternal DNA that may interfere with interpretation of the fetal results. Both cultured and uncultured amniotic fluid samples may have MCC, but uncultured amniotic fluid has a higher frequency of MCC than cultured amniocytes [7].

Question 6: What are the limitations of sequence analysis?

Analytical limitations: Rare mutations deep in an intron or in the promoter region could be missed. Large deletions encompassing one or more alleles or the whole *CFTR* gene could be missed. Genetic variants that interfere with a sequencing primer could prevent amplification of a region of the *CFTR* gene, thereby preventing detection of a mutation if one were present in that region. Other sources of false positive or false negative results include blood transfusions, bone marrow transplantation, or laboratory error. The risk of laboratory error is minimized by the use of assay controls, effective quality control systems, and independent confirmation of positive results. Interpretive limitations: Not every sequence change identified is well-characterized in terms of clinical correlations. Interpretation of sequence changes can be challenging. The American College of Medical Genetics has published standards and guidelines for the interpretation of sequence variants [8].

Question 7: What are the limitations of MCC analysis?

The analytical sensitivity of the assay should be determined by the laboratory, and this should be correlated with the amount of MCC that would result in a false negative or positive result in the relevant assay. For example, if results of sequence analysis are ambiguous when >10% of the sample tested is contaminated with maternal cells, then the analytical sensitivity of the MCC assay must be at least 10%. The number and quality of markers used can limit analysis, because not every marker may be informative for the maternal/fetal pair analyzed. The markers used should be distributed throughout the genome, and should be sufficiently polymorphic that the appropriate number of informative loci, as determined by the laboratory as necessary for a valid result, can be achieved. Other sources of false positive or negative results are similar to those listed in the answer to Question 6.

Question 8: Why was Mary's first CF mutation screening result negative?

Most likely, the mutation(s) carried by Mary were not included in the initial carrier screening mutation panel. Alternatively, it may have been a false negative result, for example because of a genetic variant under the primer or a mislabeled tube. Based on the mutations identified in the fetus, it was not possible to determine which parent carried which mutations. To answer these questions and to interpret the fetal results, it was necessary to phase the mutations by testing the parents.

Question 9: Are these sequence changes pathogenic?

The laboratory should interpret the significance of the sequence changes by using expert knowledge and experience, as well as by reviewing the literature and assessing the effect of the mutation on the protein. In this case, F508del is the most common CF mutation worldwide. It is considered a classic CF mutation and is found in individuals with a severe form of CF. I1027T and F508del have been reported as a complex allele on the same chromosome (e.g. [9]). However, there is

insufficient evidence to categorize the I1027T sequence change as either disease-causing or benign. V232D is a rare mutation that is likely to be clinically significant based on a predicted change in protein structure and its presence in individuals with CF and congenital absence of the vas deferens (e.g. [10, 11]). M470V is considered to be a benign variant and was listed as having no clinical consequences in a report from a cystic fibrosis consensus conference [4].

Question 10. Does this result explain the presence of echogenic bowel?

Yes.

Answers to Multiple Choice Questions

1. *The correct answer is E.*
2. *The correct answer is D.*
3. *The correct answer is C.*
4. *The correct answer is C.*
5. *The correct answer is E.*

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Alport Syndrome

2

Jane W. Kimani and Karen E. Weck

Clinical Background

A.K. was a 5-year-old boy who presented to the pediatric nephrology clinic with a recent finding of microscopic hematuria and proteinuria on routine screening. The analysis was repeated two weeks later with persistence of hematuria and proteinuria. A complete blood count (CBC) and a metabolic panel (Chem-7) were both normal. Renal ultrasound was performed which was also normal and without hydronephrosis. A.K. had one younger brother who was two years old with no health problems. A.K.'s father was 38 years old and had no health concerns. A.K.'s father's brother, sister, and parents were all healthy, with no renal concerns. A.K.'s father's brother had one son who was healthy at seven years. A.K.'s mother was healthy at 37 years. She had one brother and two sisters, none of whom had any renal concerns. One of her sisters had a son and a daughter; the son, who was six years old, had proteinuria found on dipstick about a year ago, but he has not been referred to a nephrologist. A.K.'s maternal grandfather was healthy and his grandmother died of myocardial infarction at the age of 60.

Question 1: Draw a three-generation pedigree for this family
Question 2: What is your differential diagnosis?

Reason for Molecular Testing

A diagnosis of X-linked Alport syndrome (XLAS) was suspected. Diagnosis of Alport syndrome is complex and requires urinalysis, renal function studies, audiometry, ophthalmic evaluation, and skin and/or kidney biopsy. Molecular testing for mutations in the *COL4A5* gene is useful for diagnosis of XLAS as other diagnostic methods may be inconclusive in the early stages of renal disease. Molecular testing is also useful for prognosis, as identification of specific mutations may be helpful to predict disease severity. In addition, molecular testing is useful for family testing to identify other male relatives who are at risk of developing symptoms and to identify female carriers. Finally, while renal transplantation is an effective treatment for Alport syndrome, identification of an unaffected living-related donor can be difficult and can be guided with molecular testing in families who have a known mutation.

Test Ordered

The physician ordered molecular testing for *COL4A5*.

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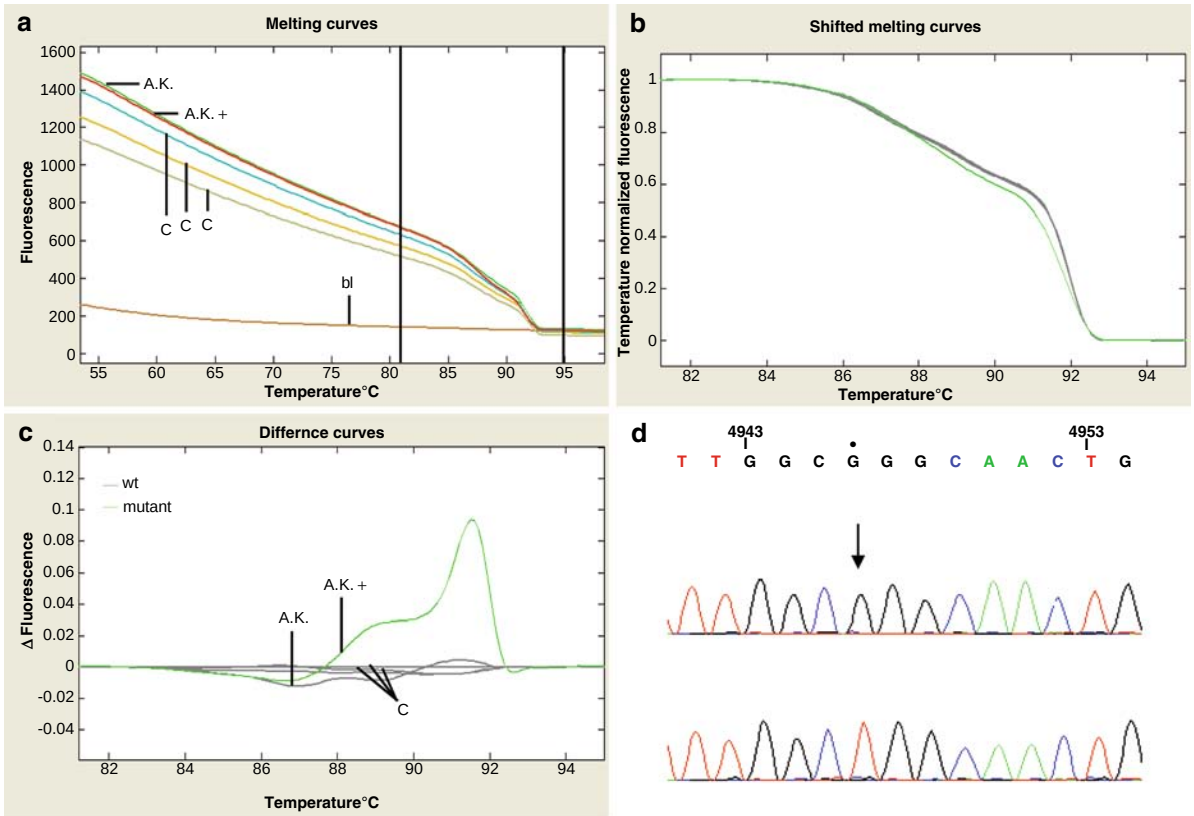

Fig. 2.1 High resolution melting curves and partial DNA sequencing analysis for *COL4A5* exon 50. (**a**) Fluorescence (*F*) *versus* temperature (*T*) melting curves using raw fluorescence data. (**b**) Temperature shifted melting curves after fluorescence normalization. (**c**) Fluorescence difference curves. (**d**) Sequencing electropherograms showing patient sample A.K. (*top panel*) and a control wild-type sample (*bottom panel*). *A.K.* patient sample (neat), *A.K.+* patient sample spiked with normal DNA, *C* control wild-type samples, *bl* blank (no template control)

Laboratory Test Performed

Mutation scanning of the exons and flanking intronic regions of the *COL4A5* gene was performed using high resolution melting analysis (HRMA) followed by DNA sequencing of any exons with an abnormal melting profile. *COL4A5* is a large 51-exon gene that spans a genomic region of approximately 250 kb on chromosome Xq22 and generates an RNA transcript of about 6.5 kb. There is no mutation hotspot and hundreds of mutations, most of them missense mutations, have been identified throughout the gene. Molecular diagnosis therefore requires analysis of the entire coding region either by direct sequence analysis or mutation scanning followed by sequence analysis of exons with putative sequence variation.

Question 3: What are the limitations and advantages of this approach?

Results with Interpretation Guideline

The results of mutation scanning by HRMA of the *COL4A5* gene demonstrated an abnormal melting profile for exon 50 (Fig. 2.1); the HRMA results for all other exons were normal.

HRMA detects sequence variation in a DNA fragment based on differences in melting properties relative to a normal control (wild-type) sample. In our case, individual exons were amplified by PCR in the presence of a saturating DNA-binding dye such as LCGreenPlus that fluoresces only in the presence of

double-stranded DNA. The PCR was followed by a "heteroduplex formation" cycle involving denaturation at 94°C for 30 s, followed by cooling to 25°C for 30 s. The amplicons were then melted slowly on a LightScanner instrument (Idaho Technology Inc., Salt Lake City, UT) by increasing the temperature to 96°C at a rate of 0.1°C/s. The decrease in fluorescence was measured as the double-stranded DNA molecules melt apart.

Figure 2.1a shows the decrease in fluorescence as a function of increasing temperature as the double-stranded DNA molecules labeled with LCGreenPlus dye melt apart for three normal control (C) samples, the patient sample (A.K.), the patient sample mixed in a 1:1 ratio with a wild-type control sample (A.K.+), and a no template water control (bl). Figure 2.1b reflects the melting curves from 81°C to 95°C after fluorescence normalization by Call-IT™ software (Idaho Technology Inc., Salt Lake City, UT). Figure 2.1c demonstrates the difference in the melting curve of each sample compared to a normal control sample. The Call-IT™ software groups samples based on the similarity of the melting curve to the normal control (shown in *gray*). Samples with significant difference in melting profile from the normal control are grouped as unknowns (shown in *green*). The neat patient sample (A.K.) clusters with the wild-type control samples, but the spiked patient sample (A.K.+) demonstrates an abnormal melting curve. This result illustrates the increase in sensitivity of HRMA for detection of a hemizygous (e.g., X-linked) mutation by mixing with normal DNA. This forces heteroduplexes of normal and mutant DNA molecules which melt more easily than homoduplexes of identical DNA molecules. DNA sequencing of *COL4A5* exon 50 was subsequently performed to identify the mutation (Fig. 2.1d).

Result Interpretation

Mutation scanning by HRMA followed by DNA sequencing revealed that the patient has a c.4946T > G (p.Leu1649Arg) mutation in the *COL4A5* gene. A single nucleotide at position 4946 of the cDNA was changed from a thymine (T) to a guanine (G). In the primary protein structure, this missense mutation results in the substitution of a leucine codon (CTG) at position 1649 by an arginine codon (CGG). This *COL4A5* L1649R mutation substitutes a conserved neutral amino acid in the non-collagenous (NC1) domain of the COL4A5 protein with a charged amino acid. This mutation has previously been reported in patients with Alport syndrome [1]. The results are consistent with a diagnosis of Alport syndrome.

Question 4: Does this result explain the patient's symptoms?

Table 2.1 Types of Alport syndrome based on the genes involved and the inheritance pattern

Mode of inheritance	Genes	Frequency (%)
X-linked	*COL4A5*	80
Autosomal recessive	*COL4A3* and *COL4A4*	15
Autosomal dominant	*COL4A3* and *COL4A4*	5

Further Testing

There is no need for further genetic testing of the patient. However, his kidney function should be monitored closely for disease progression to allow timely treatment and intervention. It is also recommended that he be referred to an ophthalmologist and audiologist for assessment of extra-renal manifestations of Alport syndrome. The identification of a disease-causing mutation in A.K. allows for molecular diagnostic testing of at-risk family members. Targeted testing of *COL4A5* exon 50 in A.K.'s mother revealed the c.4946T > G (p.L1649R) mutation in a heterozygous state, confirming that she is a carrier of XLAS. Genetic testing is recommended for the maternal cousin with proteinuria and for A.K.'s younger brother if he develops symptoms of Alport syndrome such as hematuria.

Background and Molecular Pathology

Alport syndrome (OMIM # 301050) is a heterogeneous disorder characterized by progressive renal disease, cochlear, and ocular defects. It has an estimated prevalence of approximately 1:50,000 live births [2]. Mutations in the type IV collagen genes that code for structural components of basement membranes are the underlying cause of Alport syndrome. There are three types of Alport syndrome as shown in Table 2.1.

Alport syndrome is predominantly an X-linked disease. Males present with persistent microscopic

and episodic gross hematuria from childhood, which develops into proteinuria, progressive renal insufficiency, and eventually end stage renal disease (ESRD). Other symptoms including progressive hearing loss and ocular lesions, particularly anterior lenticonus, may be present depending on the underlying mutation. However, there can be variability in the age of onset even in family members with the same mutation [3]. Clinical features in females vary from severe involvement, intermittent microscopic hematuria, to no symptoms at all. Hearing loss and ocular lesions are infrequent in female carriers. The clinical features of autosomal recessive Alport syndrome are similar to those of X-linked Alport syndrome in males, but affect males and females equally. Autosomal dominant Alport syndrome has a variable clinical phenotype that is generally milder than both X-linked and autosomal recessive Alport syndrome [4].

There are six genetically distinct type IV collagen alpha chains (α1–α6) that together with other molecules such as laminins and proteoglycans form structural components of basement membranes. The basement membrane is a sheet-like structure found between the epithelium and the tissue stroma that provides cellular support, compartmentalizes tissues, and is involved in various biological functions including growth and differentiation, tissue repair and molecular ultra-filtration. Each type IV α-chain consists of a middle triple-helical domain with the characteristic collagenous Gly-X-Y motif, flanked by an amino-terminal 7S domain and a carboxy-terminal non-collagenous (NC1) domain. The α1(IV) and α2(IV) chains have ubiquitous expression in all basement membranes, but the expression of α3(IV), α4(IV), and α5(IV) chains is specific to the basement membranes of the glomerulus, the inner ear, and the corneal epithelium. Three α-chains initiate assembly at the NC1 domain to form triple helical protomers, which form the building blocks for the self-assembly of a collagen type IV supra-structure network [5, 6].

COL4A5 mutations result in defective or deficient α5(IV) chains, which also abolishes expression of the α3(IV) and α4(IV) chains. This causes ultrastructural changes in the glomerular basement membrane (GBM) such as irregular thinning and thickening that can be observed by electron microscopy in renal biopsy specimens from affected patients. There is no mutation hotspot within the *COL4A5* gene and recurrent mutations are rarely seen. Hundreds of mutations have been reported throughout the gene including missense (40–48%), splice site (11–16%), nonsense and frameshift (25–30%), and large rearrangement (6–20%) mutations. The incidence of *de novo* mutations is 3–12% [4, 7]. The missense mutations mostly involve substitution of the glycine residue within the Gly-X-Y motif with a bulkier amino acid, which alters the secondary structure of the protein resulting in defective assembly of the corresponding α-chain. Genotype–phenotype correlations in Alport syndrome are not well established. However, large gene rearrangements, nonsense, and frameshift mutations that result in a truncated or absent protein are generally associated with a more severe phenotype and earlier onset of ESRD, compared with missense mutations. Additionally, because assembly of the collagen protomers begins at the carboxy-terminal NC1-domain, glycine missense mutations involving the 3′ end of the gene generally result in a more severe phenotype than those involving the 5′ end of the gene [8].

Multiple Choice Questions

1. Alport syndrome can result from mutations in three different genes. This is an example of:
   A. Allelic heterogeneity
   B. Cellular heterogeneity
   C. Clinical heterogeneity
   D. Locus heterogeneity
   E. Phenotypic heterogeneity
2. What is the probability that a third child born to this family would be affected with Alport syndrome?
   A. 10%
   B. 25%
   C. 50%
   D. 66%
   E. 75%
3. A 33-year-old male has a clinical diagnosis of Alport syndrome. He reports that his 60-year-old father has had recent episodes of hematuria. Which of the following sequence changes would BEST explain the phenotype in this family?
   A. *COL4A3* c.1452G > A (p.G484G)
   B. *COL4A3* c.1477G > A (p.G493S)

C. *COL4A5* c.1095G > A (p.G365G)
D. *COL4A5* c.2023G > A (p.G675S)
E. *COL4A5* c.5030G > A (p.R1677Q)

4. A.K.'s mother does not have features of Alport syndrome, but has the same mutation as her son who is affected. The clinical phenotype in females with X-linked Alport syndrome is MOST LIKELY modified by:
   A. Genomic variation
   B. Haplotype
   C. Non-penetrance
   D. Variable expressivity
   E. X inactivation
5. Which of the following mutation scanning methods would NOT be optimal for molecular diagnosis of Alport syndrome?
   A. Denaturing gradient gel electrophoresis (DGGE)
   B. Denaturing high performance liquid chromatography (DHPLC)
   C. Protein truncation test (PTT)
   D. Single strand conformational polymorphism (SSCP)
   E. Temperature gradient gel electrophoresis (TGGE)

Answers to Questions Embedded in the Text

Question 1: Draw a three-generation pedigree for this family (Fig. 2.2)

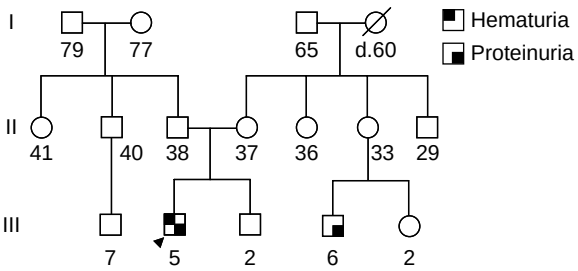

Fig. 2.2 Shown is a three-generation pedigree with the proband denoted by an arrow. Males are depicted with square symbols and females with circles. The ages of the individuals are shown. A slash through the symbol denotes a deceased individual with the age of death shown. Affected individuals are denoted by shaded blocks according to the key

Question 2: What is your differential diagnosis?

There are several causes of hematuria and proteinuria in children. The two most common causes of isolated hematuria are thin basement membrane nephropathy (TBMN) and Immunoglobulin A (IgA) nephropathy [9]. IgA nephropathy is the most common glomerulonephritis worldwide. It is an autoimmune disease in which deposition of the IgA antibody in the glomerulus results in inflammation. Because most cases of IgA nephropathy are sporadic, the diagnosis is unlikely in this family where the proband's cousin appears to be presenting with similar symptoms [10]. TBMN is associated with heterozygous mutations in *COL4A3* and *COL4A4* and may represent a mild form of Alport syndrome [4]. The presence of proteinuria in this family suggests the more severe Alport syndrome, since proteinuria is rarely observed in TBMN. Additionally, the family history appears to be consistent with an X-linked pattern of inheritance, thus implicating the X-linked *COL4A5* gene.

Question 3: What are the limitations and advantages of this approach?

A mutation scanning approach allows rapid analysis of all the exons and detection of known and novel mutations. For large genes, mutation scanning allows for a faster and less expensive method of mutation analysis than direct DNA sequencing. However, some mutation scanning approaches have limited sensitivity. HRMA has been reported to have >99% sensitivity for the detection of heterozygous variants in amplicons smaller than 500 bp [11]. HRMA has other advantages over other scanning methods: it is a closed-tube, one-step scanning method, and scanning is nondestructive so that positive amplicons can be directly analyzed by subsequent sequencing to identify the specific mutation. One limitation is that, since the sensitivity of HRMA is enhanced by the formation of heteroduplexes between wild-type and mutant DNA molecules, the sensitivity to detect homozygous or hemizygous variants is decreased. Mixing the DNA sample with an equal concentration of a normal control allows formation of heteroduplexes and increases the sensitivity of homozygote and hemizygote detection (see Fig. 2.1).

Another limitation is that mutation detection techniques such as HRMA and DNA sequencing will not detect large gene deletions or rearrangements. Sequencing analysis has a mutation detection rate of ~90% in patients with a typical presentation of Alport

syndrome and a family history consistent with X-linked inheritance [12]. Comprehensive molecular diagnosis requires additional dosage analysis for large structural rearrangements, particularly in affected females where the presence of a normal allele confounds interpretation of sequencing results.

Question 4: Does this result explain the patient's phenotype?

The reported *COL4A5* c.4946T>G (p.Leu1649Arg) mutation alters a conserved amino acid that is involved in intramolecular interactions within the non-collagenous (NC1) domain of the COL4A5 protein and is the molecular basis for the patient's renal symptoms. Mutations in the NC1 domain of COL4A5 affect the assembly of the collagen triple helical protomer. There is no clear genotype–phenotype correlation, but NC1 domain mutations may result in a more severe phenotype than glycine missense mutations, particularly those in the 5′ end of the gene [8]. *COL4A5* L1649R is a founder mutation that was initially reported at a high prevalence in a population from the western United States [1]. Affected males with this mutation have developed microscopic hematuria in childhood, but onset of renal failure was generally delayed until after 40 years of age and usually preceded hearing loss. Renal biopsy showed GBM alterations that are characteristic of Alport syndrome. A similar clinical course might be expected for this patient.

Answers to Multiple Choice Questions

1. *The correct answer is D.*

Locus heterogeneity refers to the fact that mutations in different genes (*COL4A3*, *COL4A4*, and *COL4A5)* result in the same phenotype of Alport syndrome. Choices A, C, and E are all true for Alport syndrome. Allelic heterogeneity refers to the fact that many different mutations within a given gene have been described in Alport syndrome. Clinical and phenotypic heterogeneity both refer to the presence of different symptoms and disease severity that can manifest in patients with Alport syndrome. Cellular heterogeneity refers to the presence of distinct cell types, such as within a tumor or cell culture.

2. *The correct answer is B.*

For this family, the disease-causing mutation appears to be non-penetrant in females, so only a boy inheriting the disease allele would be affected. Multiply the two independent variables: 1/2 (the probability of having a boy) × 1/2 (the probability that he will inherit the mutation) = 1/4 (25%).

3. *The correct answer is B.*

Choices A and C are benign synonymous single nucleotide polymorphisms. Choices B, D, and E are pathologic mutations that have been reported previously in association with Alport syndrome [12–14]. However, the inheritance pattern in this family from father to son excludes X-linkage, so a *COL4A5* mutation is very unlikely to be the disease-causing mutation in this family.

4. *The correct answer is E.*

X-inactivation is the mechanism by which one X-chromosome is randomly silenced in each cell of females, in order to equalize X-linked gene dosage between males and females. As a result, female carriers of X-linked diseases such as XLAS are usually unaffected or mildly affected except in cases of extremely skewed X-inactivation.

5. *The correct answer is C.*

PTT relies on identification of shortened protein fragments *in vitro*, so only nonsense or frameshift mutations can be detected by this method. Since these represent a small proportion of mutations in XLAS, PTT is not optimal for diagnosis of XLAS. The other choices are suitable mutation screening methods that can detect sequence variants based on different migration patterns of DNA molecules through an electrophoretic gel (DGGE, SSCP, and TGGE) or chromatography column (DHPLC).

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Alpha Thalassemia

3

Colin C. Pritchard and Jonathan F. Tait

Clinical Background

A pregnant couple presented for evaluation of possible alpha thalassemia trait. Because both prospective parents were of Egyptian ancestry, routine screening for thalassemia trait was indicated. Hematologic testing showed that the mother was microcytic [mean red-cell volume (MCV) 75 fL] with a HbA_2 fraction of 2.5% and a normal hemoglobin electrophoresis. The father had a similar picture (MCV 77 fL, HbA_2 2.3%, normal hemoglobin electrophoresis). Iron studies were normal, and the normal HbA_2 results effectively ruled out beta thalassemia trait. DNA testing for alpha thalassemia was therefore performed, but both parents were negative for six common deletional mutations that cause most cases of alpha thalassemia.

Question 1: Is there any need for further genetic testing? Why or why not?

Reason for Molecular Testing

In view of the still-unexplained microcytosis in both parents, DNA sequencing of the alpha globin genes was ordered to detect rare non-deletional mutations that can cause alpha thalassemia. Detection of specific mutations would clarify the risk of alpha thalassemia for the fetus and allow prenatal diagnosis if clinically indicated.

Question 2: Is this a clinically useful test to order in these circumstances? Why or why not?

Test Ordered

The test ordered was complete sequencing of the two alpha globin genes (*HBA1* and *HBA2*) to identify potential point mutations, small insertions, or small deletions.

Laboratory Test Performed

The test performed was sequencing of the two alpha globin genes (*HBA1* and *HBA2*) (Fig. 3.1). In this test, a PCR product of 1,259 bp is produced from the *HBA1* gene, and a product of 1,102 bp from the *HBA2* gene. The amplified region includes the promoter, the entire protein coding region, the two introns, and the 5′ and 3′ untranslated regions. These products are then sequenced bidirectionally with internal and flanking primers. This approach allows detection of most of the non-deletional mutations that cause alpha-thalassemia, such as Hb Constant Spring in *HBA2* (Fig. 3.1a).

Question 3: What kinds of mutations will this technical approach miss?

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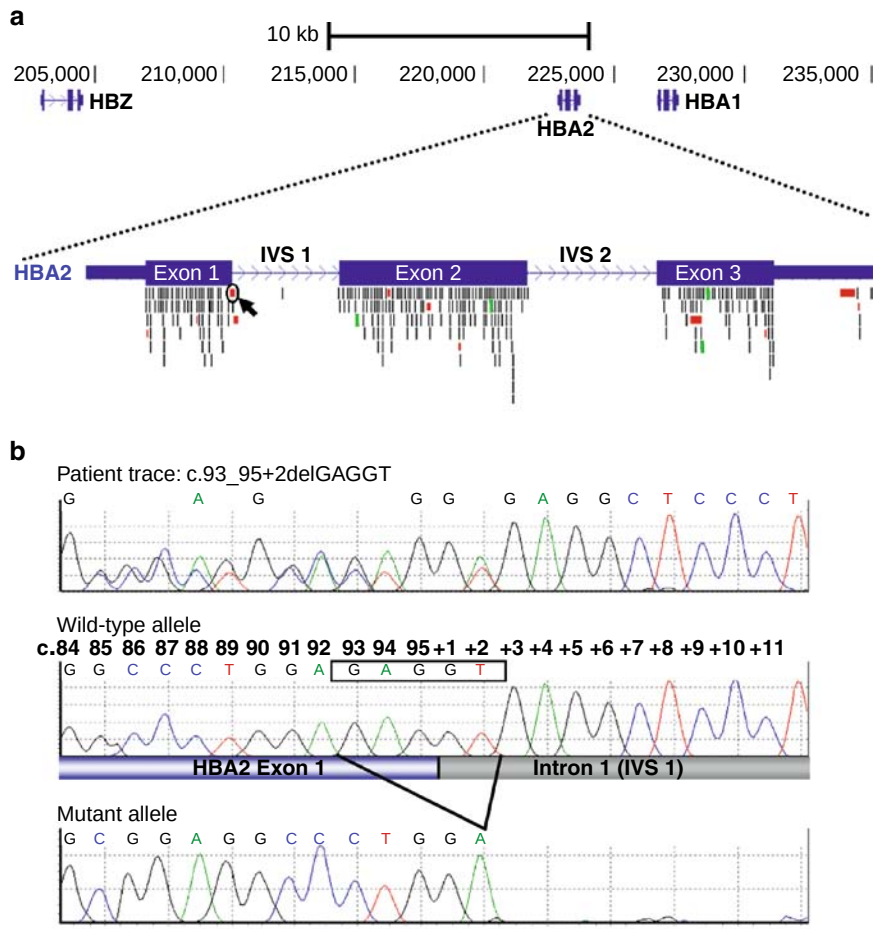

Fig. 3.1 Sequence analysis of the *HBA2* gene. (**a**) Genomic structure of the Alpha Globin Gene Cluster. There are three functional genes at this locus: *HBZ*, which produces the zeta-globin protein during embryonic life; and *HBA1* and *HBA2*, two nearly identical genes that produce the alpha-globin protein during prenatal and postnatal life. The expansion of the *HBA2* gene shows the spectrum of non-deletional alpha-thalassemia mutations reported at this locus. *Black hashmarks* indicate point mutations, *red* indicates deletions, and *green* indicates insertions. The *arrow* points to the position of the novel 5-bp deletion reported here. The numeric scale at the *top* of the figure is genomic numbering on chromosome 16, based on human genome build 19 (February 2009 build, http://genome.ucsc.edu). (**b**) Sequence analysis of a patient with alpha thalassemia trait. Sequencing was performed with a reverse primer beginning in intron 1 and proceeding in the 5′ direction into exon 1 (*uppermost trace*). At the 3′ end of the sequence, the patient sample shows a clean homozygous trace that matches the reference sequence up to base c.95 + 3 (designated "+3"). Starting from base c.95 + 2 onward toward the 5′ end of the sequence, there is a pattern of heterozygosity at most bases that suggests the presence of a frameshift mutation. Deconvolution of the sequence data revealed two components: the wild-type sequence (*middle trace*) and a mutant sequence (*lower trace*) with a 5-bp deletion that obliterates the intron 1 splice donor site in the *HBA2* gene. The normal sequence spanning the intron 1 splice donor site beginning at nucleotide 84 is GGCCCTGGAGAGgtgaggctcccctccc, where *upper case* indicates exon 1 sequence and *lower case* indicates intron 1 sequence. The patient has a deletion of GAGgt, resulting in the abnormal sequence beginning at nucleotide 84 of GGCCCTGGAgaggctccct. Using HGVS nomenclature this mutation is designated as c.93_95 + 2delGAGGT, or as NC_000016.9:g.223004_223008delGAGGT. *IVS* intervening sequence

Results with Interpretation Guideline

Sequence analysis showed an abnormal result in the *HBA2* gene for both patients (Fig. 3.1b). The sequence obtained with a reverse primer diverged from the reference sequence at the exon 1 – intron 1 boundary. Deconvolution of the data from each patient indicated that *both* were positive for a heterozygous deletion of 5 bp that obliterates the intron 1 splice donor site in the *HBA2* gene. In HGVS nomenclature (http://www.hgvs.org/) this mutation is described as c.93_95+2delGAGGT. No other actual or potential pathogenic mutations were detected in either patient.

Question 4: What are some reasons why one might see the same mutation in both members of a couple?

Result Interpretation

The first step in analyzing this result is to determine whether this mutation has been previously reported. An online mutation database [1, 2], a textbook [3], and the research literature were consulted [4, 5], but no previous reports of this mutation were identified. Therefore, it was concluded that it was novel. It was surprising to find the same novel mutation in two individuals who denied consanguinity. To exclude the possibility of a sample mixup, *HBA2* gene sequencing was repeated on both patients and Y-chromosome PCR was performed to confirm that the samples were from a man and woman. Thus, it seemed most likely that the patients shared the same mutation due to distant common descent in their ancestral homeland of Egypt.

Next, the laboratory sought to determine whether this novel mutation was likely to be pathogenic. The normal sequence spanning the exon 1–intron 1 boundary is GGAGAGgtgagg, where upper case indicates exon sequence and lower case indicates intron sequence, and the underlined bases are those deleted by the novel mutation (Fig. 3.1b). Because the mutation deletes the canonical splice donor site at the 5′ end of the intron, it is highly likely to prevent normal removal of intron 1 sequences during mRNA processing, thus resulting in an abnormal transcript from the mutant allele. A different known mutation that disrupts the splice site at the 5′ end of intron 1 of *HBA2* does cause phenotypic alpha thalassemia [4, 5]. This mutation deletes bases two through six at the 5′ end of intron 1 (c.95+2_95+6delTGAGG), and is often described in the older literature as $\alpha_2^{-5nt}\alpha_1$.

Finally, the laboratory aimed to predict the phenotypic consequences of this mutation, since this couple has a 25% chance of having a child who is homozygous for the c.93_95+2delGAGGT mutation in the *HBA2* gene. In the absence of prior reports of homozygous individuals, the actual clinical consequences of this genotype are uncertain. However, phenotypes have been reported for several patients either homozygous for the c.95+2_95+6delTGAGG mutation or compound heterozygous for the c.95+2_95+6delTGAGG mutation and a deletion of both alpha-globin genes on the other chromosome; these patients have a mild anemia (hemoglobin levels approximately 9–10.5 g/dL) [4–6]. In making a phenotypic prediction, one should keep in mind that inactivating mutations in *HBA2* (the alpha-2 gene) are generally more deleterious than mutations in *HBA1* (the alpha-1 gene), because the alpha-2 gene normally produces two to three times as much mRNA as the alpha-1 gene [3]. Thus, it would be reasonable to predict that an individual homozygous for the novel mutation present in this couple would have a mild to moderate degree of anemia. However, the uncertainties of this prediction should be clearly conveyed to the couple in follow-up genetic counseling.

Further Testing

No further genetic testing was indicated for the prospective parents because the results of the alpha globin sequence analysis were definitive. The laboratory contacted the genetic counselor involved in the patients' care to report the novel mutation and discuss the possible phenotypic consequences of a homozygous mutation in the child. After receiving the results and genetic counseling, the parents decided not to pursue prenatal diagnosis.

Other Considerations

Although DNA sequencing provided a definitive diagnosis in this case, it is worth remembering the limitations of sequence-based testing in this setting. Sequencing will not detect mutations that lie outside of the sequenced region of approximately 2 kb. In addition, large *HBA1* and *HBA2* gene deletions will be

mostly invisible to sequencing, as there is insufficient normal polymorphism in the sequenced region to provide a reliable indicator of hemizygosity at the level of an individual patient. As with other PCR-based assays, sequencing is also subject to false negative results if there is allele dropout during the amplification step, due to a missing or mismatched primer binding site.

When there is still a high suspicion of alpha thalassemia in a patient who is negative for common large deletions and point mutations, testing with additional technical approaches may be indicated. For example, chip-based comparative genomic hybridization (CGH) analysis and multiplex ligation-dependent probe amplification (MLPA) are clinically available to detect very large or novel deletions.

Background and Molecular Pathology

The thalassemias are among the most common genetic disorders worldwide [3, 7]. They result from imbalances in the synthesis of alpha and beta globin chains due to mutations in the corresponding genes. Two alpha globin genes are located on the short arm of chromosome 16, for a total of four alpha globin genes per diploid genome. Alpha thalassemia is primarily a result of alpha globin gene deletions, which can eliminate from one to all four genes, with a corresponding increase in the severity of disease (Reviewed in [7–10]). People with the one-gene deletion, known as silent alpha thalassemia carriers, have a clinically normal phenotype. Those with two genes deleted, either in *cis* as alpha thalassemia-1 (or α° thalassemia [10]) or in *trans* as alpha thalassemia-2 (or α^{+} thalassemia [10]), have mild anemia. Hemoglobin H disease occurs when three alpha-globin genes are deleted or nonfunctional, and it is characterized by moderate to severe anemia, icterus, and splenomegaly [8]. The most severe form of alpha thalassemia, hemoglobin Bart's hydrops fetalis, occurs when all four alpha globin genes are missing or non-functional, and causes stillbirth or death shortly after birth. Non-deletion mutations can occur, as in this case, but account for only about 9–10% of the mutant alleles worldwide [10].

The mode of inheritance of alpha thalassemia is complex and depends upon both the type of mutation(s) and the form of alpha thalassemia. The most clinically severe forms of alpha thalassemia, hemoglobin H disease, and hemoglobin Bart's hydrops fetalis, are inherited in an autosomal recessive pattern, which is probably why some sources report that alpha thalassemia is primarily an autosomal recessive condition [10]. However, clinically milder phenotypes can be inherited in either an autosomal dominant pattern (alpha thalassemia-1, with two genes in *cis* deletion) or in an autosomal recessive fashion (alpha thalassemia-2, with two genes in *trans* deletion). Non-deletion mutations, such as the one reported in this case, generally cause alpha thalassemia in the heterozygous state, and therefore have an autosomal dominant pattern of inheritance [3].

The highest prevalence of alpha thalassemia is seen in blacks and Southeast Asians. Approximately 25–30% of blacks are silent alpha thalassemia carriers, and 3% have alpha thalassemia-2, due most commonly to a two-gene deletion in *trans*, in which one alpha gene is deleted from each chromosome. Two-gene deletions in *cis* are very rare in blacks, which means that hemoglobin Bart's hydrops fetalis and hemoglobin H disease are very uncommon in this group. Southeast Asians have a combined prevalence of alpha thalassemia-1 and alpha thalassemia-2 carrier genotypes ranging from 5% in some populations to up to 30% in Thailand and as high as 80% in parts of New Guinea. Unlike the black population, in Southeast Asians the most common type of alpha thalassemia-1 is caused by a two-gene deletion in *cis*, which means that both alpha globin genes are missing from the same chromosome. This results in a high frequency of hemoglobin Bart's hydrops fetalis and hemoglobin H disease in this population. Other populations affected by alpha thalassemia include those from the Mediterranean, the Middle East, and the Indian subcontinent. Among Egyptians, the prevalence of alpha thalassemia alleles is as high as 16% [3], with clinical manifestations of alpha thalassemia estimated in about 8% [11].

There are currently over 800 hemoglobin variants catalogued [1, 2], of which approximately 300 are due to mutations in the alpha globin genes. Thus, sequence analysis can be indicated in the workup of known or suspected thalassemia carriers, as in this case. DNA sequencing of the alpha or beta globin genes can be indicated for several reasons in addition to detection of thalassemia carriers. Sequencing can identify the specific hemoglobin variant responsible for an unknown variant initially detected at the protein level by electrophoresis or anion-exchange HPLC (high performance liquid chromatography). It can also be useful in detecting hemoglobin variants that alter the oxygen affinity

of the hemoglobin tetramer, which can cause otherwise unexplained polycythemia.

Multiple Choice Questions

1. What is the most common type of mutation causing alpha thalassemia?
   A. Gene duplication
   B. Gene inversion
   C. Large deletion
   D. Point mutation
   E. Trinucleotide repeat expansion
2. In the case presented here, what DNA sequence results would have been expected if one of these patients had been heterozygous for the Mediterranean-type alpha thalassemia deletion, which deletes both the *HBA1* and *HBA2* genes?
   A. No sequence data could be obtained due to complete gene deletion
   B. Peak heights in the sequence chromatogram would be half the normal value due to loss of one allele
   C. The deletion would have been reliably detected due to loss of heterozygosity in *HBA1* and *HBA2*
   D. The Mediterranean-type deletion would have been detected by sequencing due to presence of novel sequence at the deletion breakpoint
   E. The patient would have normal DNA sequence in *HBA1* and *HBA2*
3. Why was beta thalassemia trait excluded as an explanation for the low MCV in the two patients presented in this case study?
   A. Beta thalassemia is not present in their ethnic group
   B. Carriers of beta thalassemia would have a much lower MCV value than seen in these patients
   C. Coexisting iron deficiency confounded the interpretation of red cell indices
   D. Hemoglobin electrophoresis was normal, ruling out beta thalassemia
   E. The hemoglobin A_2 levels were normal
4. Here we present a novel alpha globin mutation that is very likely to cause clinical disease because it abolishes a splice donor site. Which of the following types of mutations is LEAST LIKELY to cause clinical disease?
   A. A point mutation in the poly-adenylation sequence of the 3′ untranslated region
   B. A point mutation near the middle of an intron
   C. A point mutation resulting in an amino acid change at a position that is highly evolutionarily conserved
   D. A point mutation that abolishes the stop codon
   E. A small deletion that results in a frame-shift and premature stop codon
5. What is the most widely used online resource that best summarizes mutations in the globin genes?
   A. Human Genome Variation Society Database (HGVS)
   B. Online Mendelian Inheritance in Man (OMIM)
   C. The Globin Gene Server
   D. The Single Nucleotide Polymorphism Database (dbSNP)
   E. University of Santa Cruz (UCSC) Genome Browser

Answers to Questions Embedded in the Text

Question 1: Is there any need for further genetic testing? Why or why not?

The six most common large deletions account for ~90% of alpha thalassemia alleles. However, alpha thalassemia trait remains likely in both parents because the low MCV cannot be explained by iron deficiency (normal iron studies) or beta thalassemia (normal HbA_2). Additional genetic testing for non-deletional forms of alpha thalassemia is indicated for prenatal counseling.

Question 2: Is this a clinically useful test to order in these circumstances? Why or why not?

DNA sequencing of the *HBA1* and *HBA2* genes will reveal the majority of the remaining ~10% of alpha thalassemia alleles not detected by testing for common large deletions. Thus, this is a clinically useful and appropriate next test to order in this circumstance. It would be reasonable to do additional tests for rare large deletions if no pathogenic mutations had been detected by gene sequencing.

Question 3: What kinds of mutations will this technical approach miss?

Gene sequencing cannot reliably detect large heterozygous deletions due to allele drop-out, which results from an inability of the primer to bind to the (deleted) sequence. Rare mutations in far upstream (5′) or downstream (3′) regulatory elements will also be missed because they are not included in the sequenced region.

Question 4: What are some reasons why one might see the same mutation in both members of a couple?

The couple may share a common ancestry or be directly related (consanguineous). The mutation may have a very high allele frequency in a population. A sample mixup should be excluded if a very rare or novel mutation is found in both members of a couple not known to be consanguineous.

Answers to Multiple Choice Questions

1. *The correct answer is C.*

Large deletions (>1 kb) are the most common type of alpha thalassemia mutation. In beta thalassemia, small point mutations are the most common mutation type.

2. *The correct answer is E.*

The patient would appear to have a normal sequence because sequence data could only be obtained from the single allele containing a normal alpha globin locus; the allele with the Mediterranean deletion would produce no PCR product with *HBA1* and *HBA2* primers.

3. *The correct answer is E.*

A normal HbA_2 level excludes beta thalassemia, except in rare instances such as delta–beta thalassemia.

4. *The correct answer is B.*

A point mutation near the middle of the intron is usually silent, except in rare circumstances, for example when it makes a cryptic splice site fully functional and alters normal mRNA splicing patterns.

5. *The correct answer is C.*

The Globin Gene Server [2] is the most authoritative online compilation of mutations in the alpha and beta globin gene clusters.

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Charcot-Marie-Tooth Disease

4

Zhiqiang B. Wang and Jeffrey A. Kant

Clinical Background

A 10-year-old Caucasian male returned with his parents to his pediatrician's office for genetic counseling and sample acquisition to undergo genetic testing for a specific disorder. He was conceived via sperm donation and born with bilateral congenital hip dislocation to a 27-year-old G1P0 mother. Developmental delay, hyptonia, and megalocephaly were noted at six months of age, at which time computed tomography (CT) evaluation demonstrated lateral and third ventricles of high-normal size with bilateral frontal extra-axial fluid. Further magnetic resonance imaging (MRI) evaluation revealed mild hydrocephalus with excess subdural fluid and increased ventricular size. An electro-encephalogram (EEG) was normal, as was a laboratory workup which included creatine phosphokinase, lactate, and pyruvate levels as well as urine organic acids.

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Question 1: What is your differential diagnosis?

The year before, this patient had undergone anterior tibialis transfer surgery with a split left posterior tibialis tendon for a left calcovarus deformity. An electromyogram (EMG) had demonstrated decreased nerve conduction velocity of 15 m/s (normal >45 m/s). A definitive diagnosis was not made, although there was a strong candidate diagnosis of Charcot-Marie-Tooth (CMT), type 1 disease. A prior karyotype was normal as were earlier molecular genetic tests for Fragile X syndrome, Prader-Willi syndrome, and mitochondrial myopathy. He had also been diagnosed with Attention-Deficit Hyperactivity Disorder (ADHD) at age four and with Asperger's disease a year ago. Development of gross motor skills and speech were delayed, although fine motor skills were normal.

Reason for Molecular Testing

Based on the patient's age of presentation, clinical features and course as well as negative prior tests for other molecular genetic disorders, a presumptive diagnosis of CMT type 1 disease was considered. To confirm that suspicion, the primary care physician requested genetic testing.

Test Ordered

After the mother received genetic counseling and signed informed consent for testing, a 5 mL anticoagulated whole blood sample from the patient was submitted to the hospital laboratory with a

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request for a complete CMT evaluation panel. The requisition form used was one previously left in the physician's office during a visit by a sales agent for an outside reference laboratory which provides neurogenetic testing services. The complete CMT evaluation panel included assessment for duplication or deletion of the *PMP22* and *GJB1* genes as well as full gene sequence analysis for the following 15 genes: *CX32 (GJB1)*, *EGR2*, *FIG4*, *GARS*, *GDAP1*, *HSPB1*, *LITAF/SIMPLE*, *LMNA*, *MFN2*, *MPZ* (Myelin Protein Zero), *NFL* (Neurofilament Light), *PRX* (Periaxin), *PMP22*, *RAB7*, and *SH3TC2*. This panel was a send-out test which was listed, at that time, for more than $11,500. Interestingly, a sample was also received from the patient's "sister." Her biological relationship (recall that the patient was conceived via sperm donation) to the patient was not indicated.

Question 2: Is this an appropriately ordered test?

There are six recognized CMT1 subtypes, including, CMT1A, CMT1B, CMT1C, CMT1D, CMT1E, and CMT2E/1F. More than 40 genes have been associated with CMT, but duplication and deletion of a 1.5 Mb region on chromosome 17, which includes the *PMP22* gene, accounts for diagnostic abnormalities in 60–80% of patients meeting clinical criteria for CMT1 [1–3]. The pathologist reviewed the request for send-out testing and called the clinician to discuss the relative frequency of genetic abnormalities associated with CMT1. The clinician agreed to proceed first with *PMP22* gene deletion/duplication testing, which was performed using the Multiplex Ligation-dependent Probe Amplification (MLPA) technique at a charge of $695. The option was left open to pursue future CMT molecular genetic tests if initial testing results were negative.

Laboratory Test Performed

MLPA is a variation on polymerase chain reaction (PCR) amplification in which pairs of oligonucleotide probes, which hybridize to directly-adjacent DNA sites in a portion of a gene (typically an exon), are first incubated as a group with genomic DNA. One member of each probe pair is fluorescently tagged. Subsequently, adjacently-bound oligonucleotides are covalently joined to each other using the enzyme DNA ligase. The amount of oligonucleotide bound should correspond to the relative amount of genomic DNA target present. In addition to the probe segment which hybridizes specifically to genomic DNA, the oligonucleotide probes also contain sequences at their respective upstream and downstream ends which do not bind genomic DNA, but are instead complementary to a "universal" forward or reverse oligonucleotide primer used as a set to amplify covalently-joined oligonucleotides. Oligonucleotide probes that do not hybridize to the appropriate targets will not be ligated to one another and are not amplified. The total length of adjacently-bound and ligated oligonucleotide probes is designed to be unique for each target region by incorporation of a variably-sized "stuffer sequence." The number of PCR cycles is adjusted to obtain signal from PCR products during the linear phase of amplification, which allows relative quantification of the dose of each target region. By comparing the size and signal intensity patterns with those of controls, the relative abundance (deletion or duplication) of a particular region can be determined [4].

Question 3: What are the limitations and advantages of this approach?

MLPA is an efficient and cost-effective method for detection of copy number changes (deletions and duplications) of moderate to large size genomic regions because multiple regions can be interrogated simultaneously via probe binding and ligation, followed by amplification using a single pair of PCR amplification primers. In addition to being technically more straightforward than dosage analysis by Southern blot or quantitative PCR, the assessment of copy number for multiple regions can typically be performed in a single reaction. Impurities in extracted nucleic acid or nucleotide sequence variants in regions bound by MLPA probes may lead to mis-estimation of copy number. Consequently, copy number variations are supposed to be confirmed by an independent second method. MLPA is technically demanding to set up *de novo* in the laboratory. A company (MRC Holland) which specializes in this technique provides reagents for more than 300 genomic regions [4]. These reagents typically have not been cleared for clinical use in the United States (FDA review) or Europe (CE Mark). As such, they are labeled and intended "for research use only." Laboratories which choose to use such reagents validate them internally as part of a laboratory-developed test.

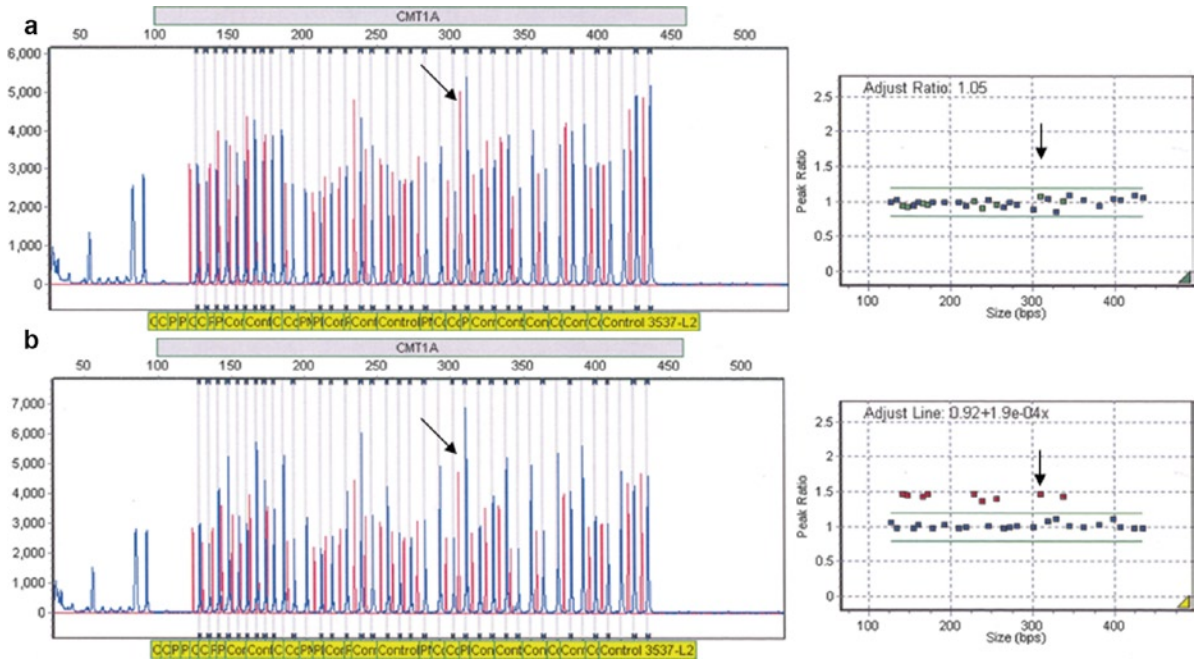

Fig. 4.1 MLPA analysis of patient sample DNA for the chromosome 17 region associated with *PMP22* gene duplication/deletion. *Panel* **a**, DNA from a normal control, *Panel* **b**, DNA from the patient described. The *arrows* point to MLPA product from the same *PMP22* gene region. The panels to the *right* of each figure represent the ratio of *PMP22* region MLPA products to control signals. It is straightforward to appreciate the copy number gain in the patient tested (*panel* **b**) (Original data provided by Sev Batish, PhD)

Results with Interpretation Guideline

Direct testing for the typical CMT1A mutation (duplication of the 1.5 Mb region which includes the *PMP22* gene) and the Hereditary Neuropathy with liability to Pressure Palsies (HNPP) mutation (deletion of this region) was performed by MLPA analysis. The accuracy, defined as the number detected in samples known to be positive, of mutation detection by the analyses was determined, by the reference laboratory, to be greater than 96%. Figure 4.1 depicts the MLPA results for this patient, generously provided by the reference laboratory.

Question 4: Is this sequence change pathogenic?

Result Interpretation

MLPA analysis identified both an abnormal *PMP22* allele (possessing a duplication mutation) and a *PMP22* allele in which no mutation was detected. Although the patient's family history is lacking and peripheral nerve biopsy was not performed, this finding is consistent with a diagnosis of CMT1A, which is supported by clinical features in this patient including decreased peripheral nerve conduction velocity and characteristic clinical features (calcovarus foot deformity).

Further Testing

Duplication of the PMP22 gene region confirms a diagnosis of CMT1A, and further testing of the patient is not indicated.

Other Considerations

MLPA is a common method of assessing dosage for discrete regions in the genome, but other methods including quantitative PCR, Southern blot, and, more recently, array hybridization will also provide such information.

The patient was most likely a *de novo* case of CMT1A due to a germline mutation or possibly due to autosomal

inheritance from the father. Because the father was a sperm donor, the paternal family history could not be assessed. The mother did not demonstrate clinical signs of CMT. If she were interested in having additional children, a neurologic examination might be desirable and in the event that features of neuropathy were identified nerve conduction studies could be performed.

In the absence of clinical symptoms via neurologic examination, no testing of the "sister's" sample was indicated. A generally-observed principle in genetic testing is to not test samples from minors to establish a diagnosis unless the child is symptomatic and confirmation of diagnosis is important for ongoing medical management of the child [5].

Background and Molecular Pathology

CMT hereditary neuropathy characterizes a group of polyneuropathy disorders sometimes referred to as the hereditary sensory and motor neuropathies (HSMN). A range of other hereditary and acquired neuropathies, including those due to mitochondrial dysfunction, may be considered depending on clinical presentation. A fifth of patients presenting with complaints associated with chronic peripheral neuropathy will have CMT1A. Symptoms of motor neuron compromise such as distal muscle weakness and atrophy dominate, but one frequently also encounters sensory loss of different degrees, depressed tendon reflexes, and severe pes cavus deformity of the feet, particularly in cases with childhood onset [1–3, 6–9].

Family history is an important element in evaluating patients for hereditary versus acquired causes of neuropathy, but can be challenging to evaluate with *de novo* cases; electrophysiologic studies on family members may sometimes be helpful. Cases with severe pes cavus deformity, as was present in this patient, may require extensive orthopedic surgery. Decreased nerve conduction velocity (NCV) is a hallmark of CMT1 and contrasts with typically-preserved NCV in CMT type 2 (CMT2), the symptoms of which, while generally less severe, may overlap with CMT1. Note that many of the genes in the "complete CMT panel" originally requested by the clinician are associated with CMT2, instead of CMT1 with which the patient's NCV studies and clinical phenotype were most consistent. Consistently decreased NCV was originally felt to be useful to differentiate hereditary from acquired (e.g., inflammatory) neuropathies, but NCV can vary inconsistently, particularly in the sex-linked forms associated with mutation in the Connexin 32 *(GJB1)* gene. Sural nerve biopsies are infrequently performed, but often demonstrate characteristic lesions [8, 9].

The predominant form (up to 60–90%) of CMT1 is CMT1A, which is inherited in an autosomal dominant manner or occurs *de novo*. Other forms of CMT may be inherited in autosomal dominant, autosomal recessive, and sex-linked forms. Over 40 different genes and an even larger number of chromosomal loci have been associated with CMT [1–3]. Molecular genetic testing is available on a clinical basis for various types of CMT. Clinical and electrophysiologic features may be helpful in suggesting which gene(s) to test initially, a fact which pathologists or other laboratorians should be willing to pursue to promote a cost-effective approach to diagnosis when physicians or genetic counselors request testing on large panels of genes [6–9]. Genetic counseling is recommended and prenatal testing is available if desired, when a disease-causing mutation is known [1–3].

The basis of CMT1A is typically a localized dosage increase of a 1.5 Mb region on the short arm of chromosome 17, resulting from duplication of this region on one chromosome 17 during meiosis. Duplication arises from unequal crossover between two 24 kb repeat regions which flank this region, which includes the *PMP22* gene. With the exception of CMT1B, which accounts for 5–10% of CMT1 and is due to abnormalities of the myelin P_0 protein produced by the *MPZ* gene, other forms of CMT1 resulting from abnormalities in other genes are rare, and it may be appropriate to question the cost-benefit of molecular genetic testing if there is a firm clinical diagnosis of CMT1 [10].

Of note, a separate disorder, HNPP, is caused by deletion of the *PMP22* gene to realize a final germline copy number of one.

Multiple Choice Questions

1. The mechanism for gene duplication seen in patients with CMT1A is best described by which of the following concepts.
   A. Gene amplification
   B. Isodisomy
   C. Overexpression

D. Polyploidy
E. Unequal chromosomal crossover

2. CPT code 83898 is currently used to bill for PCR amplification in molecular procedures performed in the United States. For the MLPA procedure which employs hybridization probes directed at the five exons of the *PMP22* gene, followed by PCR amplification using universal primers, how many units of 83898 are you allowed to code for the amplification portion of the MLPA procedure?
   A. 1
   B. 2
   C. 3
   D. 4
   E. 5
3. The typical patient with CMT1A disease is most likely to demonstrate:
   A. Compromise of motor and sensory neural function and decreased nerve conduction velocity
   B. Compromise of motor neuron function only, pain, and decreased nerve conduction velocity
   C. Compromise of motor neuron function only, pain, and normal nerve conduction velocity
   D. Compromise of sensory neural function only and decreased nerve conduction velocity
   E. Compromise of sensory neural function only and normal nerve conduction velocity
4. If it were shown through other studies that the CMT-affected boy in this case inherited the duplicated *PMP22* gene on a maternal chromosome, then what is the most likely risk of the mother having another CMT-affected child?
   A. <10%
   B. 25%
   C. 33%
   D. 50%
   E. 100%
5. A neurologist with whom you have developed a relationship over the years to discuss appropriate indications for genetic testing calls for advice on a three-generation family with a history of a CMT-like disorder seen in several branches of the pedigree but affecting only middle-aged males. Which gene would you probably suggest testing for first?
   A. Connexin 32 *(GJB1)*
   B. *MFN2*
   C. *MPZ*
   D. *PMP22* (deletion/duplication analysis)
   E. *PMP22* (full gene sequence analysis)

Answers to Multiple Choice Questions

1. *The correct answer is E.*

Unequal crossover during meiosis has been convincingly demonstrated to be the mechanism for dosage abnormalities associated with CMT1A. Two homologous regions, roughly 1.5 Mb apart, permit misalignment of chromosome 17 sister chromatids such that when crossover occurs, one chromatid ends up with two copies of the *PMP22* gene and the other with no copies. Individuals who inherit the double copy end up with functional trisomy for this region and demonstrate CMT1A. While it occurs less frequently, inheritance of the "null" chromosome with no *PMP22* copy results in a different neurologic syndrome, HNPP. There is no evidence for localized gene amplification, overexpression of the *PMP22* gene, or improper chromosomal segregation which could potentially lead to isodisomy.

2. *The correct answer is A.*

The correct answer is 1. Universal means exactly what it implies. Universal PCR amplification primers bind to the same sequences engineered at the 5′ and 3′ ends of each DNA species produced when adjacently-bound probes, targeting various regions associated with deletion/duplication of the *PMP22* gene or other genomic regions used to normalize for diploid gene dosage, are ligated.

3. *The correct answer is A.*

While motor neuron function is most affected, it is not uncommon to also see loss of sensory nerve function in patients with CMT1A. Decreased nerve conduction velocity is a hallmark of CMT1A.

4. *The correct answer is A.*

There appears to be no clinical evidence that the mother has CMT; hence, she is unlikely to be a germline carrier with the standard one in two risk of passing an affected chromosome 17 to offspring for an autosomal dominant disorder. The possibility exists that a population of her oocytes contains a duplicated region on chromosome 17 with *PMP22* duplication (and perhaps even a population containing a deleted chromosome without *PMP22*!). This phenomenon is known as gonadal mosaicism, and the risk for gonadal mosaicism is typically derived from empiric experience with families such as this one. It is likely to be low. Hence, <10% is the best answer and the information a genetic counselor would use in talking with the mother.

5. *The correct answer is A.*

The family history provided is strongly suggestive of a sex-linked disorder. CMTX is associated with mutations in the Connexin 32 *(GJB1)* gene on the X chromosome. It would be important to confirm with the clinician the absence of any male-to-male transmission in the pedigree. If identified, this would suggest the likelihood of autosomal dominant transmission with a serendipitous distribution affecting only males. When a large number of male-only CMT cases are observed in a family, the statistical likelihood of autosomal dominant transmission becomes very small.

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Hearing Loss

5

Iris Schrijver

Clinical Background

A 1-year-old girl with profound congenital hearing loss presented to the pediatric genetics clinic because her parents wanted to know why their child was deaf. They were also contemplating another pregnancy and sought to learn about the risk of having a second child with hearing loss. The patient's mother had northern European ancestry, whereas the father was of Ashkenazi Jewish extraction.

The patient was born at 40 weeks, after an uneventful pregnancy. The delivery was without complications, birth weight was normal at 3.5 kg, and her appearance was not dysmorphic. However, while still in the hospital, she failed her newborn hearing screen by otoacoustic emission testing. She received a follow-up hearing test, in which her auditory brainstem response was measured, a few weeks later. This test confirmed that she had bilateral, profound sensorineural hearing loss. Upon review, there was no history of environmental risk factors for congenital hearing loss. These include positive TORCH (toxoplasma, other or syphilis, rubella, cytomegalovirus, and herpes simplex virus) titers during gestation, asphyxia at birth, complications that warrant admission to a neonatal intensive care unit, craniofacial anomalies, hyperbilirubinemia, neonatal infection, and the use of ototoxic medications. There also was no history of hearing loss in the family, except for the maternal grandmother whose hearing loss only became noticeable after age 80. The physical examination was appropriate for age and interpreted as completely normal with exception of the hearing loss. There were no physical features that would suggest that the hearing loss was part of a clinical syndrome. An ophthalmology evaluation was performed because ocular abnormalities can be identified in up to half of children with severe to profound hearing loss [1], but it was normal.

Question 1: What is the most likely pattern of inheritance?
Question 2: Can molecular testing be helpful, given that it is already known that the child is deaf and management can be initiated?

Reason for Molecular Testing

There are many valid reasons for molecular diagnostic testing in both children and adults with hearing loss. Whereas hearing loss can be caused by environmental factors, genetic defects, or a combination of both, in western nations at least 50% of prelingual hearing loss is estimated to have a genetic etiology. The cause remains obscure in approximately 25%, but most of these cases are assumed to have a genetic basis as well. Thus, genetic causes account for the largest proportion of

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prelingual hearing loss [2]. Of the estimated minimum of 50% of cases with inherited hearing loss, ~70% are non-syndromic and ~80% are autosomal recessive [3].

Molecular diagnostic testing can be helpful because an etiology cannot be otherwise established in the majority of individuals with genetic hearing loss. In those patients, extra-auditory features associated with a syndrome are not identified, imaging studies are negative or inconclusive, and the hearing loss phenotype is not unique to a certain etiology. Molecular analysis can also be beneficial for the diagnosis of syndromic hearing loss, especially prior to the emergence of additional syndromic features (examples are Jervell and Lange-Nielsen syndrome or Pendred syndrome). In addition, it can identify mitochondrial mutations, which increase the risk of iatrogenic hearing loss when aminoglycosides are administered. Molecular testing is essentially non-invasive and may reduce the need for more extensive and costly evaluations, which sometimes require sedation or general anaesthesia of infants and children. Finally, molecular analyses can contribute to an accurate and early diagnosis, which supports optimal cognitive and social development. Upon identification of a genotype that can explain the hearing loss, the associated knowledge of the pattern of inheritance enables accurate genetic counseling.

Question 3: Which molecular genetic test would you order?

Mutations in the *GJB2* gene (OMIM number *121011) on chromosome 13q11-12 are the most common cause of sporadic and recessive non-syndromic sensorineural hearing loss (SNHL). Mutations have been identified in populations all over the world, and account for approximately half the cases in the USA and several other geographic areas [4]. Autosomal recessive non-syndromic SNHL at this locus (DFNB1) is prelingual, ranging from mild to profound, and most often not progressive. DFNB1 contains two genes, *GJB2* and *GJB6* (OMIM number *604418), which respectively encode the gap junction proteins connexin 26 (Cx 26) and connexin 30 (Cx 30). These two genes share a sequence identity of 77% [5].

Test Ordered

The clinical geneticist ordered direct DNA sequencing of the *GJB2* gene.

Laboratory Test Performed

The laboratory-developed assay was designed to detect a wide range of mutations in the *GJB2* gene. This gene consists of 681 basepairs, which are translated into a protein with 226 amino acids. Mutations and sequence variants in *GJB2* are associated with both syndromic and nonsyndromic SNHL. They are dispersed throughout the coding region of the gene, which is encompassed in the second exon. Exon 1 is contained in the 5′-UTR (untranslated region). The *GJB2* sequencing assay includes isolation of DNA, generation of an 830 bp amplicon via the polymerase chain reaction (PCR), an amplification check on a 2% agarose gel, and direct DNA sequencing with four individual fluorescent sequencing primers that cover the entire sequence in both the forward and reverse directions. The sequences have sufficient overlap to obtain an electropherogram with adequate signal strength and an excellent signal to noise ratio for the coding region (exon 2) and its splice sites with intronic flanking sequences. Sequence analysis is facilitated by Mutation Surveyor, a sequence analysis software program.

Results with Interpretation Guideline

Figure 5.1 displays the results of one of the reverse sequence tracings generated in the course of the *GJB2* sequencing assay. The signal strength of this assay was good and technical issues were not reported. The beginning of the coding exon (exon 2) is indicated by the directional arrow and the sequence must be read in reverse. The coding region of this gene can be viewed on Genatlas: http://genatlas.org/. On this website, select "Gene database," type "*GJB2*" into the "Symbol name" field, and select "See the exons" on the next web-page. The exon will be displayed in bold and the initiating methionine codon, as well as the termination codon, are shown in red. Published *GJB2* mutations and polymorphisms are listed on the Connexin-deafness homepage (http://davinci.crg.es/deafness/). All sequence variants are compared to those listed on the website and to the current literature in order to determine clinical significance.

Question 4: Which change is present at the black arrow in this sequence tracing?

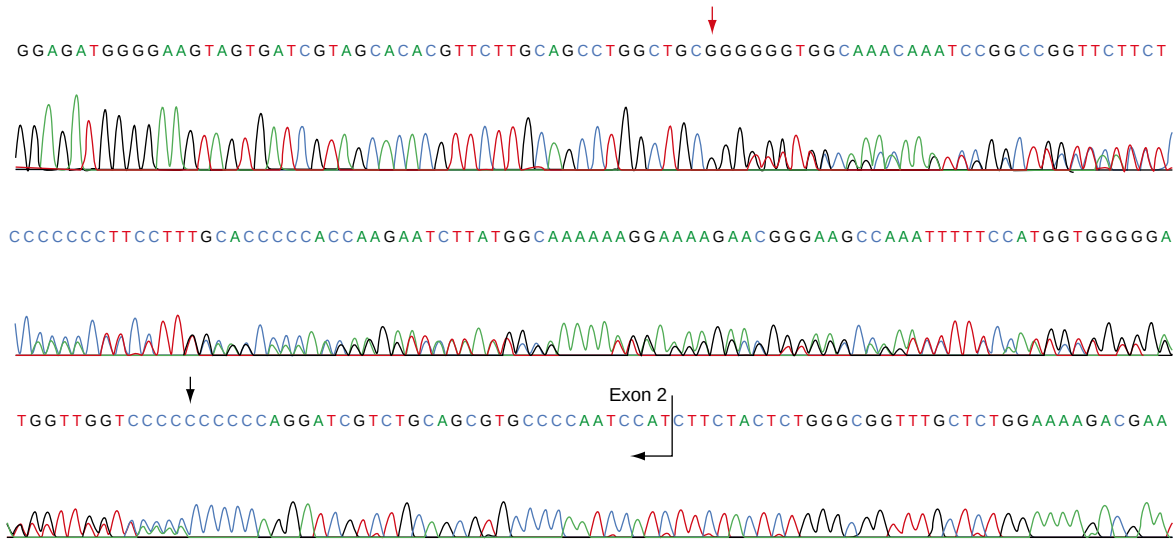

Fig. 5.1 Partial electropherogram of the *GJB2* sequencing test performed

Question 5: How can you explain the apparently clean sequence toward the end of the exon (red arrow)?

Result Interpretation

The impression of the sequence is that of a frameshift mutation. At 35 nucleotides from the start of the exon, the signal appears to decrease and a double sequence is present at most nucleotide positions. When the normal sequence is written down first, the complementary second signal can be recorded below it. Thus, it becomes readily apparent that, in the forward direction, one guanine is missing out of the normal string of six. This mutation represents the c.35delG mutation. It is the most prevalent *GJB2* mutation in Caucasians. In the USA, its carrier frequency is approximately 2.5% but actual frequencies depend on ethnic origin [6, 7]. According to guidelines of the HGVS (Human Genome Variation Society, http://www.hgvs.org/), the nomenclature at the protein level is p.Gly12fs for the short description and p.Gly12ValfsX2 for the complete description of this frameshift mutation.

Near the red arrow (Fig. 5.1), the double sequence signal seems to end. This phenomenon is caused by a frameshift mutation on the other allele, which eliminates the appearance of the mixed sequence. From this nucleotide position in the electropherogram onward, both alleles have a single nucleotide deletion. The effect on the sequence tracing is a correction of the frameshift pattern. This is a consequence of the mixture of both of the patient's alleles in the test tube. In the body, however, each frameshift mutation results in an independent premature stop codon and the defect at the protein level is not corrected.

Question 6: Which change is present at the red arrow in this sequence tracing, and what is its correct HGVS nomenclature?

Comparing the normal and mutant sequences, a deletion of a thymine at nucleotide position 167 can be identified. This frameshift mutation, c.167delT, has a carrier frequency of approximately 4% in Ashkenazi Jews [8]. According to guidelines of the HGVS, the amino acid change associated with this mutation should be described as p.Leu56fs (short form) or p.Leu56ArgfsX26 (long form).

Further Testing

The hearing loss in this patient can be explained by the compound heterozygosity for two premature termination mutations. Therefore, further molecular

testing is not clinically warranted. The mutations identified are compatible with the ethnicities of the parents and they were counseled that they have a risk of 25% for a child with hearing loss, with each pregnancy. They were also told that, even though both these mutations are considered severe and are predicted to result in severe to profound SNHL, there is inter- as well as intra-familial variability with *GJB2*-related phenotypes. Therefore, an exact prediction of the level of hearing loss cannot be made. The patient was referred for consideration of cochlear implant surgery.

Background and Molecular Pathology

Hearing loss is one of the most common birth defects that affects child development, education, medical needs, and social life [9]. It is present in 6–8% of the population overall [10]. Universal newborn screening has revealed that, in the USA, approximately 1 in 300 children are born with hearing loss and 1 in 1,000 are deaf at birth. Before becoming adults, an additional 1 in 1,000 develop profound hearing loss [11, 12]. Once the hearing loss has been diagnosed, early intervention with hearing aides and/or cochlear implant can drastically improve the ability to communicate and contribute to the quality of life.

Genetic hearing loss is autosomal recessive in approximately 80% of cases, dominant in 10–20%, and X-linked in 1–2% [13, 14]. The exact frequency of mitochondrial deafness has yet to be determined. Most prelingual hereditary hearing loss is transmitted as an autosomal recessive trait, whereas autosomal dominant inheritance is more commonly identified with postlingual onset [15]. Syndromic hearing loss is associated with complex phenotypes that affect other organs, as well. In contrast, non-syndromic hearing loss is isolated. It also is the most common type, present in up to 80% of all individuals with congenital hearing impairment [13]. Most often sensorineural, it can be categorized as DFNA (autosomal dominant), DFNB (autosomal recessive), DFN (X-linked), and mitochondrial hearing loss.

The genetics of hearing loss are intricate and it is estimated that several hundred genes are required for the physiologic process of hearing [16]. The *GJB2* gene at the DFNB1 locus encodes Cx 26, a

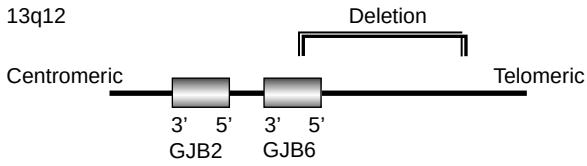

Fig. 5.2 The orientation of the DFNB1 locus with the position of the large deletions involving *GJB6*

Gap Junction protein of the Beta group with a molecular mass of 26 kD. The characterization of this gene and the subsequent identification of more than 100 sequence changes highlighted the importance of cochlear gap junctions, which enable transportation of ions between cells. Individual mutations within *GJB2* are responsible for recessive and dominant, as well as syndromic and non-syndromic hearing loss. Two frameshift mutations, c.35delG and c.167delT, are, respectively, the most commonly observed sequence changes in Caucasians and Ashkenazi Jews. However, *GJB2* mutations have been reported in many populations and are the most commonly recognized cause of sporadic and autosomal recessive non-syndromic SNHL. Sequence changes reported in *GJB2* are primarily mutation types which can be detected by direct DNA sequencing, and include nonsense, missense, splicing, and frameshift mutations.

The DFNB1 locus also contains the *GJB6* gene (which encodes Cx 30). It is located ~35 kb telomeric from the *GJB2* gene (Fig. 5.2). In contrast to *GJB2*, however, large deletions, primarily del(*GJB6*-D13S1830), are the most common mutation type in and around this gene. Such deletions are relatively uncommon in the USA; among individuals heterozygous for a mutation in the *GJB2* gene, the del(*GJB6*-D13S1830) accounted for no more than 2.8% of mutations, and homozygous *GJB6* deletions are exceedingly rare [17]. *GJB6* is implicated in non-syndromic SNHL when homozygous for deletions, or when a deletion is heterozygous and on the opposite allele of a *GJB2* mutation [18]. Although it was originally thought that mutations in these two genes cause hearing loss through a digenic mechanism of inheritance, it is now clear that deletions in *GJB6* cause allele-specific loss of *GJB2* expression on that same allele [19, 20]. It is therefore likely that the deletions eliminate a regulatory element for *GJB2*.

Multiple Choice Questions

1. From the list below, identify the most frequent pathogenic sequence change in the *GJB2* gene that is associated with non-syndromic hearing loss:
   A. c.35delG
   B. c.79G > A
   C. c.167delT
   D. c.235delC
   E. c.223C > T
2. Which type of sequence change is most likely pathogenic?
   A. Frameshift mutation
   B. In-frame deletion
   C. Missense mutation
   D. Nonsense mutation
   E. A and D
3. Select the correct statement regarding mutations in the *GJB6* gene:
   A. Mutations in the *GJB6* gene are more common than those in *GJB2*
   B. Non-syndromic SNHL can be caused by heterozygosity for a point mutation in *GJB2* combined with heterozygosity for a deletion in *GJB6*
   C. The *GJB6* gene is a pseudogene located next to the *GJB2* gene
   D. The most common *GJB6* mutations are point mutations
   E. The most common mutations in the *GJB6* gene are associated with syndromic hearing loss
4. A woman with normal hearing seeks genetic counseling. She has a sister with non-syndromic autosomal recessive SNHL, who is homozygous for c.35delG. What is the client's risk of being a carrier?
   A. 1
   B. 1 in 2
   C. 1 in 4
   D. 2 in 3
   E. Impossible to determine without information about ethnicity
5. A deaf patient with the autosomal recessive Pendred syndrome carries two mutations in the *SLC26A4* gene. One allele carries a missense mutation in exon 4, the other allele carries a deletion of exon 5. What will the sequencing electropherograms demonstrate?
   A. A heterozygous nucleotide change in exon 4 and a mixed sequence pattern in exon 5
   B. A heterozygous nucleotide change in exon 4 and no sequence of exon 5
   C. A heterozygous nucleotide change in exon 4 and the normal sequence of exon 5
   D. A homozygous sequence pattern in exon 4 and a mixed sequence (frameshift pattern) in exon 5
   E. A mixed sequence (frameshift) pattern throughout exon 4 and the normal sequence of exon 5

Answers to Multiple Choice Questions

1. *The correct answer is A.*

c.35delG is the most common mutation in the *GJB2* gene overall. c.79G > A is a clinically benign polymorphism. c.167delT is the most common mutation in Ashkenazi Jewish individuals. c.235delC is the most commonly identified pathogenic variant in Asian populations, with a carrier frequency of approximately 1%. c.223C > T is a *GJB2* mutation associated with dominant inheritance and syndromic, instead of non-syndromic, hearing loss.

2. *The correct answer is E.*

Both a frameshift mutation and a nonsense mutation result in premature termination of the protein. These mutations are typically pathogenic. In-frame deletions leave the reading frame intact and may have small effects on the protein made. It is often challenging to determine the pathogenicity of missense mutations. Segregation with the phenotype, studies of population frequency, conservation across species, amino acid change and location within the protein, as well as functional studies can help elucidate the likelihood of a pathogenic effect.

3. *The correct answer is B.*

Mutations in the *GJB2* gene are much more common than those in *GJB6*. The *GJB6* gene is not a pseudogene, and the most common *GJB6* mutations are large deletions that remove part of the *GJB6* gene. The *GJB2* gene is left intact. These deletions are associated with non-syndromic hearing loss when homozygous, or when heterozygous in the presence of a heterozygous *GJB2* mutation on the opposite allele.

4. *The correct answer is D.*

According to Mendelian inheritance, an individual can be affected (AA), unaffected (aa), or a carrier (Aa or aA). Given that this client has normal hearing, we know that she is unaffected. Her risk of being a carrier, therefore, is two out of three.

5. *The correct answer is C.*

This individual is compound heterozygous for two mutations in the *SLC26A4* gene, which encodes the pendrin protein. These mutations will result in a heterozygous nucleotide change in exon 4, with two different nucleotide signals visible at the nucleotide that is affected with the point mutation. A complete exon deletion will prevent amplification of the deleted exon if the primers were not able to anneal because the primer sequences on that allele were deleted. As a consequence, the amplification will include only the unaffected allele, which will demonstrate the normal sequence of exon 5. Sequence analysis alone will typically not identify whole exon deletions. Methods such a MLPA (Multiplex Ligation-dependent Probe Amplification) are able to detect such changes.

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Polycystic Kidney Disease

6

Ying-Cai Tan, Jon Blumenfeld, and Hanna Rennert

Clinical Background

A 42-year-old woman presented for additional evaluation of autosomal dominant polycystic kidney disease (ADPKD). She was initially diagnosed with ADPKD at age 36 after developing fever and flank pain. At that time, ultrasonography showed bilateral renal cysts, and liver and ovarian cysts. The serum creatinine level was normal. Hypertension was diagnosed at the age of 32 years and has since been controlled by treatment with an angiotensin converting enzyme inhibitor. There have been multiple, uncomplicated urinary tract infections, but no history of kidney stones or abdominal wall hernia. Episodes of abdominal and flank discomfort were treated successfully with non-narcotic medications. She was not screened for intracranial aneurysm. There have been no pregnancies, by choice, and there was no history of oral contraceptive drug use. Although the patient was unaware of a family history of ADPKD, her mother underwent unilateral nephrectomy during childhood for an unknown indication. The patient had three siblings; both sisters had negative screening renal ultrasonography after age 40 and her 36-year-old brother had not been evaluated. There was a three pack-year history of tobacco use that ceased at age 26.

Her physical examination was significant for blood pressure 124/84 mmHg, heart rate 70 beats/min, a 2/6 systolic murmur, and palpable kidneys and liver. The serum creatinine was 0.74 mg/dL and 24-hour urine creatinine clearance was 103 mL/min; complete blood count, liver function tests, and urinalysis were normal.

Magnetic resonance imaging of the abdomen showed innumerable cysts in the liver and in both kidneys (Fig. 6.1). Right and left kidney lengths were 19 and 22.3 cm, respectively. Total kidney volume was 1,925 mL, total cyst volume was 1,289 mL, and cyst fraction (total cyst volume/total kidney volume) was 67%. Liver volume was 1,431 mL, liver cyst volume was 174 mL, and cyst fraction was 12.2%. There were no cysts in the pancreas or spleen.

Reason for Molecular Testing

The patient requested *PKD* gene analysis to improve her understanding of her disease and to potentially inform the process of future kidney donor selection from a family member.

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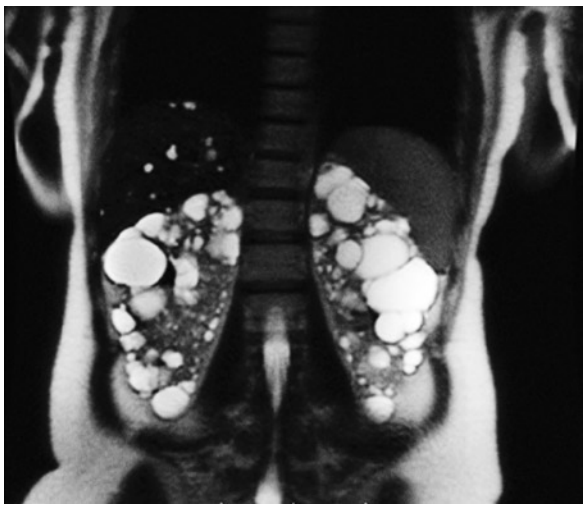

Fig. 6.1 Magnetic resonance image of the abdomen in the patient with ADPKD. The massively enlarged kidneys, each with innumerable cysts of varying sizes, represent the typical ADPKD phenotype. Polycystic liver disease, also present in this patient, is highly prevalent in ADPKD

Test Ordered

Initially the clinician ordered mutation analysis of the *PKD* genes from a commercial reference laboratory that had exclusive license for the *PKD1* and *PKD2* gene patents in the USA. The test involved direct sequencing of the entire coding regions of both *PKD1* and *PKD2*. However, due to the unusual result of a homozygous nonsense mutation (c.8095C>T; p.Gln2699Stop) for this autosomal dominant disease, the clinician requested re-analysis of the patient's DNA for *PKD1* and *PKD2* mutations, as part of a prospective study conducted by The Rogosin Institute of patients with autosomal dominant polycystic kidney disease. These tests were performed in a diagnostic molecular pathology laboratory on a research basis, using a mutation screening approach coupled with sequencing.

Question 1: In which clinical situations is ADPKD genetic testing useful?

Laboratory Test Performed

Initial testing by the commercial reference laboratory included mutation analysis of the entire coding sequence of *PKD1* and *PKD2* genes by sequencing analysis, using peripheral blood lymphocyte DNA. Repeat mutation analysis was performed by heteroduplex analysis, using a novel mismatch-specific DNA endonuclease (SURVEYOR™ nuclease that cleaves both strands of heteroduplex DNA at the 3′ side of any mismatch site, including all substitutions and insertions and deletions up to 12 bp in length) and the Transgenomic WAVE® Nucleic Acid High Sensitivity Fragment Analysis System (Transgenomic, Inc. Omaha, NE). This was followed by sequencing of variant gene segments [1]. Specifically, the duplicated region of *PKD1* (exons 1–33) was first amplified with the GeneAmp High Fidelity PCR System (Applied Biosystems, Foster City, CA) as five distinct long-range PCR fragments (exon 1, exons 2–12, exons 13–15, exons 15–21, and exons 22–33), using PCR primers anchored in the single-copy DNA or mismatched with homologue genes (HG), which are more than 98% identical to *PKD1* sequence, thus exclusively amplifying *PKD1* sequences. Subsequently, dilutions of *PKD1* long-range PCR products or the single-copy areas of *PKD1* (exons 34–46) and the entire *PKD2* gene (exons 1–15), including splice junctions and 5′- (*PKD1* only) and 3′-untranslated regions of the genes, were amplified in a second set of reactions, using primers positioned approximately 80–100 bp from the intron–exon boundaries to allow optimal detection of splice site variants, generating PCR fragments of approximately 250–550 bp. Due to the numerous polymorphisms in *PKD1*, and to minimize allele dropout due to primer mismatch, primers were positioned in regions for which no polymorphisms were reported. Both *PKD* genes were amplified in separate PCR reactions, using similar PCR conditions and a touchdown PCR amplification with a hot-start protocol. Following PCR amplification, all PCR products were denatured and slowly reannealed to form heteroduplexes, followed by SURVEYOR® nuclease digestion and WAVE® HS analysis according to the manufacturer's instructions. PCR samples demonstrating digestion products were sequenced on an automated ABI 3100 Genetic Analyzer.

Question 2: What are the main advantages and limitations of complete gene sequencing compared to mutation scanning methods?
Question 3: What is a major concern when designing primers for molecular testing of highly polymorphic and duplicated genes?

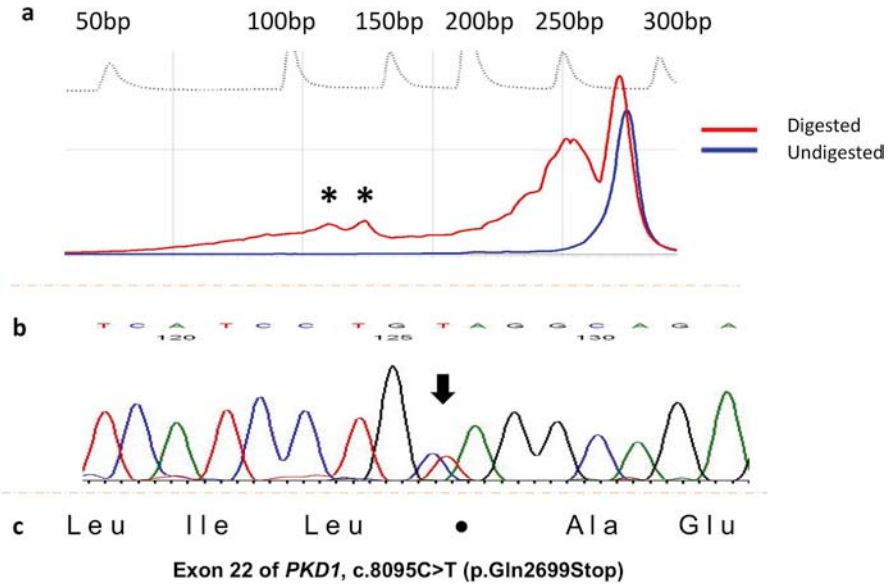

Fig. 6.2 Genetic Analysis of *PKD1* exon 22 by SURVEYOR WAVE HS-Sequencing. Surveyor digested PCR products of exon 22 were separated on the WAVE HS system using the non-denaturing sizing application. (**a**) Digested patient samples, an uncut control and a size marker of 50 bp; the *stars* indicate the specific digested peaks. (**b**) Sequencing results from the SURVEYOR nuclease-positive samples; the *arrow* indicates the location of the C > T heterozygous change. (**c**) The reading frame of the mutated allele; the *bold dot* indicates a stop codon

Results with Interpretation Guideline

Genetic testing using SURVEYOR–WAVE-Sequencing analysis demonstrated two unexpected peaks (Fig. 6.2a), compared to a normal control sample (not shown), on the SURVEYOR chromatogram. These peaks correspond to digested 270 bp PCR products into approximately 130 and 140 bp fragments, respectively, supporting the presence of a heterozygous mutation in this patient, which was confirmed by sequencing (Fig. 6.2b). Analyzing the reading frame of the mutated allele confirms the presence of the previously reported nonsense mutation, *PKD1* c.8095C > T (p.Gln2699Stop) (reference sequence: PKD1: NM_000296.2). The concordance between SURVEYOR–WAVE analysis and sequencing results obtained in the repeated testing verifies that this is, however, a heterozygous nonsense mutation.

Question 4: How do you explain the discrepant results between the initial sequencing analysis and the subsequent mutation screening and sequencing methods?

Result Interpretation

ADPKD is caused by a heterozygous mutation in either the *PKD1* or the *PKD2* gene. Homozygosity of *PKD1* or *PKD2* mutations in *PKD* orthologous mouse models results in an embryonic lethal phenotype [2, 3]. Therefore, it is unlikely that our patient has a homozygous germline mutation. Although hypomorphic alleles have been reported, the associated phenotype tends to differ from typical ADPKD [4]. Additional DNA testing confirmed the presence of a heterozygous nonsense mutation in *PKD1* exon 22, which causes truncation of polycystin 1, the gene product of *PKD1*. This result correlated well with the patient's phenotype.

Further Testing

Recommended future testing of this patient includes total kidney volume measurements by magnetic resonance imaging, which reportedly provides prognostic information regarding the risk of progression of chronic kidney disease [5]. Genetic testing can be offered to family members with negative or equivocal renal ultrasonography screens, in whom a diagnosis of ADPKD is uncertain. The role of genetic testing may expand significantly in the future for early identification of affected individuals, particularly if effective or preventive therapies are developed [6].

Other Considerations

The diagnosis of ADPKD requires an age-specific cystic renal phenotype together with a 50% risk of inheritance determined by a positive family history [7]. These criteria

were initially defined in patients with *PKD1* mutations, based on the detection of renal cysts by ultrasonography [8, 9]. The diagnostic sensitivity is ~90% between ages 15–30 years and approaches 100% for older patients. By contrast, in patients with *PKD2* mutations who are younger than 30 years, renal ultrasonographic criteria have a sensitivity of only approximately 67% [10]. Renal CT scans and magnetic resonance imaging techniques are more sensitive than ultrasonography. However, the diagnostic performance characteristics of these methods have not been defined for ADPKD. This often poses a management issue, especially when considering potential kidney donors who are at risk for ADPKD. In these individuals, the merits and limitations of PKD genetic testing should be considered [9, 11].

Background and Molecular Pathology

ADPKD is the most common inherited kidney disease in the USA occurring in approximately 1:1,000 individuals worldwide [12]. It is characterized by an abnormal proliferation of renal tubular epithelial cells, which manifests as cysts that increase gradually in size and number, leading to massive kidney enlargement and progressive decline of renal function. About one-half of all ADPKD patients reach end-stage renal disease (ESRD) by the sixth decade, accounting for ~5% of all individuals requiring dialysis or kidney transplantation. Extra-renal manifestations of ADPKD, which are a major cause of morbidity, include polycystic liver disease and vascular aneurysms [13].

ADPKD is a genetically heterogeneous dominant disease caused by mutations in two genes: *PKD1* (MIM# 601313) located on chromosome 16p13.3 [14], which accounts for 85% of cases and *PKD2* (MIM# 173910) located on chromosome 4q21 [15], which accounts for the remaining 15% of cases. *PKD1* consists of 46 exons spanning ~52 kb of genomic DNA, encoding a 4,033 amino acid protein [16, 17]. The 5′-end (exons 1–33) of the gene is duplicated in at least six homologous genes that show 97–99% homology with *PKD1* and significantly complicate genetic testing [16]. *PKD2* has 15 exons, with a 5.3-kb transcript encoding a 968 amino acid protein [18]. *PKD1* and *PKD2* genes encode polycystin 1 (PC1) and polycystin 2 (PC2), respectively. These transmembrane proteins localize to epithelial cilia and interact to produce cation-permeable currents that may be important in mechanoreception [19].

One pathogenic mechanism that has been demonstrated in ADPKD is the “two-hit” phenomenon, in which a germline mutation combines with a somatic mutation within the renal cells to inactivate both *PKD* genes [20], leading to loss of function and promoting cell proliferation and apoptosis, cyst formation, and chronic kidney disease [21]. Individuals with mutations in *PKD1* have a more severe clinical phenotype, progressing to ESRD on average 20 years earlier than *PKD2* patients. However, the ADPKD phenotype is characterized by considerable intrafamilial and interfamilial variability, which has been attributed to various mechanisms, including allelic heterogeneity, modifying genes that are as yet unidentified, and locus heterogeneity [22].

Genetic testing is useful for diagnosis and prognosis of ADPKD, particularly in asymptomatic individuals or those without a family history [9]. However, the *PKD* genes are highly polymorphic. More than 290 mutations have been described in *PKD1* and more than 90 in *PKD2* (Human Gene Mutation Database, HGMD, http://www.hgmd.cf.ac.uk/ac/index.php), the majority of which are private mutations located throughout both genes. Currently, definite pathogenic mutations (nonsense, truncation and canonical splice defects) are identified in only approximately 60% of the cases. Comprehensive analyses using computational analysis tools identified a large number of variants of uncertain significance that may account for the disease in an additional 22–37% of ADPKD patients [23].

Multiple Choice Questions

1. ADPKD disease severity has been primarily attributed to which of the following genetic variations?
   A. Allelic variations
   B. Locus heterogeneity
   C. Hypomorphic alleles
   D. Modifier genes
   E. Structural variations
2. A 28-year-old man with a paternal family history of ADPKD is being evaluated as a potential living-related kidney donor. Renal ultrasonography of his kidneys was negative. His father was diagnosed with bilateral enlarged kidneys with innumerable cysts, and with liver cysts at age 51 years. His paternal grandmother was diagnosed with ADPKD in her 40s and received a deceased donor kidney

transplant at age 70 years. ADPKD mutation studies in the father were negative. To exclude ADPKD in the proband, you would recommend to:
A. Perform linkage analysis studies
B. Reevaluate the family for ADPKD because no mutation has been identified
C. Recommend using the proband as a donor since he screened negative by ultrasonography
D. Screen the proband for ADPKD mutations
E. Use a more sensitive renal imaging method such as MRI or CT

3. You refer the family above (question 2) for genetic testing. What are the chances that this family carries a mutation in *PKD1*?
A. 25%
B. 50%
C. 70%
D. 85%
E. 100%

4. Approximately 5% of the patients with ADPKD have large gene rearrangements that are missed by direct sequencing or mutation screening strategies. The most common method currently used for identifying dosage changes is:
A. Comparative Genomic Hybridization (CGH) arrays
B. Fluoresce In-Situ Hybridization (FISH)
C. Multiplex Ligation-dependent Probe Amplification (MLPA)
D. Protein Truncation Testing (PTT)
E. Southern blot

5. The contiguous gene syndrome in which both ADPKD and tuberous sclerosis are clinically manifested is typically due to:
A. Chromosomal deletions involving both *PKD1* and *TSC2* on 16p13.3
B. Chromosomal duplication of 16p13.3 region
C. De novo mutations in *PKD1* and *TSC2*
D. Germline mutations in *PKD1* and *TSC2*
E. Translocations involving chromosome 16

Answers to Questions Embedded in the Text

Question 1: In which clinical situations is ADPKD genetic testing useful?

Genetic testing for ADPKD is useful in young patients in whom renal ultrasonography or other imaging modalities may not be conclusive or when the family history is negative or unknown. Also, genetic testing is potentially useful during pre-transplant evaluation of prospective kidney donors who are at risk for ADPKD by family history.

Question 2: What are the main advantages and limitations of complete gene sequencing compared to mutation scanning methods?

Direct sequencing is still considered the method of choice for mutation detection in many laboratories, because conventional screening methods cannot detect homozygous changes and may not detect all sequence variations. However, due to its limited analytical sensitivity of ~15–20%, sequencing may not detect low-signal heterozygous changes attributed to DNA species present at low levels in the reaction, potentially yielding erroneous results. This is a significant concern in acquired genetic diseases such as leukemias, when small populations of malignant cells may be circulating.

Question 3: What is a major concern when designing primers for molecular testing of highly polymorphic and duplicated genes?

A major concern in PCR primer design is that single nucleotide polymorphisms (SNPs) in primer binding regions may affect the amplification efficiency of the PCR. Therefore, it is recommended that PCR primers be designed using specific software that masks known areas with sequence variation to assure unique PCR amplification of the gene regions to be analyzed.

Question 4: How do you explain the discrepant results between the initial sequencing analysis and the subsequent mutation screening and sequencing methods?

The false homozygosity for the nonsense mutation detected by sequencing was probably caused by allele drop-out during PCR amplification. Allele drop-out or pseudo-homozygosity has been well documented and is likely due to the presence of a SNP in the primer binding region of one of the two alleles resulting in lower, or complete lack of, amplification of one allele. If the allele that can be amplified carries a mutation, the genotype may appear homozygous [24]. By contrast, enzyme-based screening methods are considered very sensitive (down to 1–5%) for detection of mutations in an impure population of DNA [25].

Answers to Multiple Choice Questions

1. *The correct answer is B.*

The main distinction between populations of patients with *PKD1* versus *PKD2* mutations is that the latter is associated with a milder phenotype, including a later age at diagnosis, decreased prevalence of hypertension, and later onset of ESRD. This distinction may not be evident for the individual patient because of the significant clinical heterogeneity of ADPKD.

2. *The correct answer is A.*

Linkage analysis is used to follow the segregation of chromosomal markers flanking the disease gene(s) in family members in whom the clinical status (affected or unaffected) is known. Using this approach, it is almost always possible to determine if the at-risk subject is an obligate carrier without the need to know the pathogenic mutation. However, this approach requires several affected and unaffected family members.

3. *The correct answer is D.*

PKD1 mutations are more common than *PKD2* mutations, accounting for approximately 85% of the cases.

4. *The correct answer is C.*

MLPA is currently the method of choice for identification of gross rearrangements (insertions and deletions) in most genes. FISH and array-CGH are currently lacking the resolution required for identifying small dosage changes in DNA.

5. *The correct answer is A.*

Concurrent manifestations of ADPKD and tuberous sclerosis occur in the contiguous gene syndrome involving a chromosomal deletion of both *PKD1* and *TSC2*, which are located in close proximity on chromosome 16p13.3. Patients with this syndrome may present during infancy with rapid progression to ESRD. The absence of a family history of ADPKD in these patients is common as their parents are somatic mosaics, or the disease is caused by a *de novo* mutation.

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Fragile X Syndrome

7

Ruth A. Heim

Clinical Background

Linda Jones was a healthy, 35-year-old lawyer who was pregnant for the second time. Her first child was a healthy, now 10-year-old girl. At Linda's initial prenatal visit she discussed fragile X testing with her physician, having read about it in a popular magazine. Although Linda's first child did not have fragile X syndrome and there was no history of mental retardation or autism-spectrum disorders in her family, she requested carrier screening for fragile X syndrome.

Reason for Molecular Testing

Fragile X syndrome is the most common inherited cause of mental retardation in males and females and the leading known single gene cause of autism. Linda's reason for fragile X carrier screening was parental anxiety.

Test Ordered

Fragile X expansion mutation analysis was ordered. Most cases of fragile X syndrome are caused by expansion of an unstable trinucleotide repeat sequence (CGG) located in the 5′-untranslated region of the *FMR1* gene on the X chromosome. Affected individuals with a full mutation have an expansion with more than 200 repeats. This expansion is methylated by the cell and causes the gene to be inactivated. Individuals who are carriers of fragile X syndrome have a gene with 55–200 repeats; this repeat size range is referred to as the premutation range. An *FMR1* gene with a trinucleotide repeat size in the premutation range is unstable and, therefore, may expand to a full mutation as the X chromosome is passed on to the next generation. Carriers are at risk of having a child with fragile X syndrome. In rare cases an individual is a carrier of fragile X syndrome if there is a loss of an active *FMR1* gene because of a point mutation, translocation, or deletion.

Individuals with repeats in the intermediate range of 45–54, or repeats in the normal range of <45, are not considered to be carriers of fragile X syndrome. Both intermediate and premutation alleles can be unstably transmitted from parent to child. However, only premutation alleles are known to expand to full mutations in a single generation. An intermediate allele typically may change by only a few repeats in each generation and can eventually reach a premutation size. The majority of expansions occur during transmission through a carrier woman, and the risk of expansion to a full mutation increases as the size of the premutation increases.

Question 1. Is this an appropriately ordered test?

Laboratory Test Performed

The number of CGG repeats in Linda's *FMR1* genes was determined by amplification of the repeat region using the polymerase chain reaction (PCR), size fractionation

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of the PCR product by capillary gel electrophoresis, and fluorescence detection. The methylation status of her *FMR1* genes was determined by restriction enzyme digestion of extracted DNA using methylation sensitive enzymes and Southern blot analysis.

Question 2: After PCR analysis, how many repeat length results are expected?
Question 3: What are the limitations and advantages of PCR and Southern blot analyses?

Results with Interpretation Guideline

PCR analysis identified 33 CGG repeats, which is in the normal size range. By Southern blot analysis Linda had one unmethylated allele in the normal size range (Fig. 7.1, lane 5). For Southern blot analysis, DNA was digested simultaneously with the restriction enzymes *Eco*RI and the methylation sensitive *Eag*I. *Eag*I restriction sites are digested only when they are unmethylated. In Fig. 7.1, the normal methylated (inactive) sequences are seen as larger bands of approximately 5.2 kb, and the normal unmethylated (active) sequences are seen as smaller bands of approximately 2.8 kb.

Question 4. Are the PCR and Southern results consistent?

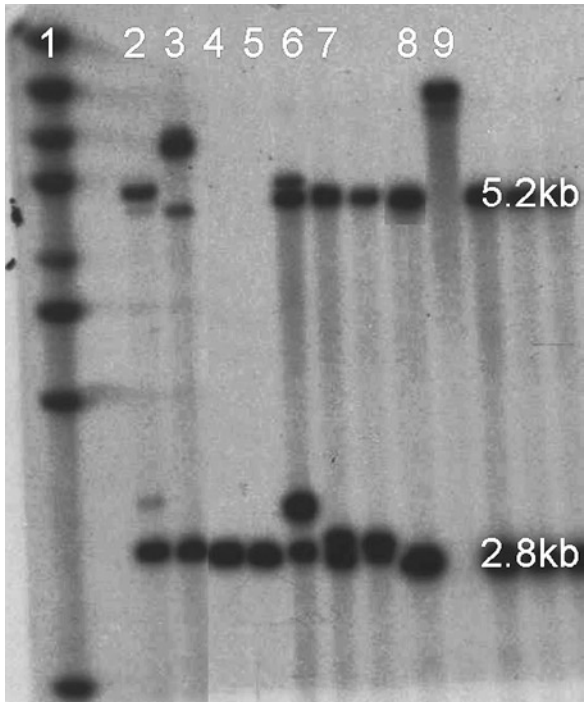

Fig. 7.1 Lane 1: Molecular size marker, Lane 2: Control (female with premutation), Lane 3: Control (female with full mutation), Lane 4: Male with normal result, Lane 5: Linda's result, Lane 6: Linda's fetus' result, Lane 7: Female with intermediate result, Lane 8: Female with normal result, Lane 9: Male with full mutation result

Result Interpretation

The results indicated that only one *FMR1* gene was present. This was unexpected, based on Linda's female sex and phenotype. The laboratory director questioned the accuracy of the result and initiated an internal investigation.

Question 5. What steps can be taken by the laboratory to investigate this apparent discrepancy?

After an internal investigation confirming that only one *FMR1* gene was present in the sample provided, the laboratory contacted the ordering physician, offering to test a new sample and recommending cytogenetic analysis. The physician sent a new sample from Linda for fragile X testing, which yielded identical results. Subsequently, the ordering physician called to say that, while Linda's karyotype was normal, by FISH analysis she had a small deletion on one X chromosome that encompassed the *FMR1* region.

Question 6. Is Linda a carrier of fragile X syndrome?

The interpretation of these results is that Linda is indeed a carrier of fragile X syndrome. There is a 50% chance (for each pregnancy) that the X chromosome with the deletion would be inherited by the next generation. No *FMR1* gene product would be made from the deleted gene. Based on Linda's fragile X status, Linda's physician sent an amniotic fluid sample for prenatal fragile X testing.

Question 7: Is amniotic fluid an appropriate specimen type for prenatal fragile X testing?
Question 8: Is any additional testing required to interpret a prenatal fragile X test result?

PCR analysis of the fetal sample demonstrated one CGG repeat of 33 and one of 110 (Fig. 7.2). No maternal cell contamination was present. Together with the fetal Southern blot result (Fig. 7.1, lane 6) this was consistent with a female fetus having inherited Linda's

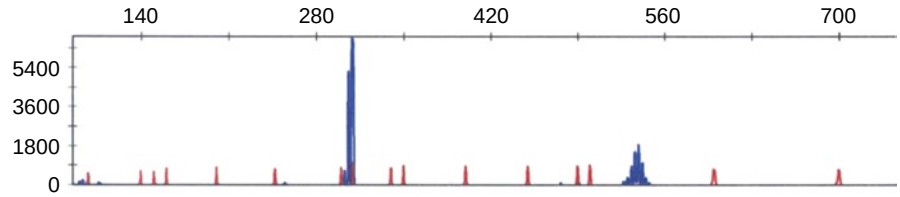

Fig. 7.2 In this electropherogram, the larger peak on the left corresponds to 33 CGG repeats, and the smaller peak on the right corresponds to 110 CGG repeats. The difference in peak height is the result of the analysis software flattening larger peaks on the instrument

normal allele of 33 CGG repeats, and having inherited a premutation allele of 110 CGG repeats, presumably from the father. The fetus being female was consistent with results of cytogenetic testing (normal female fetus, 46, XX). The fetus, therefore, was predicted not to be affected with classic fragile X syndrome.

Question 8: Should these results alleviate Linda's parental anxiety?

Further Testing

No further molecular testing was indicated for this patient.

Other Considerations

Genetic counseling was recommended to discuss the implications of these results. Linda's deletion carrier status implied that any future pregnancies were at risk for fragile X syndrome. Linda's first child was at risk of carrying a premutation. Other members of her and her husband's family were at risk of carrying a fragile X deletion mutation or a premutation, respectively. Consultation with a medical specialist was recommended to learn more about the medical implications of Linda's fetus' and her husband's premutation carrier status.

Background and Molecular Pathology

Classic symptoms of fragile X syndrome include cognitive and behavioral problems, facial dysmorphism, connective tissue anomalies, epilepsy, and macroorchidism (reviewed in [1]). Fragile X syndrome is the most common single-gene cause of autism-spectrum disorders. Female carriers of premutations (but not full mutations) are at risk of premature ovarian failure. Male premutation carriers, and more rarely, female premutation carriers, are at risk of an age-dependent, late-onset, progressive neurodegenerative disorder: fragile X-associated tremor/ataxia syndrome (FXTAS). Features of FXTAS include progressive cerebellar gait ataxia and intention tremor, cognitive changes, and psychiatric symptoms, including anxiety and depression [1]. Among affected males, earlier ages at onset of tremor or ataxia are correlated with larger premutation repeat sizes. In children, premutation carrier status may be associated with learning difficulties [1].

Fragile X syndrome is caused by transcriptional silencing of the *FMR1* gene, but the pathogenesis of the disease is not fully understood. Silencing can be caused by expansions and deletions of the CGG repeat sequence in the 5′-untranslated region of the *FMR1* gene, as well as by point mutations. Expansions are thought to account for at least 95% of mutations, but because routine clinical assays typically monitor only expansions, the relative frequency of the different mutation types is not yet known [2]. The risk of expansion to a full mutation increases as the size of the premutation increases, and is virtually certain if the repeat is larger than 100 [3].

Many psychiatric and neurological symptoms of fragile X syndrome may be the result of excessive activity of mGluR5, a metabotropic glutamate receptor. mGluR5 was shown to contribute significantly to the pathogenesis of the disease in mice [4], and a reduction of mGluR5 signaling in mice was shown to reverse some of the symptoms associated with the syndrome [5]. These findings have therapeutic implications for fragile X syndrome and autism. Several clinical trials are currently in progress to assess the safety and tolerance of mGluR5 antagonists (e.g., [6]).

Multiple Choice Questions

1. Which of the following does NOT cause fragile X syndrome?
   A. A deletion of the *FMR1* gene
   B. A methylated allele of 365 CGG repeats in the *FMR1* gene
   C. A methylated full expansion in the 5′-untranslated region of the *FMR1* gene
   D. A point mutation in the *FMR1* gene
   E. An unmethylated allele of 185 CGG repeats in the 5′-untranslated region of the *FMR1* gene
2. Which individual is at risk for FXTAS?
   A. A female with 54 CGG repeats
   B. A female with 374 CGG repeats
   C. A male with 54 CGG repeats
   D. A male with 156 CGG repeats
   E. A male with 450 CGG repeats
3. Which of the following is true of Southern blot analysis of the *FMR1* gene?
   A. Analysis is not labor-intensive
   B. A small amount of DNA is required
   C. Full expansion mutations are typically not detected
   D. Mosaic full mutations can typically be detected
   E. The number of CGG repeats can be accurately determined
4. Which of the following is NOT true of PCR analysis of the *FMR1* gene?
   A. Analysis is faster than Southern blot analysis
   B. A small amount of DNA sample is required
   C. Females with two CGG repeats of the same size in the normal range would have the same result as females with one CGG repeat and an undetectable full mutation
   D. The number of CGG repeats can be accurately determined
   E. The upper limit of size detection is typically in the full mutation range
5. Which of the following is NOT an appropriate clinical indication for fragile X testing?
   A. Carrier testing because of a family history of autism
   B. Carrier testing because of a family history of Turner syndrome
   C. Diagnostic testing because of developmental delay
   D. Diagnostic testing because of late-onset ataxia
   E. Diagnostic testing because of premature ovarian failure

Answers to Questions Embedded in the Text

Question 1. Is this an appropriately ordered test?

Parental anxiety alone has not been considered to be a sufficient basis from which to give informed consent for testing. Therefore, Linda's physician referred her to a genetic counselor to learn more about the genetics of fragile X syndrome. The counselor discussed the risks and benefits of being tested, in terms of Linda's own potential genetic results as well as the associated potential risks to her fetus, and also discussed the limitations of testing. After counseling, Linda continued to request testing. Population screening for fragile X syndrome was not recommended by the American College of Medical Genetics in 2005 [7], in part because of the complex implications of a positive test result. However, offering fragile X carrier screening to pregnant women or women considering pregnancy has become more prevalent [8, 9], and an increasing number of molecular diagnostic laboratories routinely provide fragile X carrier screening [10].

Question 2: After PCR analysis, how many repeat length results are expected?

Linda had a previous healthy child and did not have any features of Turner syndrome. Therefore, she was expected to have two X chromosomes. Because one *FMR1* gene is expected to be located on each X chromosome, two repeat sizes were expected. In practice, Linda could have two repeats of the same size or two different repeat sizes. She could also have more than two repeat sizes, although this is much less likely. Possible explanations for having more than two repeat sizes would include the presence of more than two X chromosomes, somatic mosaicism for the CGG repeat, a structural rearrangement of the X chromosome involving the *FMR1* gene, low-level chromosome mosaicism, or sample contamination. When more than two repeat sizes are identified, a laboratory would typically request a new sample for testing and/or recommend cytogenetic analysis.

Question 3: What are the limitations and advantages of PCR and Southern blot analyses?

The number of CGG repeats can be accurately determined by PCR analysis: the number is somewhat more accurate in the lower repeat ranges, and the upper limit of size detection by PCR is typically in

the premutation range. Accuracy and detection limits vary and should be validated by the laboratory. PCR analysis is faster than Southern analysis and requires a minimal amount of sample. Southern blot analysis permits identification of large premutations and full mutations as well as determination of the methylation status of the gene. Southern analysis is slower, more labor-intensive, and requires much more DNA than PCR analysis. Fragile X testing has been considered to be most accurate and reliable when both approaches are used in the laboratory. For a female with a full mutation undetectable by PCR analysis, the PCR result looks the same as for a female with two normal alleles of the same size. Southern blot analysis is essential for correct interpretation of this result. Similarly, there is a very small risk of missing a mosaic full mutation if fragile X screening is performed solely by PCR analysis.

Analytical limitations: Rare point mutations would be missed by both PCR and Southern analyses. Large deletions encompassing the whole *FMR1* gene could be missed by PCR analysis but are likely to be seen by Southern analysis. Genetic variants that interfere with an amplification primer could prevent amplification of the *FMR1* repeat region, causing allele drop-out and preventing analysis of that allele by PCR (e.g., [11]). Other sources of false positive or false negative results include blood transfusions, bone marrow transplantation, or laboratory error. The risk of laboratory error can be minimized by the use of assay controls, effective quality control systems, and independent confirmation of positive results.

Question 4. Are the PCR and Southern results consistent?

The results are consistent with each other, but are not consistent with Linda's sex or phenotype. These results would typically be seen in males who have one *FMR1* gene on their single X chromosome.

Question 5. What steps can be taken by the laboratory to investigate this apparent discrepancy?

Laboratories typically establish investigation protocols to address this type of apparent discrepancy. Some of the ways a laboratory can perform an investigation include: (1) Re-examining the original blood tubes to confirm that the client's label is correct and includes two unique identifiers (for example, name and date of birth), and that the client's label matches the laboratory's label. (2) Re-extracting DNA from all blood tubes received, to address the possibility that DNA had been extracted from a tube belonging to a different individual. (3) Obtaining and testing a new sample, to address the possibility that the sample received belonged to a different individual but was labeled with the patient's name before receipt by the laboratory. (4) Reviewing all documentation to address the possibility of a transcription error. When investigating a possible discrepancy it is as important to assess pre- and post-analytical processes as it is to assess analytical processes.

Question 6. Is Linda a carrier of fragile X syndrome?

Yes. Whether the mutation is a deletion or a methylated expansion, the gene product is absent [12].

Question 7: Is amniotic fluid an appropriate specimen type for prenatal fragile X testing?

Yes. The methylation status of the *FMR1* gene as well as the number of CGG repeats can be accurately and reliably determined using DNA from amniotic fluid. Sufficient DNA for PCR analysis can typically be obtained from amniocytes. However, cultured amniocytes are typically required to obtain sufficient DNA for Southern blot analysis. Culturing cells may add weeks to the testing protocol. The number of CGG repeats can also be accurately and reliably determined using DNA from chorionic villus sampling (CVS). However, methylation may not be complete in placental (CVS) tissue at 10–12 weeks gestation and may not reflect methylation status after birth. It can be difficult to distinguish large unmethylated premutations and small methylated full mutations in a CVS sample [8].

Question 8: Is any additional testing required to interpret a prenatal fragile X test result?

Maternal cell contamination studies are required to interpret a fetal result [13]. Knowledge of the fetal sex can be helpful when interpreting results, but is not typically required by laboratories. In Linda's case, in addition to the fragile X carrier test, her physician had ordered a fetal karyotype because of advanced maternal age.

Question 9: Should these results alleviate Linda's parental anxiety?

Linda is no longer anxious about whether her fetus has fragile X syndrome, but as a parent she has new questions to consider, among them: What are the implications for her new daughter of being a premutation

carrier? What is her first child's fragile X status? Should she test her 10-year-old now or should Linda wait until her daughter is old enough to choose testing herself? How will Linda talk to her family about these results?

Answers to Multiple Choice Questions

1. *The correct answer is E.*
2. *The correct answer is D.*
3. *The correct answer is D.*
4. *The correct answer is E.*
5. *The correct answer is B.*

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Recommended Reading

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Malignant Hyperthermia

8

Raju K. Pillai and Jeffrey A. Kant

Clinical Background

A 17-year-old Caucasian female was admitted for a minor surgical procedure under general anesthesia. Fifteen minutes into the procedure, the patient experienced an acute hypermetabolic episode manifested by hyperthermia (41.6°C), tachycardia (heart rate 250), and increasing end-tidal carbon dioxide ($ETCO_2$ 65 mmHg). The procedure was terminated. A provisional diagnosis of malignant hyperthermia (MH) was made, and the patient was treated with a loading bolus of 2.5 mg/kg intravenous dantrolene, an antidote to MH-triggering agents. Subsequent bolus doses of 1 mg/kg were administered intravenously until the signs of acute MH abated and the $ETCO_2$ normalized. The patient had an unconfirmed family history of a similar episode in a maternal uncle.

Question 1: What is your differential diagnosis?

The differential diagnosis of conditions manifesting with clinical features similar to MH includes, but is not limited to, hypoventilation, sepsis, hypoxic encephalopathy, meningitis, intracranial bleed, thyrotoxicosis, pheochromocytoma, brain injury, neuroleptic malignant syndrome, serotonin syndrome, contrast-induced neurotoxicity, anticholinergic syndrome, cocaine toxicity, amphetamine toxicity, sympathomimetic toxicity, drug/alcohol withdrawal, lethal catatonia, salicylate toxicity, heatstroke, absorption of CO_2 during laparoscopy, and extrapyramidal syndrome.

Reason for Molecular Testing

A sample was submitted for genetic testing to evaluate possible malignant hyperthermia susceptibility (MHS).

Test Ordered

The test ordered was *RYR1* gene-targeted sequence analysis.

Question 2: Is this an appropriately ordered test?

Genetic linkage studies estimate that more than 50% of MHS cases are associated with the *RYR1* gene on the long arm of chromosome 19 (19q13.1) [1, 2]. Susceptibility to MH has also been associated with five other loci – the DHP receptor on 17q11.2-q24 (MHS2), the alpha-2/gamma subunit of the DHP receptor on 7q21-q22 (MHS3), a locus on 3q13.2 (MHS4), the alpha-1 subunit of the DHP receptor (MHS5), and a locus on 5p (MHS6).

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Laboratory Test Performed

Genomic DNA from this individual was used for PCR amplification of 18 exons (2, 6, 9, 11, 12, 14, 15, 16, 17, 39, 40, 44, 45, 46, 95, 100, 101, and 102) in the three regions of the *RYR1* gene which, at the time of assay, had known disease-causing mutations, defined according to criteria of the European Malignant Hyperthermia Group (EMHG) [3]. The regions analyzed included the full coding sequence of each exon as well as exon–intron boundaries, and varying amounts of adjacent intron sequence, not less than 25 nucleotides and as much as >200 nucleotides. Direct sequence analysis of *RYR1* PCR amplification products was performed in forward and reverse directions with an automated fluorescence dideoxy sequencing method using dye-terminator nucleotide labels (Sanger analysis). The data were analyzed by at least two independent observers, using both a software analysis program (Mutation Surveyor, v3.24) as well as visual inspection.

Question 3: What are the limitations and advantages of this approach?

The assay does not detect potential disease-causing nucleotide changes in unsequenced exons of the *RYR1* gene, the *RYR1* gene promoter, deep intronic or extended 3′-untranslated regions of this gene, or in other genes that may cause malignant hyperthermia. Sequencing also will not detect larger scale partial (e.g., whole exon) or full gene deletion mutations.

Results with Interpretation Guideline

During data analysis, the reviewer examines sequence data on paper printouts or on a computer monitor to verify the presence of (a) minimal or no background signal and (b) clearcut peaks and strong signals for each of the four dideoxynucleotides. Satisfactory sequence should be observed over the full length of the region of interest sequenced. Areas within the sequence which demonstrate "broad" signal peaks of one dideoxynucleotide color are thought to represent "dye blobs"; the normal sequence can typically be "read" below the dye blob. Heterozygous nucleotide substitutions will typically result in a "non-call" ("N") by base-calling software used with primary sequence tracings. Such a non-call results from the overlap of different signals from two nucleotides. Occasionally, base-calling software will not make this call although a heterozygous change is obvious from visual inspection. However, in the laboratory where our patient was tested, the experience is that Mutation Surveyor will almost always detect such changes even if missed by the other base-calling software.

Sequence variants (heterozygous or homozygous) are compared with known databases (e.g., dbSNP, locus-specific databases; see reference [4]) and laboratory experience to make a preliminary judgment whether they are likely to be benign polymorphisms, pathogenic mutations, or variants of uncertain significance (VUS). One potentially pathogenic sequence change was identified, and confirmed in the opposite direction in this patient's sample (Fig. 8.1). A straightforward interpretation of this result might be: "A heterozygous previously unreported DNA sequence variant of unknown significance (c.6343G > A, p.Glu2115Lys) was identified in exon 39 of the *RYR1* gene." This is a category 3 variant according to American College of Medical Genetics (ACMG) criteria. Formally, missense variants of any type that have not been previously reported are classified as category 3 nucleotide sequence variations according to recommended ACMG criteria [4], i.e., "may or may not cause disease." Category 3 variants are sometimes referred to as variants of uncertain significance (VUS), and conservative interpretation is recommended. Additional approaches to further evaluate such changes for possible pathogenicity are discussed under "Other considerations."

In addition, comparison to the reference sequence revealed three individual nucleotide sequence changes of varying frequency in control populations that were also identified bidirectionally and interpreted as benign polymorphisms. These included:

1. Heterozygous c.1668A > G (p.Ser556Ser), exon 15, rs2288888
2. Heterozygous c.1672 + 29C > G, intron 15, rs2288889
3. Heterozygous c.14646 + 113C > T, intron 101, rs7254175

Note: an "rs" number, sometimes known as a reference SNP ID, is a cataloging number assigned by dbSNP to uniquely identify a particular reported nucleotide change, often accompanied by population frequency data.

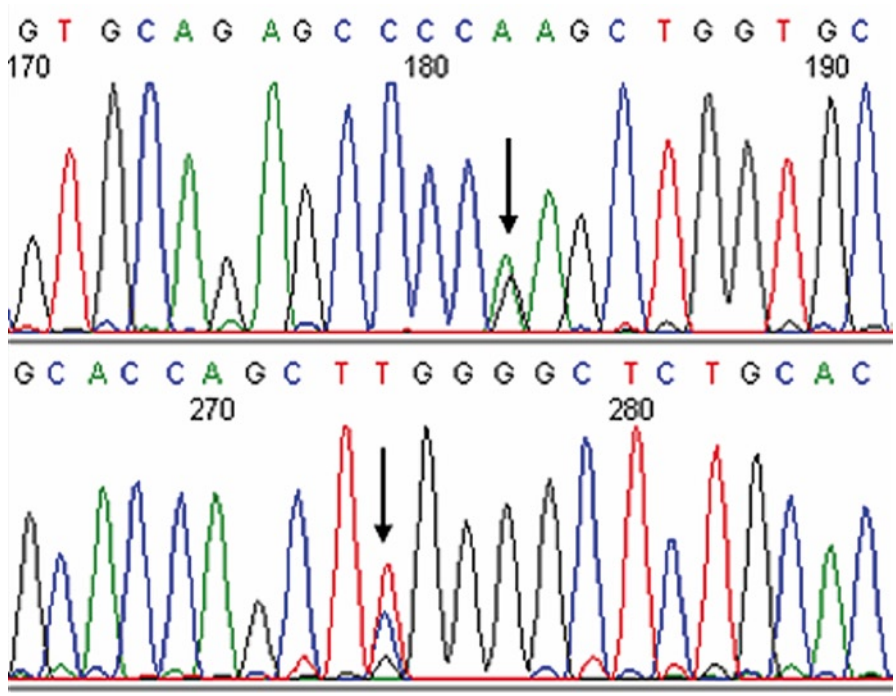

Fig. 8.1 Exon 39: region of concern. The forward (*top*) and reverse (*bottom*) sequencing reactions are shown. Note the heterozygous variant (A/G in forward and T/C in reverse) at the identical nucleotide position. Although visually obvious, the sequence base-calling software does not indicate a "non-call" at either position. Mutation Surveyor software identified the heterozygous variant in both directions

Result Interpretation

Question 4: Is this sequence change pathogenic?

VUS are, not surprisingly, likely to be frustrating for both clinician and patient, and it is important in pre-test counseling and acquisition of informed consent that the patient and/or family understand the possibility that such a result may be obtained. For this reason, the clinician may wish to involve a genetic counselor or medical geneticist in the pre-test evaluation and in post-test disclosure meetings with the patient.

In the case of MH, a "gold standard" functional assay for MHS, the caffeine-halothane contracture test (CHCT) is available to independently assess MHS status. Because the CHCT involves travel to a biopsy center, invasive direct muscle biopsy, and a cost that is 10–15 times as high as genetic analysis, it is not uncommon for patients to first pursue genetic testing.

Taking into account such factors, a more extended interpretation for this patient might read as follows:

"Whereas this nucleotide change leads to a non-conservative amino substitution which also demonstrates evolutionary conservation across a range of species from human to zebrafish, it is predicted to likely not affect protein function using two research-based calculation models, SIFT and Polyphen. Moreover, the substitution score using a BLOSUM 62 matrix is neither negative nor positive. Hence, it should still be regarded as of uncertain significance. Of interest, a single example of substitution to asparagine has also been demonstrated at this position in one other pedigree evaluated for MHS in our laboratory but not in over 100 controls. Genetic testing to assess segregation of the p.Glu2115Lys variant in MHS-affected (if any) or unaffected relatives might be of value. More definitive functional contracture testing to assess MHS status as well as the possible pathogenic significance of this variant should be considered if clinically warranted."

Further Testing

Further genetic testing of other affected members in a family may help to understand the significance of previously unreported sequence variants. In the case of MHS, it is the exception for samples submitted for analysis to come from such pedigrees. Failure to co-segregate suggests that the suspect VUS is not likely to cause disease, although co-segregation data must be

interpreted with caution because variants which are not disease-associated have a one in two probability of "chance" passage to the next generation.

MHS can typically be identified in patients using the CHCT which involves a muscle biopsy followed by in vitro testing at a series of different caffeine and halothane concentrations. All diagnostic centers in North America follow the protocol for in vitro testing published by the North American Malignant Hyperthermia Group [5]; a slightly different protocol using halothane only is performed in European centers. The halothane and caffeine contracture tests are done in triplicate. An abnormal response in any muscle strip is considered diagnostic for MHS. An abnormal muscle contracture response is defined as one of the following:

(a) Halothane contracture test: 0.2–0.7 g contracture after exposure to 3% halothane for as long as 10 min
(b) Caffeine contracture test: ≥0.2 g contracture at 2 mM caffeine, or a >7% increase in tension compared with maximal tension generated at 32 mM caffeine

Contracture testing has a high sensitivity (>95%), thus negative results generally rule out a diagnosis of MHS [6]. A few individuals with known causative MH mutations have a negative CHCT, and anesthetic management should consider the clinical features that prompted testing as well as genetic testing results. False positive results have been reported in up to 22% of patients [7].

Other Considerations

As widespread DNA sequencing becomes available, even to the point of whole genome sequencing, interpretation of VUS will constitute a significant challenge for laboratorians [8]. The first question to be addressed is whether the variant, in fact, has been previously reported as a polymorphism or a pathogenic mutation. The largest repository of single nucleotide polymorphisms is dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/), which is hosted by the National Center of Biotechnology Information. The HapMap project is a multi-country effort to identify and catalog genetic similarities and differences in human beings (http://hapmap.ncbi.nlm.nih.gov/). In the initial phase of the HapMap project, genetic data are being gathered from four populations with African, Asian, and European ancestry, and the data will be available in dbSNP. These two databases are very helpful to assess whether a variant has been previously reported and, when available, its frequency in other ethnic groups.

Many Internet databases have been constructed to catalog known mutations affecting specific genes. The Cardiff Human Gene Mutation Database (www.hgmd.cf.ac.uk) and Online Mendelian Inheritance in Man (OMIM) (www.ncbi.nlm.nih.gov) are central mutation databases. Locus-specific databases such as the European Malignant Hyperthermia Group (EMHG) database on *RYR1* mutations concentrate on specific genes or diseases. The EMHG database currently lists 30 causative *RYR1* mutations (and 74 non-pathogenic variants) as of June 2010 (http://www.emhg.org/nc/genetics/mutations-in-ryr1/). The listing of variants as mutations in such databases and their subsequent withdrawal upon further study is not unheard of, so evaluation of the supporting data and interpretative caution is prudent.

In general, it is desirable to demonstrate a "disease-causing" variant in two or more independent families, and segregation data add further credibility.

Variants that have not been documented in the literature can also be assessed using a range of phylogenetic, biophysical, and structural techniques. If an amino acid residue in a peptide sequence is conserved among a wide range of evolutionarily diverse species, it is more likely to be functionally important. The NCBI Homologene tool (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=homologene) can be used to examine sequence alignments of the same gene from different species. The BLOSUM (Blocks of Amino Acid Substitution Matrix) matrices are based on local alignments of amino acid sequences. A log-odds score is calculated for each of the possible substitutions of the standard 20 amino acids and provides an estimate of their substitution probabilities.

The significance of a variant depends on the protein domain and its role in protein function. Differences in biophysical properties of substituting amino acids can be assessed by the Grantham score, which is derived from volume, polarity, and side chain composition of the amino acid [9]. Grantham variation (GV) is a quantitative measure of the observed range of variation at a position in a multiple sequence protein alignment, and Grantham Deviation (GD) is a quantitative measure of the distance between a missense substitution and the range of variation observed at its position in the alignment. These parameters have been used to calculate

the probability that a variant is deleterious [10]. Computational methods such as SIFT (Sorting Intolerant from Tolerant) and PolyPhen use domain information, evolutionary conservation, and biophysical properties to predict the impact of a variant. For this patient, both these software tools predicted the possible impact of the p.Glu2115Lys variant as benign. For previously unidentified amino acid substitution variants where all tools give a consistent indication, it is probably reasonable to indicate that the variant is likely to be a disease-associated variant even though further family studies or functional testing (if a functional test is readily available – which it typically is not) would be more definitive. Research groups which have devoted many years to studying a protein may be a valuable source of perspective on a VUS, but reference to any such conversations should be qualified with an appropriate disclaimer that such information should be regarded as investigational.

Future developments in protein modeling and molecular dynamics simulation will provide more insight into determining the functional effects of mutations in coding regions. Several groups have explored the use of protein modeling techniques, especially homology modeling, to understand the impact of individual amino acid substitutions [11]. For proteins with published crystal structures, homology modeling attempts to map residues in the query sequence to residues in the template sequence and to predict the structural alteration at the atomic level.

Background and Molecular Pathology

MHS is an autosomal dominant pharmacogenetic disorder triggered by administration of commonly used halogenated volatile anesthetics (halothane, enflurane, isoflurane, desflurane, and sevoflurane) or the depolarizing muscle relaxant succinylcholine.

Muscle cells utilize the transverse tubule system to propagate depolarization signals. Dihydropyridine (DHP) receptors are voltage-dependent calcium channels located within the transverse tubule membrane, which triggers the ryanodine receptors (RYR1) embedded in the sarcoplasmic reticulum to release calcium into the intracellular space, resulting in muscle contraction. Mutations in RYR1 or DHP receptors trigger an unregulated intracellular calcium flux and sustained muscle contraction, which leads to the clinical manifestations of MH. The exact mechanisms by which halogenated anesthetics trigger these calcium channels are not well understood. Dantrolene, which is used to treat episodes of malignant hyperthermia, binds to the RYR1 receptors to inhibit the release of calcium.

Multiple Choice Questions

1. The inheritance of malignant hyperthermia susceptibility (MHS) is best described as:
   A. Autosomal dominant
   B. Autosomal recessive
   C. Mitochondrial
   D. Multifactorial
   E. Sex-linked
2. Which type of pathogenic nucleotide sequence variant is least likely to be detected by bidirectional sequencing as described for this case?
   A. Consensus splice donor/acceptor variant
   B. Cryptic splice site variant
   C. Frameshift variant
   D. Missense variant
   E. Nonsense variant
3. Which of the following features in a genetic testing report from a sequence-based assay would likely be of least use to a clinician, genetic counselor (or patient)?
   A. Description of the methodology including which region(s) of the gene (e.g., specific exons/introns) were analyzed
   B. Description of variants identified using Human Genome Variation Society (HGVS)-recommended nomenclature
   C. Information on the analytic as well as clinical sensitivity of the assay to identify a pathogenic mutation
   D. The reference sequence used for interpretation of sequence data
   E. Variants with a significant frequency in the general population
4. Which feature is most likely to provide definitive interpretation of a substitution variant of uncertain significance (VUS)?
   A. Evolutionary conservation
   B. Grantham score
   C. Homology modeling
   D. Polyphen prediction

E. Segregation in family studies

5. Given the potential to find a variant of uncertain significance (VUS) in DNA sequence-based assays, which of the following is likely to be of greatest initial importance in the management of an individual undergoing genetic testing for MHS?
   A. Caffeine-halothane contracture testing
   B. Detailed family history
   C. Informed consent for genetic testing
   D. Serum creatine kinase level
   E. Thyroid function studies

Answers to Multiple Choice Questions

1. *The correct answer is A.*

Inheritance is best characterized as autosomal dominant because the major genetic locus associated with MHS, the *RYR1* gene, is located on chromosome 19 and a single *RYR1* mutant allele is sufficient to cause disease. This is of importance to genetic counseling because children will have a one in two (50%) risk of MHS. Expressivity and penetrance of MHS mutations is somewhat more complex in that a malignant hyperthermia episode may or may not occur upon exposure to a triggering agent. Thus, it is important that a genetics professional or a healthcare professional experienced with MHS speak with the patient (and perhaps other family members) at the appointment where genetic testing results are disclosed.

2. *The correct answer is B.*

Sequence-based assays almost always examine the full coding sequence of an exon and the sequence of adjacent exon–intron boundary areas to include splice donor and acceptor regions. Moreover, these regions typically are sampled bi-directionally by sequencing both DNA strands from opposite directions. Such an approach should detect all missense, nonsense, frameshift, splice donor/acceptor, and other intronic variants. Sometimes, particularly for very large genes such as *RYR1*, only "hotspot" exons will be sequenced. Other exons, sequences deep within introns which could potentially develop a variant that leads to unexpected (cryptic) splicing, and usually 5′-promoter or 3′-untranslated sequences are not interrogated. Larger scale duplications or deletions of portions of the gene (e.g., an exon) are also not typically detected in sequence-based assays. Splice donor/acceptor mutations, while described, are very rare in *RYR1*; the vast majority of changes reported to date are missense variants.

3. *The correct answer is E.*

The sensitivity of the assay to identify an affected patient, whether the "full gene" has been tested, and if not the whole gene then which regions have been examined, are invariably of interest. A description of variants which are unknown or interpreted as pathogenic using standardized nomenclature is crucial for comparison with other published references in the literature or mutation databases. For this reason, inclusion of the reference DNA and/or cDNA sequence number used for interpretation is helpful. Sequence variants which occur at significant frequency in the general population are almost certainly benign polymorphisms of no functional consequence to the patient. Some laboratories do not include such variants in reports to avoid any possibility of "confusing" the clinician or patient.

4. *The correct answer is E.*

Failure to co-segregate in other affected individuals of the same family would suggest that a variant is not likely to be related to the phenotype, although reduced or incomplete penetrance must always be kept in mind because it is known that some patients with pathogenic variants do not develop an MH episode on initial exposure to triggering agents. Conversely, segregation of a variant with disease (and/or the absence of the variant in normal individuals) progressively increases the likelihood that the variant is disease-associated. In a sense, this is a variation on the answer and discussion of the previous question. Unfortunately, many individuals presenting for testing do not have relatives with well-documented MHS episodes.

5. *The correct answer is C.*

Informed consent obtained from the patient is very important for DNA-sequence based assays. This includes general discussion of the likelihood of a positive or negative result given the clinical circumstances and family history as well as the possibility of an indeterminate result due to identification of a previously unknown or incompletely understood nucleotide sequence variant. Consent should also include discussion of potential benefits and limitations (as well as costs) of genetic versus contracture testing, and the likely recommendation to proceed to contracture testing if a negative or

VUS result is reported. Some investigators advocate contracture testing even if a known pathogenic *RYR1* gene mutation is found. Additional items of importance include: the possibility that testing of individuals in the family may be recommended and desirable and the availability (and cost) of follow-up full gene sequence analysis for exons not tested in the "screening" assay. Because of ease, some states require the laboratory to collect informed consent (or evidence of such). However, informed consent should be seen as a primary role of the referring clinician assisted by the laboratory, because it must include information about clinical and social implications of a disorder as well as the genetic test. Indeed, "fill-in-the-blank" test consent forms which lack this information are not considered, by some, to be "informed."

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Rh Incompatibility

9

Daniel B. Bellissimo

Clinical Background

A 25-year-old African American RhD-negative woman presented in the 15th week of her first pregnancy. Her spouse was known to be RhD-positive. Her antibody screen was negative at 15 weeks and remained negative during repeat screens at monthly intervals. The patient was treated with Rh immune globulin (RhIG) at 28 weeks. She went into labor at 40 weeks. The infant was RhD-positive and RhIG was administered to the mother. One year later, the patient became pregnant again and anti-D was detected at a titer of 1:8 at 15 weeks. The titer increased to 1:64 at 18 weeks. The pregnancy was followed using Doppler measurements of the peak velocity of systolic blood flow in the middle cerebral artery (MCA) every one to two weeks starting at 24 weeks. The peak MCA velocity was elevated at >1.5 MOM (multiples of the mean) at 30 weeks. Cordocentesis was performed. Testing indicated a fetal hematocrit of 23%, elevated reticulocytes, hyperbilirubinemia and an RhD-positive phenotype with a 3+ direct anti-globulin test (DAT) result. The DAT result, also known as the direct Coombs test, indicated that the fetal red cells were coated with maternal alloantibodies. The fetus was treated with intrauterine blood transfusion. At 36 weeks gestation, the fetus was delivered and was treated with exchange transfusion and phototherapy. In the following year, the patient became pregnant for the third time.

Question 1: What is the differential diagnosis?

This case illustrates a typical presentation of hemolytic disease of the fetus and newborn (HDFN). The RhD-negative mother is alloimmunized by exposure to fetal RhD-positive red cells. In subsequent pregnancies, maternal anti-D antibodies cross the placenta into the fetal circulation. The antibodies may lead to the destruction of the red blood cells in an antigen-positive fetus, leading to hemolytic disease.

Question 2: How could molecular testing have been used to help manage this case?

Reason for Molecular Testing

Molecular testing for paternal zygosity and prenatal testing of the fetus plays an important role in the proposed algorithms for the management of HDFN [1]. The goal is to minimize invasive procedures in these patients because additional exposure to fetal red cells can cause further sensitization. Paternal zygosity is used to predict the risk of HDFN in each pregnancy. If the paternal sample is homozygous for the *RHD* gene (*RHD/D*), then the fetus is predicted to be RhD-positive and the fetus can be appropriately monitored and invasive procedures may be avoided. In the case of a homozygous father, prenatal testing of the fetus may still be indicated if nonpaternity is a possibility. If the paternal sample is heterozygous (*RHD/d*) for the *RHD* gene, fetal DNA testing through amniocentesis,

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chorionic villus sampling (CVS) or the testing of free fetal DNA in maternal plasma can be used to determine whether the fetus is *RHD*-positive or *RHD*-negative. If the father is *RHD*-negative then the fetus is not at risk for HDFN related to anti-D.

Zygosity determination is straightforward by serological or molecular methods in most biallelic antigen systems except for *RHD*, in which zygosity must be determined by gene copy number. *RHD* zygosity can be predicted by the Rh phenotype and haplotype frequencies. However, the predictions are not reliable in some ethnic groups, especially African Americans. *RHD* zygosity is most accurately determined using molecular methods.

Genotyping assays for red cell blood group antigens can help identify variant alleles that may or may not be identified in the immunohematology laboratory or blood bank by their usual phenotypic characteristics. It must be recognized that some individuals will be typed as RhD-negative by serology yet may have a weak or partial D that is not detected by the antibody reagent being used. Molecular testing is useful in detecting these variants. In addition, molecular assays are useful in predicting blood group phenotype in the transfused patient [2].

Test Ordered

The maternal–fetal medicine physician ordered *RHD* genotyping on direct amniotic fluid at 18 weeks gestation.

Question 3: Was the appropriate test ordered? Were all the necessary samples provided?

Although testing on direct amniotic fluid can be performed, a backup culture is recommended in case the laboratory needs additional sample or if the direct fluid is contaminated with maternal cells. In conjunction with molecular analysis of a prenatal sample, a molecular assay for maternal cell contamination should be performed.

Testing of parental samples should also be performed given the genetic variants in the Rh system. This is especially relevant in this case because the couple is African American and Rh variants are more common in this ethnic group. In order to reduce the risk of false-negative and false-positive results, the parental samples should be tested by serology and genotyping to identify any possible discrepancies caused by variant alleles. Variants will be easily identified in RhD-negative mothers who test positive for the *RHD* gene. Allelic variants can be masked in paternal samples by a "normal" *RHD* allele. *RHD* zygosity testing should be ordered on the paternal sample to predict the risk of HDFN for this fetus.

The fetal *RHD* genotype can also be determined from the fetal DNA present in maternal plasma [3]. Fetal DNA accounts for approximately 3% of the total cell-free DNA in maternal plasma during the second trimester and increases throughout pregnancy. This method avoids invasive techniques that cause risk to the fetus and prevents further sensitization of the mothers. Laboratories outside the United States have successfully performed these assays for almost 10 years. However, due to intellectual property issues, the maternal plasma assays have only recently become available in the United States, and their sensitivity and specificity have not yet been thoroughly evaluated. Caution is warranted with this potentially exciting technology. The fetal DNA represents a minority of the total DNA in plasma, so it is important to have an internal control to demonstrate that fetal DNA is present and detectable. Y chromosome-specific sequences can be used when the fetus is male but other paternal-specific polymorphisms, dose- or fetal-specific markers may be required to provide the necessary internal control.

Laboratory Test Performed

RHD genotyping was performed using a laboratory-developed allele-specific PCR-based method. Testing of two or more regions of the *RHD* gene is required in order to recognize allelic variants. A variety of assays have been described that take advantage of the various sequence differences in exons 3–7 and 9, introns 4 and 7 and the 3′ UTR of exon 10 [4, 5]. The assay used in this case targeted exon 4, intron 4, exon 7 and intron 7 and the p.Trp16X mutation. These targets have a high sensitivity and specificity for *RHD*-positive genes and specifically detect the 37 bp insertion found in exon 4 of RHD_{ψ} [6]. Detection of the pseudogene is important to avoid false-positive results. The amplification products are analyzed on a 2% agarose gel.

A laboratory-developed assay was used for zygosity determination. Quantitative fluorescent PCR (QF-PCR) was used to detect *RHD* exons 5 and 7,

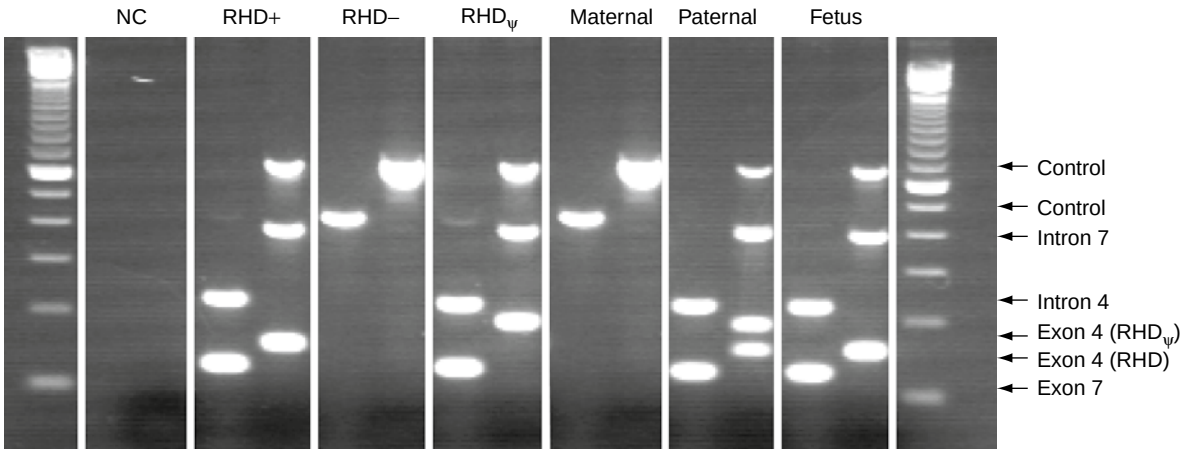

Fig. 9.1 RHD genotyping assay. There are two reactions per sample. The first PCR reaction detects exon 7 and intron 4. The second reaction detects exon 4 and intron 7. If the *RHD*$_{\psi}$ is present, the size of the exon 4 PCR product is increased by 37 bp. Each PCR reaction also contains primers to a control gene product. The control products for reactions 1 and 2 are of different sizes. The PCR products are identified by the *arrows*

using *RHCE* exon 7 as an internal two copy control. The use of exons 5 and 7 improves the detection of common Rh variants in Caucasians and African Americans. Variants are recognized when the *RHD* exon 5 copy number is discordant with exon 7. The exon 5 primers are specific for the *RHD* gene; *RHD*$_{\psi}$ is not detected. The exon 7 primers detect both the *RHD* and *RHD*$_{\psi}$ genes. The fluorescent PCR products were analyzed by capillary electrophoresis. The genotype and zygosity were determined from the peak area ratios of *RHD* exon 5 or 7 to *RHCE* exon 7. The precise site of the *RHD* deletion has been defined making it possible to detect the deletion with a PCR-based assay [7]. Although this is the most common RhD-negative allele in Caucasians and African Americans, this assay will not correctly determine zygosity in the presence of some *RHD* variant alleles. Real-time PCR assays can potentially be used for determining zygosity, as long as as more than one region of the *RHD* gene is detected.

Results with Interpretation Guideline

The agarose electrophoresis of the *RHD* multiplex PCR reactions for the maternal, paternal and fetus samples includes four controls: a negative control (NC), and *RHD*-positive, *RHD*-negative and *RHD*$_{\psi}$ controls (Fig. 9.1). There are two reactions per sample. The first PCR reaction detects exon 7 and intron 4. The second reaction detects exon 4 and intron 7. If the *RHD*$_{\psi}$ is present, the size of the exon 4 PCR product is increased by 37 bp. Each PCR reaction also contains primers to a control gene product. If the sample is *RHD*-negative, the control PCR product rules out a failed PCR reaction.

Figure 9.2 displays the results of *RHD* zygosity testing. After PCR and capillary electrophoresis, the peak ratios of exon 5 and exon 7 to exon 7 of the *RHCE* gene (the two copy control) are calculated. The mean ratio ±3 SD is indicated for the heterozygous (D/d) and homozygous (D/D) genotype.

Question 4: In RHD zygosity analysis of the paternal sample, the exon 5 ratio indicates that the sample is heterozygous but the exon 7 ratio indicates that the sample is homozygous. Is this consistent with the results observed in the RHD genotyping assay?

Result Interpretation

In the maternal sample, only the control PCR products were detected, similar to the *RHD*-negative control and consistent with her RhD-negative phenotype. A deletion of the *RHD* gene is the most common *RHD*-negative allele. It is important to genotype the maternal sample because there are RhD-negative variants that may genotype as *RHD*-positive (Fig. 9.3). Clearly, this would affect the interpretation of the prenatal sample.

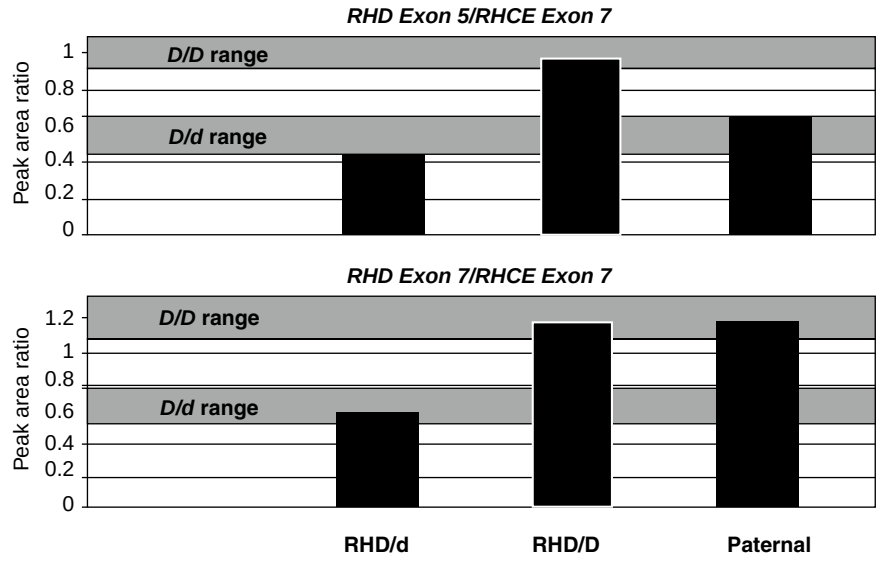

Fig. 9.2 RHD zygosity determination. Quantitative fluorescent PCR was used to detect *RHD* exons 5 and 7 using *RHCE* exon 7 as a two copy internal control. The genotype and zygosity were determined from the peak area ratios of *RHD* exons 5 and 7 each to *RHCE* exon 7. The paternal sample appears discrepant between exon 5 (one copy) and exon 7 (two copies)

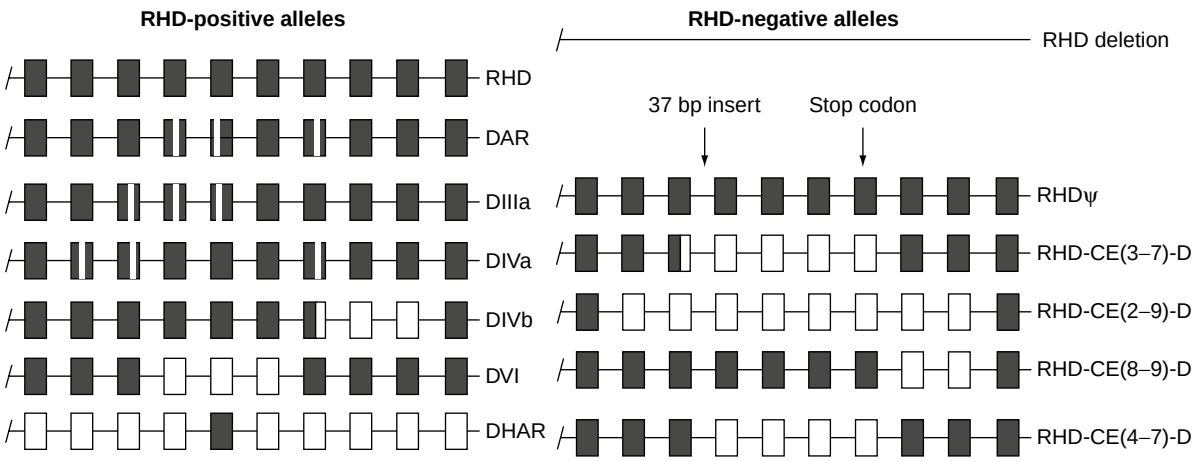

Fig. 9.3 RHD-positive and RHD-negative variant alleles. The listing of the most common *RHD* variant alleles illustrates why *RHD* genotyping assays require multiple targets within the *RHD* gene. Most variants are *RHD/RHCE* hybrid genes. *RHD* exons are indicated by the square boxes. *Black boxes* indicate *RHD*-specific sequences and white *boxes* indicate *RHCE*-specific sequences. The RHD_{ψ} and *RHD-CE*(3–7)-*D* are frequently seen in the African American population and may lead to false-positive results if not specifically targeted and detected

The paternal sample tested positive for all of the *RHD*-specific targets indicating the sample was *RHD*-positive. It is important to note that the sample had two exon 4 products. This indicates that the paternal sample had one *RHD* gene and one *RHD* pseudogene (RHD_{ψ}). The RHD_{ψ} gene is an RhD-negative allele that is not expressed. In *RHD* zygosity analysis of the paternal sample, the exon 5 ratio indicated that the sample was heterozygous, but the exon 7 ratio indicated that the sample was homozygous (Fig. 9.2). This was consistent with the genotyping result. The exon 5 product is specific for the *RHD* gene and one copy was detected. The exon 7 product detects both *RHD* and RHD_{ψ}, so two copies were detected. Both the genotyping and zygosity assays are consistent with the paternal sample being heterozygous for a functional *RHD* gene. In this case, the paternal zygosity could have been determined without a separate zygosity test. The chance that offspring from this father will be *RHD*-positive is 50%. If the pseudogene was not specifically detected, it would appear that the paternal sample was homozygous, and an

incorrect prediction would be made that all fetuses would be *RHD*-positive. In addition, such a homozygous genotype would make the couple ineligible for preimplantation genetic diagnosis.

The fetus tested positive for all of the *RHD*-specific targets, indicating that the sample was *RHD*-positive. This fetus is at risk for hemolytic disease of the newborn related to anti-D. When maternal and paternal samples are not available, interpretation should include the possibility of a false-positive or false-negative result. The fetus will be monitored by MCA Doppler throughout the pregnancy, following the algorithms described in the background section.

Further Testing

No further testing was indicated.

Background and Molecular Pathology

HDFN is caused by alloimmunization of the mother by exposure to fetal red cells that display a paternally inherited form of an antigen that is different from those in the mother. HDFN can occur from fetomaternal incompatibilities in a number of different red cell antigen systems including the RhD, C/c, E/e, Kell, Kidd, Duffy and M antigen systems. Fetomaternal hemorrhage is the most common cause for sensitization. Unrecognized miscarriage, transfusion, amniocentesis, chorionic villus sampling and cordocentesis also increase the risk of alloimmunization. In subsequent pregnancies, maternal anti-D antibodies cross the placenta into the fetal circulation and may cause the destruction of red blood cells of an antigen-positive fetus, which results in hemolytic disease. The severity of HDFN is variable; mild cases either require no treatment or phototherapy, whereas more severe cases may result in fetal hydrops and require exchange transfusion at birth or possibly intrauterine transfusion.

Anti-D accounts for the majority of HDFN cases followed by anti-K, anti-c and anti-E [8]. The routine use of RhIG prophylaxis has decreased the incidence of Rh immunization. Rh sensitization affects 6.8 newborns per 1,000 live births [9]. The frequency of Rh-negative individuals is 15% in Caucasians, 5% in African Americans, 8% in Hispanics, and low in Inuit, Native Americans, Japanese and other Asians.

Algorithms for managing a first sensitized pregnancy and patients with a previously affected fetus or newborn have been described [1]. Maternal antibody titers, ultrasound, MCA Doppler, cordocentesis and molecular testing are used to monitor these pregnancies. MCA Doppler has a sensitivity of 88% and specificity of 82% for the detection of severe hemolytic disease [10]. MCA Doppler has largely replaced the use of amniotic fluid ΔOD^{450} which required serial amniocentesis and put these patients at risk for additional alloimmunization.

Rh antigens are expressed on proteins from two highly homologous genes, *RHD* and *RHCE*, on chromosome 1p34.3–36.1 [11]. The *RHD* and *RHCE* genes are tandemly arranged and likely arose through duplication of a single ancestral gene [12]. The D antigen is expressed from *RHD* and the C/c and E/e antigens are expressed from *RHCE*. The alleles are inherited as a haplotype in eight possible combinations: DCe, dce, DcE, Dce, dCe, dcE, DCE and dCE (in order of frequency in Caucasians). The *RH* genes are more than 95% homologous at the nucleotide sequence level and both consist of 10 exons spanning more than 75 kb [11]. Sequence variations can be used to distinguish these two highly similar genes. However, it is important to be aware of the variant alleles that exist in this genetic system as a result of gene conversion events between the *RHD* and *RHCE* genes and as a consequence of point mutations. A database (dbRBC) of these variants is available at the NCBI website (www.ncbi.nlm.nih.gov/projects/gv/rbc). The hybrid *RHD-CE-D* alleles may result in either RhD-positive or RhD-negative haplotypes. Most variant RhD-positive alleles encode proteins that do not express all the RhD epitopes and are referred to as partial D antigens. It is possible for an RhD-negative mother to be alloimmunized by a partial D antigen but hemolytic disease in these cases is rare. In addition, a mother with a partial D can be alloimmunized by a normal D antigen. The frequency of these variant alleles is low in the Caucasian population, but in some ethnic groups, these alleles can be common [4, 11]. The DAR allele is found in 17% of South African blacks and $D^{III a}$ has been found in 18% of African Americans in New York and 28% of black individuals from Brazil.

The most common RhD-negative allele results from the deletion of *RHD*; however, there are other sequence variations that may result in the loss of expression of the D antigen. Some of these RhD-negative haplotypes are positive for at least portions of the *RHD* gene

(Fig. 9.3). In the African population, there are two RhD-positive antigen D-negative alleles that are common and must be considered in any genotyping strategy. The *RHD*-pseudogene (RHD_{ψ}) is present in 66% of RhD-negative Africans and 24% African Americans and contains a 37 bp insertion at the junction of intron 3 and exon 4 that disrupts the reading frame and results in a stop codon [6]. RHD_{ψ} contains another stop codon in exon 6. The Cde^{s} allele (*RHD-CE(3–7)-D)*, found in 15% of RhD-negative Africans, is a hybrid containing exons 1–2, part of exon 3 and exons 8–10. In addition, a significant percentage of RhD-negative Asians (27%) are positive for the *RHD* gene.

Multiple Choice Questions

1. Which statement regarding the *RH* genes is not correct?
   A. All individuals who are *RHD*-negative have a deletion of the *RHD* gene
   B. Most variant *RH* alleles are the result of gene conversion events between the *RHD* and *RHCE* genes
   C. *RHD* and *RHCE* alleles are inherited as a haplotype
   D. *RHD* and *RHCE* genes are highly homologous genes that arose from a gene duplication event
   E. The *RHD* pseudogene is common in the African American population
2. True or False: A pregnant *RHD*-positive woman with anti-D is not at risk for HDFN
3. True or False: A physician is considering prenatal testing for a pregnant RhD-negative woman with anti-D. Because the mother is RhD-negative there is no reason to order *RHD* genotyping on the maternal sample
4. What is the most common cause of alloimmunization during pregnancy?
   A. Amniocentesis/Chorionic villus sampling
   B. Cordocentesis
   C. Fetomaternal hemorrhage
   D. Transfusion
   E. Unrecognized miscarriage
5. In Caucasians, 85% of the population is *RHD* positive. What percent of the population are homozygous and heterozygous for *RHD*?
   A. 15% DD and 70% Dd
   B. 25% DD and 50% Dd
   C. 28% DD and 57% Dd
   D. 38% DD and 47% Dd
   E. None of the above

Answers to Multiple Choice Questions

1. *The correct answer is A.*

The deletion of the *RHD* gene is the most common reason for being *RHD*-negative but there are *RHD*-negative alleles that contain at least part of the *RHD* gene. RHD_{ψ} and *(RHD-CE(3–7)-D)* are common in African Americans. Furthermore, 27% of RhD-negative Asians are positive for the *RHD* gene.

2. *The correct answer is False.*

A pregnant, serologically RhD-positive woman who develops anti-D most likely has a partial D antigen. HDFN is typically less severe in these cases but prenatal diagnosis is still performed. A thorough serological and molecular workup can usually identify the partial D gene. The molecular assay used for prenatal analysis must be able to distinguish the partial *RHD* and a normal *RHD* gene. When this is not possible, zygosity analysis of the paternal sample and the fetus may help predict whether the fetus inherited the *RHD* gene.

3. *The correct answer is False.*

Although the majority of *RHD*-negative individuals will genotype as *RHD*-negative, there are variant alleles that do not express the D antigen but genotype as *RHD*-positive for at least part of the *RHD* gene. Some of these individuals will later be found to weakly express a D antigen or express a variant D antigen that was not detected by serology. *RHD* variant alleles are not infrequent in the African American and Asian populations. Given that the fetus may inherit these variants, a molecular evaluation of the maternal sample is highly recommended. The maternal sample is also used for maternal cell contamination studies.

4. *The correct answer is C.*

Answers A–E are each causes of alloimmunization but fetomaternal hemorrhage is the most common cause. RhIg is administered at 28 weeks and at birth (if the fetus is RhD-positive) to help prevent alloimmunization. RhIg therapy will not be given for an unrecognized hemorrhage or miscarriage, so alloimmunization can still occur despite receiving treatment at 28 weeks.

Invasive techniques such as amniocentesis, chorionic villus sampling and cordocentesis may cause bleeding events that result in further alloimmunization.

5. *The correct answer is D.*

The answer can be calculated using the Hardy–Weinberg equation ($p^2 + 2pq + q^2$) where p is the frequency of the *RHD*-positive allele and q is the frequency of the *RHD*-negative allele. The 85% *RHD*-positive individuals include both homozygous and heterozygous genotypes. The remaining15% of the population is *RHD*-negative ($q^2 = 0.15$). Therefore, $q = 0.387$ and $p = 0.612$ and the frequency of each genotype is DD is 38% (p^2), Dd is 47% ($2pq$) and dd is 15% (q^2).

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Pharmacogenetics

10

Kenneth L. Muldrew and Karen E. Weck

Clinical Background

A 57-year-old white male presented to the emergency room with substernal chest pressure/tightness, left forearm pain, and dyspnea at rest. Two weeks prior, the patient had noted an increase in fatigue. He had a past medical history of peptic ulcer disease, hypertension, and hypercholesterolemia. Medications included enalapril for hypertension, atorvastatin for hypercholesterolemia, and omeprazole for peptic ulcer disease. The social history was significant for smoking a pack of cigarettes a day for 30 years (30 pack-years). On examination, a systolic murmur and 2+ pitting edema were appreciated. Chest X-ray revealed diffuse infiltrates and an electrocardiogram exhibited 7 mm ST elevation in the anterior leads. Laboratory results were significant for a mildly elevated white blood cell count, creatine kinase of 541 U/L (normal 70–185 U/L), CK-MB of 78.4 ng/mL (normal <6.0 ng/mL), and troponin I of 4.53 ng/mL (normal <0.034 ng/mL), consistent with myocardial infarction.

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The patient underwent cardiac catheterization, which revealed two stenotic coronary arteries (60% and 95%). He had a bare metal stent placed in the left anterior descending coronary artery. The patient was discharged after five days on aspirin, and clopidogrel to prevent thrombosis, in addition to his usual medications. One month later the patient presented with similar signs and symptoms. Cardiac enzymes were elevated indicating acute myocardial infarction, and cardiac catheterization showed thrombosis of the previously stented region of coronary artery. Molecular testing for *CYP2C19* gene variants associated with clopidogrel resistance was ordered. Coronary artery bypass grafting was successfully performed.

Reason for Molecular Testing

This patient had a secondary coronary artery thrombosis despite treatment with the standard platelet inhibiting medications (aspirin and clopidogrel) utilized to prevent thrombotic events post-stent placement. Genetic variability in the *CYP2C19* gene affects the pharmacokinetics and response to clopidogrel treatment. Metabolism by the enzyme CYP2C19 is important for conversion of clopidogrel to the active metabolite. It has been demonstrated that certain *CYP2C19* variant alleles with reduced enzymatic function are associated with in-stent rethrombosis and increased morbidity and mortality. Testing to evaluate the presence of reduced-function alleles of *CYP2C19* can be useful to identify patients who are resistant to clopidogrel and who may benefit from increased dosage or use of alternative platelet-inhibiting drugs such as prasugrel.

I. Schrijver (ed.), *Diagnostic Molecular Pathology in Practice*,
DOI: 10.1007/978-3-642-19677-5_10,

Test Ordered

CYP2C19 genotyping was ordered to identify allelic variants associated with clopidogrel resistance.

Question 1: Is CYP2C19 genotyping appropriate in this patient?

Laboratory Test Performed

CYP2C19 genotyping using an allelic discrimination assay based on the use of TaqMan hydrolysis probes was performed.

This assay was designed to detect the most common *CYP2C19* allelic variants associated with altered clopidogrel response: CYP2C19*2 (g.19154G>A), CYP2C19*3 (g.17948G>A), and CYP2C19*17 (g.–806C>T). The assay was adapted from the TaqMan Drug Metabolism Genotyping Assays (Applied Biosystems, Inc). Genomic DNA extracted from a patient blood sample is amplified by PCR using primers specific to CYP2C19*2, *3, and *17 targets and detected by TaqMan hydrolysis probes specific to wild-type or mutant sequences using an ABI 7900 or 7500 HT real-time PCR instrument. The results are analyzed by Sequence Detection System (SDS) software and plotted in an allelic discrimination plot (Fig. 10.1). For each of the allelic variants, the fluorescence of the wild-type probe is plotted on one axis and the fluorescence of the mutant probe is plotted on the other axis. A determination is made as to mutant, heterozygous, or wild-type based on the relative fluorescence of the mutant versus the wild-type probes. A separate assay is preformed for each of the three alleles interrogated (*2, *3, and *17).

Question 2: What are the limitations of the TaqMan CYP2C19 assay?

Results with Interpretation Guideline

Figure 10.1 shows the results of the *CYP2C19* TaqMan genotyping assay for CYP2C19*2.

Those samples showing fluorescence with the wild-type probe and little or no fluorescence with the *2 probe are negative for the *2 allele (region around control A). Samples showing fluorescence with the *2 probe and little or no fluorescence with the wild-type probe are homozygous for the *2 allele (region around control C). Samples showing fluorescence with both the normal and *2 mutant probes are heterozygous for the *2 allele (region around control B). For a run to be valid, the no template control (control D) must have little or no fluorescence, and positive controls (normal, *2 heterozygous, and *2 homozygous) must have the appropriate fluorescence patterns as indicated by the allelic discrimination plot.

Question 3: Are the results of the controls appropriate in this assay run?

The patient sample (E) showed fluorescence with both wild-type and mutant *2 probes, consistent with heterozygosity for CYP2C19*2. The results of the CYP2C19*3 and *17 allele assays were negative for this patient (data not shown). These results indicate that this patient has one copy of the normal allele CYP2C19*1 and one copy of the reduced function allele CYP2C19*2 (genotype CYP2C19*1/*2).

Question 4: Does the CYP2C19 assay result explain the patient's complications?

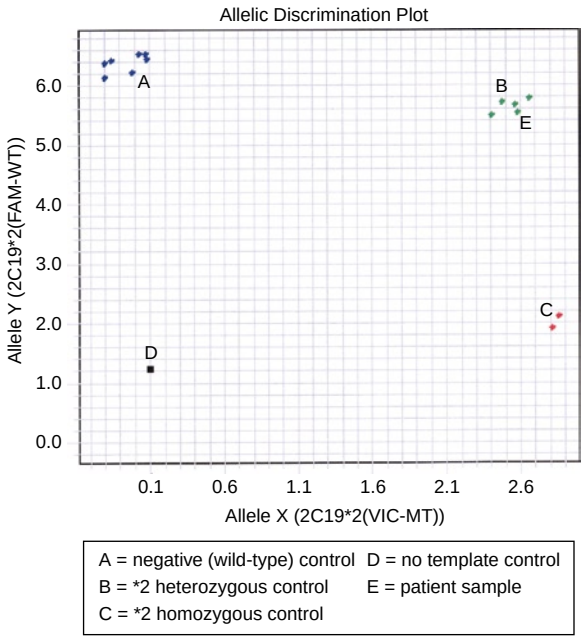
Result Interpretation

This patient was heterozygous for the CYP2C19*2 allele and negative for CYP2C19*3 and *17 alleles. CYP2C19*2 is associated with decreased effectiveness of clopidogrel and an increased risk of thrombotic cardiovascular events [1–10]. This may have contributed to stent thrombosis and subsequent myocardial infarction in this patient, while he was being treated with platelet inhibitor therapy. Another factor that should be considered is that this patient was also taking the proton pump inhibitor omeprazole. This medication inhibits the action of the CYP2C19 enzyme which results in lowering the level of the active metabolite of clopidogrel.

Further Testing

No further laboratory testing was performed. Based on the results of *CYP2C19* genotyping, the patient's antiplatelet medication was changed from clopidogrel to prasugrel. Coronary artery bypass grafting was successfully performed and the patient recovered with no further complications.

a



b

2C19*2 AD

Analysis Type: Automatic

Quality Value Threshold: 95.0

Well	Sample	Marker	Task	Call	Quality Value	Call Type	Allele X Rn	Allele Y Rn	Passive Ref Rn
25	WT	2C19*2	Unknown	2C19*2(FAM-WT)	99.63	Automatic	-0.21722497	6.41585	1421.4131
26	MT	2C19*2	Unknown	2C19*2(VIC-MT)	99.31	Automatic	2.8354933	2.1558304	1687.9546
27	HET	2C19*2	Unknown	Both	99.9	Automatic	2.4561133	5.752024	1619.2703
28	NTC	2C19*2	NTC	NTC	100.0	Automatic	0.08394075	1.2784727	1890.7416
29	10-2411	2C19*2	Unknown	Both	99.46	Automatic	2.3921936	5.535531	1687.9861
30	10-2412	2C19*2	Unknown	2C19*2(FAM-WT)	99.43	Automatic	-0.034085162	6.273004	1628.8779
31	10-2413	2C19*2	Unknown	Both	99.98	Automatic	2.543933	5.7108374	1863.669
32	10-2437	2C19*2	Unknown	2C19*2(FAM-WT)	99.84	Automatic	0.013726129	6.5751767	1729.3517
33	10-2455	2C19*2	Unknown	Both	99.52	Automatic	2.640294	5.827586	1801.9839
34	84/2386	2C19*2	Unknown	2C19*2(FAM-WT)	99.79	Automatic	0.0564406	6.568964	1745.3955
35	85/2429	2C19*2	Unknown	Both	99.94	Automatic	2.5663095	5.578654	1939.1005
36	86/2430	2C19*2	Unknown	2C19*2(FAM-WT)	99.89	Automatic	0.06113549	6.4825563	1894.6176
37	87/2431	2C19*2	Unknown	2C19*2(FAM-WT)	98.68	Automatic	-0.21566287	6.186189	1449.7893
38	88/2432	2C19*2	Unknown	2C19*2(FAM-WT)	99.79	Automatic	-0.17640586	6.461223	1432.9015
39	89/2433	2C19*2	Unknown	2C19*2(VIC-MT)	100.0	Automatic	2.7952645	1.9563382	1688.5829

Fig. 10.1 Results of the TaqMan genotyping assay for CYP2C19*2. The allelic discrimination plot shown in (**a**) depicts fluorescence of the CYP2C19*2 probe on the *X*-axis and of the wild-type probe on the *Y*-axis for each sample. A: normal wild-type control (WT), B: CYP2C19*2 heterozygous control (HET), C: CYP2C19*2 homozygous mutant control (MT), D: no template control (NTC), E: patient sample. The unlabeled data points are the results of other patient samples on the assay run. (**b**) The fluorescence data and genotype calls for each sample as analyzed by Sequence Detection System (SDS) software

Other Considerations

Multiple drugs are metabolized by the CYP2C19 enzyme. Many of these can act as competitive inhibitors of the enzyme and interfere with the activation of clopidogrel [11]. Of particular relevance is the proton pump inhibitor class used to treat peptic ulcer disease and gastroesophageal reflux (omeprazole, lanzoprazole). Many patients who are treated with clopidogrel are also treated with this class of medication. Pharmacodynamic and mechanistic data indicate a significant interaction between clopidogrel and the proton pump inhibitor class which is most evident between clopidogrel and omeprazole [12]. Much debate, however, still exists regarding the clinical importance of this interaction [13–15]. Pantoprazole does not appear to affect treatment with clopidogrel and may be an appropriate alternative to other proton pump inhibitors [14, 16]. Another option is to use an alternative platelet inhibitor such as prasugrel.

Background and Molecular Pathology

Pharmacogenetics is the study of differences in drug response due to variation in genes that affect drug metabolism (pharmacokinetics), efficacy (pharmacodynamics), and toxicity. Multiple genes have been associated with variability in drug response including genes that encode drug metabolizing enzymes (e.g., cytochrome p450 genes, thiopurine methyltransferase [*TMPT*]), drug targets (e.g., vitamin K epoxide reductase [*VKORC1*]), proteins involved in drug uptake (e.g., P-glycoprotein [*ABCB1*]), and immune system components (e.g., HLA haplotypes).

Genetic variants of the cytochrome p450 2C19 gene (*CYP2C19)* have been associated with individual variability in response to the antiplatelet medication clopidogrel bisulfate (Plavix®) [1–10]. Clopidogrel is a thienopyridine class antiplatelet agent used in the treatment of coronary artery disease, peripheral vascular disease, and cerebrovascular disease. Clopidogrel is a prodrug which requires activation to have significant antiplatelet activity. It is converted into an active metabolite (R130964) by CYP2C19 and other enzymes in the liver, resulting in irreversible inhibition of the platelet $P2Y_{12}$ ADP receptor (*P2RY12*) [17]. This results in inhibition of platelet aggregation by preventing activation of the glycoprotein IIb/IIIa receptor, which binds fibronectin, von Willebrand factor, fibrinogen, and vitronectin and is integral for fibrin cross-linking and platelet aggregation. Genetic variability in *CYP2C19* affects the pharmacokinetics and clinical efficacy of clopidogrel [17]. In the setting of clopidogrel pharmacotherapy, the presence of reduced function *CYP2C19* variants is associated with a significantly increased risk for cardiovascular events including stroke, stent thrombosis, myocardial infarction, and death due to insufficient platelet inhibition.

Table 10.1 Allele frequencies of common *CYP2C19* variants

	Allele frequency		
	*2	*3	*17
General population	15%	1.5%	19%
Caucasian	15%	0.04	25%
African American	20%	1%	18%
Hispanic	15%	<0.1%	?
Asian	30%	5%	4%

Approximate frequencies, compiled from various sources [18–21]

Clinically relevant genetic variants of *CYP2C19* associated with altered CYP2C19 enzymatic activity include CYP2C19*2, *3, and *17 and are relatively common in most populations (Table 10.1). CYP450 gene nomenclature designates the *1 allele as the wild-type (normal) allele. Variant alleles are designated *2, *3, *4, and so on, in order of discovery. At least 25 different allelic variants of *CYP2C19* have been described, many of which affect enzymatic activity [22]. The CYP2C19*1 allele is the most common allele in most populations and has normal enzymatic activity. The CYP2C19*2 and *3 alleles are the most common variant alleles with complete loss of enzymatic activity [22–24]. The absence of enzymatic activity in CYP2C19*2 results from a splicing defect due to a single nucleotide change at the junction of intron 4 and exon 5 (g.19154G > A, c.681G > A, rs4244285) which alters the reading frame resulting in premature protein truncation. The CYP2C19*3 allele is associated with a single nucleotide change (g.17498G > A, c.636G > A, rs4986893) that abrogates enzymatic activity by changing a tryptophan amino acid to a premature stop codon (p.Trp212X or W212X) resulting in protein truncation. Several clinical studies have demonstrated that CYP2C19*2 and *3 are associated with reduced efficacy of clopidogrel, higher residual platelet aggregation, and more frequent cardiovascular events [1–4, 10].

The CYP2C19*17 allele is due to a mutation in the 5′ untranslated region (g.-806C > T) that results in increased transcription of the normal gene, resulting in increased enzymatic activity and an ultrarapid metabolizing phenotype. This results in increased conversion of clopidogrel into the active metabolite. CYP2C19*17 has been associated with an increased risk of bleeding in patients on clopidogrel [18]. Other variants of *CYP2C19* (*4, *5, *6, *7, *8, *9, and *10) have been described with reduced or absent enzymatic activity, but these are rare in the general population [22]. Limited clinical data exist for the significance of these variant alleles in clopidogrel-treated patients. Because they are rare it has been more difficult to describe the risk of each of these variants individually, particularly for those alleles with reduced rather than absent enzymatic activity.

Individuals who lack CYP2C19 enzyme activity as a result of inheriting two inactive *CYP2C19* alleles are described to be poor metabolizers. Those with reduced but functional CYP2C19 enzymatic activity are intermediate metabolizers, while those with two alleles with normal enzymatic activity are extensive metabolizers. Physiologically, it appears that there is a gene dosage–dependent effect on active metabolite levels of clopidogrel and platelet function, but questions still exist regarding the frequency of adverse events on clopidogrel therapy in individuals heterozygous for CYP2C19*2 or *3 inactive alleles [3]. It is controversial whether, compared to wild-type, an increase in adverse events results from a single inactive allele (*2 or *3 heterozygous) or only in poor metabolizers with two inactive alleles [2–4, 10].

Recently the US Food and Drug Administration (FDA) added a warning to the clopidogrel (Plavix) package insert to emphasize the variability in response to the drug due to *CYP2C19* variants with reduced metabolism. The FDA label states "Tests are available to identify a patient's *CYP2C19* genotype and can be used as an aid in determining therapeutic strategy. Consider alternative treatment or treatment strategies in patients identified as CYP2C19 poor metabolizers" [19]. Alternative treatment strategies that have been suggested for patients with reduced function alleles include the use of an alternative platelet inhibitor, such as prasugrel, or an increase in the dose of clopidogrel [9–13, 20, 21, 25]. Prasugrel inhibits the same platelet receptor but is not as dependent on CYP2C19 for activation. It has better clinical efficacy but is more expensive and has an increased risk of bleeding compared to clopidogrel [6, 7]. An increase in the maintenance dose of clopidogrel from 75 to 150 mg in patients with *CYP2C19* reduced function genotypes (*2, *3) has been associated with an improvement in both platelet function studies and active metabolite levels [8, 9, 21, 25]. However, the clinical efficacy of an increase in the maintenance dose of clopidogrel in patients with reduced function *CYP2C19* genotypes and the frequency of adverse cardiovascular events has not been fully clarified.

In addition to *CYP2C19*, genetic variability in other genes may affect response to clopidogrel. The efflux pump P-glycoprotein (P-gp) affects intestinal uptake and bioavailablity of the drug, and genetic variability in the P-gp gene *ABCB1* has been associated with clopidogrel pharmacokinetics and clinical response [10]. In addition, genetic variability in the platelet receptor $P2Y_{12}$, which clopidogrel inhibits, has been implicated in altered drug response. However, these associations require further validation. Genetic variants of other enzymes associated with clopidogrel metabolism, including CYP3A4/5, CYP2C9, CYP2B6, and CYP1A2, have not shown a significant association with clopidogrel response [17]. To date, *CYP2C19* is the only gene that has been demonstrated to have significant association with clinical response to clopidogrel and with the risk of adverse events.

Multiple Choice Questions

1. Clopidogrel is
   A. A gpIIb/IIIa inhibitor
   B. A postdrug
   C. A prodrug
   D. A thrombin inhibitor
   E. Inactivated by CYP2C19*17 alleles
2. Based on the allele frequencies shown in Table 10.1, what percentage of African Americans are expected to be poor metabolizers of clopidogrel?
   A. <1%
   B. 3–5%
   C. 10–20%
   D. 20–30%
   E. 30–40%
3. CYP2C19*2 and CYP2C19*3 allelic variants:
   A. Are deletions
   B. Are detected by protein assays
   C. Are gene duplications
   D. Are point mutations
   E. Can show normal enzyme activity

4. The CYP2C19*17 allele is associated with
   A. Decreased clopidogrel active metabolite levels
   B. Increased clopidogrel levels
   C. Increased CYP2C19 protein production
   D. Increased risk of thrombosis
   E. Intermediate metabolism of clopidogrel
5. The CYP2C19*3 allele:
   A. Causes thrombocytopenia in patients on clopidogrel
   B. Causes thrombocytopenia in patients on prasugrel
   C. Is associated with variability in *ABCB1*
   D. Is less frequent than the *17 allele
   E. Is more frequent than the *2 allele

Answers to Questions Embedded in the Text

Question 1: Is CYP2C19 genotyping appropriate in this patient?

Yes. This patient was being treated with clopidogrel and had coronary artery stent thrombosis, which is one of the complications that can be seen in patients with *CYP2C19* inactive alleles. This testing guided the decision to change antiplatelet therapy to prevent future adverse events associated with resistance to clopidogrel.

Question 2: What are the limitations of the TaqMan CYP2C19 assay?

A limitation of the assay is that it is designed to detect only the most common *CYP2C19* allelic variants (*2, *3, and *17) and will not detect other more rare alleles with reduced or absent enzymatic activity (*4–*10). Another limitation is that a separate reaction must be performed for each variant interrogated. Other alternative *CYP2C19* genotyping assays are available, including the Autogenomics INFINITI CYP2C19 assay which is an array genotyping platform that detects multiple *CYP2C19* variants simultaneously [9]. Compared to other platforms, the TaqMan genotyping assay described here has comparatively low cost, fast turnaround time, low labor, and ease of use. Since the other alleles are much less frequent and their clinical significance is not as well understood at this time, the TaqMan assay is a reasonable choice.

Another limitation is that the presence of the rare CYP2C19*10 variant produces interference in the CYP2C19*2 TaqMan assay. The CYP2C19*10 variant (g.19153C > T) is due to a single base change in the nucleotide just upstream of the CYP2C19*2 variant (g.19154G > A) and thus prevents hybridization of the probes used in the CYP2C19*2 assay. In the case of a CYP2C19*2/*10 compound heterozygote, the CYP2C19*2 allele will be detected appropriately by the *2 probe, but the CYP2C19*10 allele will not be detected by either the *1 (wt) probe or the *2 probe. This will result in only the *2 probe binding, resulting in a false-positive CYP2C19*2 homozygous result. The *10 allele is relatively rare, with an allele frequency of 0.005 and 0.021 in the general and African American populations, respectively. Thus, rare patients who are CYP2C19*2/*10 compound heterozygous (less than 1% of the population) will be misclassified as CYP2C19*2/*2. This would affect less than 1% of African Americans (*10 allele frequency × *2 allele frequency × $2 = 0.021 \times 0.2 \times 2 = 0.84\%$) and less than 0.2% of Caucasians ($0.005 \times 0.15 \times 2 = 0.15\%$). Because the *10 allele is rare and has significantly reduced enzymatic activity, compared to the *2 allele which has no enzymatic activity, misidentification of CYP2C19*2/*10 patients as CYP2C19*2/*2 has minor clinical implications for clopidogrel response.

Question 3: Are the results of the controls appropriate in this assay run?

Yes. The no template control (NTC) appears in the lower left hand corner of the allelic discrimination plot indicating no fluorescence with the wild-type or mutant probes. If the NTC is positive, demonstrating significant fluorescence with either probe, this may indicate contamination in the assay. The normal (wild-type) control appeared in the upper left corner of the plot showing fluorescence with the wild-type probe but not the *2 probe indicating the absence of the *2 allele. The homozygous *2/*2 control was located in the lower right quadrant of the plot and had strong fluorescence with the *2 probe but low fluorescence with the wild-type probe, which indicates the presence of two copies of the *2 allele. The heterozygous control was located in the upper right quadrant indicating the presence of both the *2 and normal alleles. The control results demonstrate appropriate binding and fluorescence of both probes in this assay run.

Question 4: Does the CYP2C19 assay result explain the patient's complications?

The patient has a reduced function *CYP2C19* allele (CYP2C19*2) and was taking a known CYP2C19 inhibitor (omeprazole). Both of these may have contributed to his stent thrombosis and acute myocar-

antibodies which can be produced with specificity for almost any foreign antigen. In these rearrangements, one of 27 diversity (DH) segments is combined with one of 6 joining (JH) segments, resulting in removal of the DNA between them. Subsequent rearrangement joins this DH–JH junction product with one of 66 variable (VH) segments [1]. In a normal population of B cells, there will be many different rearrangements and therefore, the sizes of the rearranged loci in different B cells will vary. If a clonal proliferation occurs, a single rearrangement will be overrepresented within the population. Therefore, analysis of the *IGH* gene rearrangements may be performed to assess clonality.

The recommended sample types for this testing include fresh blood, bone marrow aspirate, and frozen or paraffin-embedded tissue. While peripheral blood and fresh or frozen tissue yield higher quantity and quality DNA compared to formalin-fixed paraffin-embedded (FFPE) tissue, many lymphoma cases are not referred for molecular analysis until histologic evaluation has been performed. For this reason, the most commonly used specimen in lymphoma diagnosis is FFPE. EDTA is the preferred anticoagulant for blood submitted for PCR analysis, because heparin is known to interfere with DNA amplification.

A large interlaboratory development program, the BIOMED-2 concerted action, has provided a standardized series of assays for the assessment of clonality in the immunoglobulin and T-cell receptor genes [2]. For the *IGH* locus, a series of five PCR reactions are performed to interrogate complete rearrangements involving the VH, DH, and JH segments, as well as incomplete rearrangements that involve only DH and JH segments. The first three tubes have six to seven forward primers complementary to relatively conserved segments, the VH frameworks (FR1, FR2, and FR3). A single consensus JH primer is used as the reverse primer which is complementary to a conserved area present in the 3′ region of all six JH segments. Tubes four and five contain multiple primers complementary to DH1-6 and a single primer for DH7, respectively, with a single reverse JH primer. The five reactions are summarized in Table 11.1.

Only the forward primers are fluorescently labeled. This could lead to poor resolution of products as the electrical field and heat produced during electrophoresis may partially denature the products, altering their mobility. Therefore, the products are denatured prior to further analysis. Samples are heated to 95°C in the presence of formamide, ensuring that the products will remain single stranded upon cooling. Analysis of the single stranded PCR products is performed by capillary electrophoresis.

Table 11.1 *IGH* gene rearrangement PCR amplifications

Forward primers (fluorescent label)	Reverse primer	Expected product sizes in nucleotides (bp)
Six FR1 primers (6FAM)	JH	310–360
Seven FR2 primers (NED)	JH	250–295
Seven FR3 primers (HEX)	JH	100–170
Six DH1-6 primers (HEX)	JH	110–290, 390–420
One DH7 primer (6FAM)	JH	100–130

Question 4: Which concerns arise regarding PCR-based IGH clonality assays when very few B cells are present in the sample?

Another molecular finding of importance, present in a subset of B-cell lymphomas, is the *IGH-BCL2* translocation involving chromosomes 14 and 18. This translocation is characteristic of follicular lymphoma and is identified, with an appropriately sensitive assay, in 90% of cases [3]. This translocation may also be detected in diffuse large B-cell lymphoma, where it occurs in 20% of cases. The t(14;18) places the *BCL2* gene under control of the *IGH* enhancer region resulting in overexpression of the normal BCL2 protein. This protein normally functions to antagonize apoptosis and to promote cell survival. Overexpression can lead to cell survival despite substantial damage from chemotherapeutic agents or ionizing radiation [4].

The typical translocations involve the rearranged *IGH* locus, and approximate the JH region to the *BCL2* gene. The breakpoints in the immunoglobulin heavy chain gene occur in one of the relatively conserved JH segments. The breakpoints in the *BCL2* gene are considerably more variable. Most occur in the 3′ untranslated portion of exon 3. This region is designated the major breakpoint region (MBR). A second cluster, 20 kb downstream of the MBR, includes the minor cluster region (mcr) and a smaller group just upstream (the 5′-mcr). A third area, the 3′-MBR, is located between the MBR and the mcr, approximately 4 kb downstream of the MBR. In addition, there are other possible sources of BCL2 dysregulation that can lead to B-cell lymphoma, making it very difficult for a single test to detect all possible sources of BCL2 overexpression.

dial infarction, due to reduced efficacy of clopidogrel and ineffective platelet inhibition.

Answers to Multiple Choice Questions

1. *The correct answer is C.*

Clopidogrel is inactive by itself and needs to be converted into its active metabolite by CYP450 enzymes including CYP2C19. Thus, it is a prodrug and not a postdrug. Clopidogrel's active metabolite binds to and inhibits the $P2Y_{12}$ ADP platelet receptor and does not inhibit thrombin. It is true that clopidogrel affects gpIIb/IIIa receptor activation and therefore platelet activation, but this occurs through downstream signaling events related to the irreversible inhibition of the $P2Y_{12}$ ADP receptor. The CYP2C19*17 allele is an ultrarapid metabolizing allele associated with increased activation of clopidogrel.

2. *The correct answer is B.*

The percentage of African Americans calculated to be poor metabolizers based on the allele frequencies shown in Table 10.1 includes individuals homozygous for CYP2C19*2 plus those homozygous for CYP2C19*3 plus those compound heterozygous for CYP2C19*2/*3, or $(0.2 \times 0.2) + (0.01 \times 0.01) + (0.2 \times 0.01 \times 2) = 0.044 = 4.4\%$. The estimate of 3–5% correlates well with the percentage of African Americans measured to be poor metabolizers by phenotypic assays. The contribution of other rarer inactive alleles is not significant.

3. *The correct answer is D.*

The CYP2C19*2 and *3 alleles result from point mutations in the *CYP2C19* gene, not gene duplication or deletions. The mutations each result in lack of CYP2C19 enzyme activity. Clinically, these alleles are detected by nucleic acid-based assays, not protein assays. Although a CYP2C19 enzymatic function assay could measure enzymatic activity, such assays could not distinguish between different poor metabolizing variants and are not readily available.

4. *The correct answer is C.*

The CYP2C19*17 allele is associated with a mutation in the 5′ untranslated region (5′UTR) of the *CYP2C19* gene resulting in increased gene expression. This results in increased CYP2C19 protein production, increased levels of the active metabolite of clopidogrel (not increased clopidogrel prodrug), an ultrarapid metabolism phenotype, and increased bleeding risk (not thrombosis).

5. *The correct answer is D.*

The CYP2C19*3 allele is much less frequent than either the CYP2C19*2 or the *17 alleles. The *3 allele is associated with an increased risk of thrombotic cardiovascular events in patients treated with clopidogrel and is not associated with thrombocytopenia in patients treated with clopidogrel or prasugrel. Both CYP2C19*3 and *ABCB1* variabilities have been associated with decreased response to clopidogrel, but they are not associated with each other.

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Part II
Hematopathology

B-Cell Lymphoma

11

Charles E. Hill

Clinical Background

A 70-year-old male presented to his primary care physician complaining of a left neck mass. He was otherwise healthy and asymptomatic. He was referred for fine needle aspiration to further characterize his neck mass. The fine needle aspiration revealed that the mass was an enlarged lymph node with an admixture of small lymphocytes and scattered highly atypical lymphoid cells consistent with lymphoma. Subsequently, an excisional biopsy was performed of the left neck lymph node. A touch preparation of the lymph node showed small mature lymphocytes and larger degenerated lymphoid cells. Sections of the node revealed a nodular proliferation of atypical lymphoid cells with an increased number of large atypical cells (Fig. 11.1). Flow cytometric immunophenotyping failed to identify any unique cell populations. Immunostaining for CD3 (pan T-cell marker), CD10 (common acute lymphoblastic leukemia antigen), CD20 (mature B-cell marker), CD21 (part of the B-cell coreceptor complex, positive on follicular dendritic cells), CD30 (marker of activated B and T cells), CD45 (leukocyte common antigen), CD57 (human natural killer-1 protein), and BCL2 (B-cell lymphoma-2 protein, antiapoptotic protein) was performed. The stains for CD3 and CD20 demonstrated that both B and T cells were present. CD3 stained many small lymphocytes within the nodules. CD20 stained both small and large atypical lymphoid cells. CD10 and CD21 highlighted the follicular dendritic network. In addition to staining for CD20, the large atypical cells also stained for CD30, CD45, and BCL2. CD57 positive cells were increased in the nodules but did not occur as a rim around the larger atypical cells.

Question 1: After reviewing this preliminary information, which neoplastic hematologic diseases are in the differential diagnosis?
Question 2: Which molecular studies could be ordered?

Based on the described findings, there are two primary considerations: follicular lymphoma and diffuse large B-cell lymphoma. Due to the follicular architecture and presence of large, atypical cells which stain for B-cell markers, testing for immunoglobulin heavy chain gene (*IGH*) rearrangements and the *IGH-BCL2* translocation were ordered. In addition, material from the lymph node was submitted for traditional karyotyping. A bone marrow biopsy was also performed and demonstrated a paratrabecular lymphoid aggregate with a few larger atypical lymphoid cells.

Question 3: How is molecular testing used as an aid in the diagnosis of lymphoma?

Reason for Molecular Testing

Molecular testing in this case can establish whether the atypical B cells identified in the histologic sections represent a clonal proliferation. Clonal rearrangements

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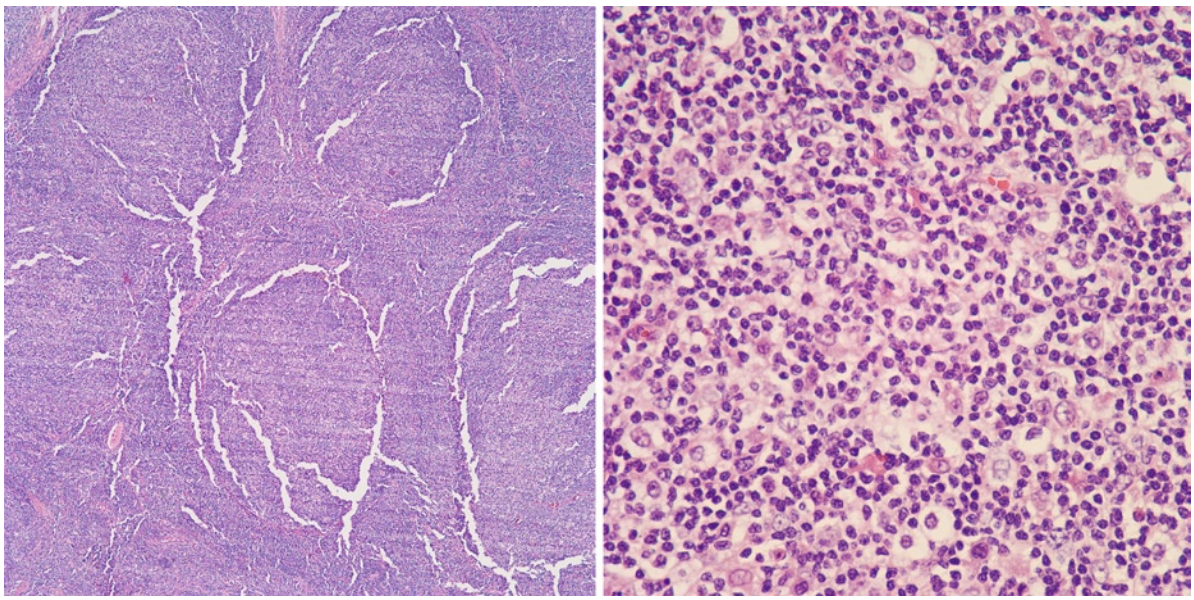

Fig. 11.1 Lymph node biopsy. The *left panel* is a low power view showing an abundance of closely packed, enlarged lymphoid follicles. The *right panel* is a higher power image demonstrating a mixture of large atypical cells (centroblasts) with vesicular chromatin and one or more prominent nucleoli and smaller follicle center cells (centrocytes)

of the immunoglobulin heavy chain gene are commonly associated with mature B-cell lymphomas. In addition, testing for the *IGH-BCL2* translocation can aid in the diagnosis of follicular lymphoma. The t(14;18), which yields the *IGH-BCL2* transgene, is commonly detected in follicular lymphoma and to a lesser extent in diffuse large B-cell lymphoma. Typically, follicular lymphoma has rearrangement of the immunoglobulin genes, the t(14;18) translocation, and a variety of other chromosomal gains and losses. If a clonal proliferation is detected in the lymph node, molecular testing of the bone marrow can establish whether the lymphoid aggregate identified by morphology is an unrelated process or the same as that in the lymph node.

Test Ordered

After reviewing the laboratory data and after histologic analysis of the lymph node, the reviewing pathologist ordered molecular diagnostic testing for immunoglobulin heavy chain gene rearrangements and for t(14;18) on both the lymph node and bone marrow. Clonality assessment using immunoglobulin heavy chain gene rearrangement testing may assist in establishing that a clonal B-cell proliferation is present but does not necessarily indicate malignancy. The t(14;18), in which the *BCL2* gene is overexpressed due to dysregulation as it comes under control of the regulatory elements of the immunoglobulin heavy chain promoter, provides a survival advantage to the transformed cells.

Laboratory Test Performed

Assessment of clonality has become a common molecular test for the diagnosis of both B- and T-cell malignancies. Historically, analysis of these loci for clonality was performed by Southern blotting. While still performed in some laboratories, Southern blotting has largely been supplanted by PCR-based methods. Although heteroduplex analysis is capable of detecting relatively small clonal populations, it too has largely been replaced by PCR with analysis of the products by capillary electrophoresis.

The biology of the *IGH* locus is an important consideration for clonality assessment. During normal B-cell maturation, the locus is rearranged to provide different specificities for the immunoglobulins. These rearrangements, followed by subsequent somatic mutation, provide the means for the immense variety of

Table 11.2 Primers for t(14;18) PCR

Forward primers	Reverse primer	Expected product sizes in nucleotides (bp)
Two MBR primers	JH	Variable
Four 3′ MBR primers	JH	Variable
One 5′ mcr primer	JH	Variable
Two mcr primers		

Testing for t(14;18) may be accomplished by a variety of methods. Traditional karyotyping can detect the translocation in many circumstances but may not be sufficiently sensitive in some cases. Fluorescent in situ hybridization is also commonly employed. This is more sensitive than traditional karyotyping due to the ease of screening many cells and the ability to detect the translocation in interphase cells, eliminating the requirement of culture and generation of metaphase cells required for traditional karyotyping. Most commonly, however, due to the ease of testing and the possibility to rapidly produce results, PCR-based methods are used to detect the *IGH-BCL2* translocation.

As part of the BIOMED-2 concerted action, a multiplex PCR assay has been developed to detect the majority of *IGH-BCL2* translocations. Due to the complexity of the possible *BCL2* breakpoints, three PCR reactions are performed to detect *IGH-BCL2* translocations in the MBR, 3′-MBR, and 5′-mcr/mcr. Similar to the *IGH* clonality assay, this assay employs a single consensus JH primer (the same as the one used in the *IGH* clonality assay). The PCR for translocations involving the MBR includes two forward primers directed toward the 3′ end of exon 3 in *BCL2*. The 3′-MBR PCR reaction utilizes four forward primers complementary to a region downstream of those used in the MBR reaction. Lastly, the 5′-mcr/mcr reaction includes three forward primers. PCR is followed by a detection method for PCR products, usually agarose gel electrophoresis or capillary electrophoresis (Table 11.2). The variety of possible *BCL2* gene breakpoints leads to great variability in the possible PCR product sizes, ranging from 100 bp up to more than 2,500 bp.

Results with Interpretation Guideline

DNA isolated from the patient's lymph node (FFPE) and bone marrow aspirate (fresh) were tested for both a clonal rearrangement of the *IGH* gene and the t(14;18). The results for FR1–3 are shown in Fig. 11.2a–f. *IGH-BCL2* results are presented in Fig. 11.2g.

Interpretation of these tests is generally straightforward. The *IGH* rearrangement assay is reviewed for peaks that are much higher than any polyclonal rearrangements that produce products with a variety of sizes. Because the primers used in the *IGH-BCL2* translocation assay should not be in close proximity in normal cells, any PCR product is considered positive.

The BIOMED-2 initiative also includes a multiplex primer set to detect a series of housekeeping genes (lanes 4 and 8, Fig. 11.2g). These markers are used to determine whether sufficient quantity and quality of amplifiable DNA is present in the samples. As is clear from the lower intensity and lack of larger amplification products in lane 4, the quality of DNA acquired from the FFPE sample was not as good as that from the bone marrow aspirate (lane 8).

Question 5: After reviewing the patient's results, can a relationship be established between the process in the lymph node and the lymphoid cells in the bone marrow?

Result Interpretation

In the *IGH* assay there are clearly peaks well above the polyclonal background present in both the lymph node and the bone marrow. Any peak detected that is significantly higher than the peaks expected for polyclonal rearrangements should be considered positive. There are no established criteria for identification of a clonal population; therefore, results must be interpreted in the context of the sample being analyzed. The results for FR1 and FR2, presented in Fig. 11.2a and b, demonstrate a polyclonal distribution of rearrangements commonly associated with normal B-cell maturation. Figure 11.2c depicts a distinct peak at 108 bp indicating that the rearrangement resulting in that PCR product is overrepresented which implies a clonal proliferation of B cells. No clonal rearrangements were detected for DH1-6 or for DH7. The observed rearrangements indicate aberrant or incomplete maturation and are not expected in polyclonal processes that involve normal B-cell maturation. The results for the bone marrow demonstrate a peak in FR3. While the peak identified in FR3 is 107 bp instead of 108 bp, the findings taken together indicate that the same clonal process is present in the bone marrow that was

detected in the lymph node. Due to the variability of assigning sizes using capillary electrophoresis, a variation of ±1 bp is within the error of the measure and represents an acceptable amount of variation.

In addition, both samples have peaks visible in the first PCR reaction containing primers complementary to the MBR. Any product in any of the lanes is considered positive. For the lymph node sample, a distinct PCR product is identified in lane 1, corresponding to the MBR. This indicates that a t(14;18) is present, involving the MBR of *BCL2*. For the bone marrow sample, a peak is visible in the MBR lane for the t(14;18) (lanes 4–8, Fig. 11.2g).The findings are summarized in Table 11.3.

While identification of a clonal process is relatively straightforward when polyclonal B cells are present in the sample that contains a clonal population, interpretation can be complicated if very few B cells are present in the sample. In these cases, "pseudoclonality" is a real concern. Individual rearrangements may be efficiently amplified, resulting in distinct peaks in the absence of a true clonal proliferation (a pseudoclone). Distinguishing whether a sample yielding a peak in the absence of a polyclonal background contains a true clone versus a pseudoclone can be quite difficult. For this reason, results should be interpreted in the context of other findings, including morphologic examination to determine the quantity of B cells in the sample submitted for analysis.

Further Testing

In addition to the molecular testing described, traditional karyotyping was performed on the lymph node tissue. The karyotype revealed three abnormal

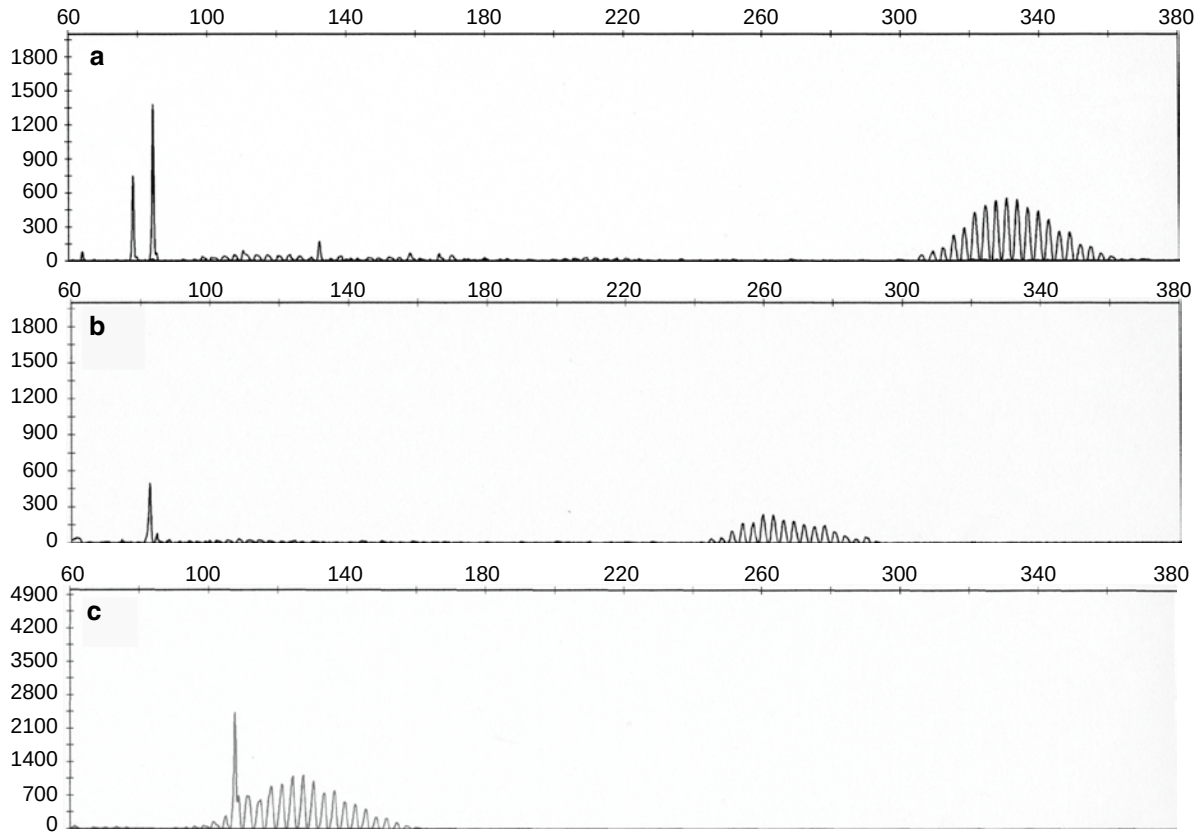

Fig. 11.2 (**a**–**f**) *IGH* gene rearrangement studies (**a**–**c**: lymph node, **d**–**f**: bone marrow). Results in **a**, **b**, **d**, and **e** indicate a polyclonal pattern of gene rearrangements. (**c**) The peak at 108 bp is much higher than expected in this region and represents a clonal rearrangement. A similar peak is present in (**f**) at 107 bp. (**g**) Demonstrates the results for t(14;18) testing. From left to right, lanes 1–4 represent the lymph node and 5–8 the bone marrow. Lanes 1 and 5 are MBR, lanes 2 and 6 are 3′MBR, lanes 3 and 7 are mcr, and lanes 4 and 8 are control amplifications to verify quality/quantity of DNA

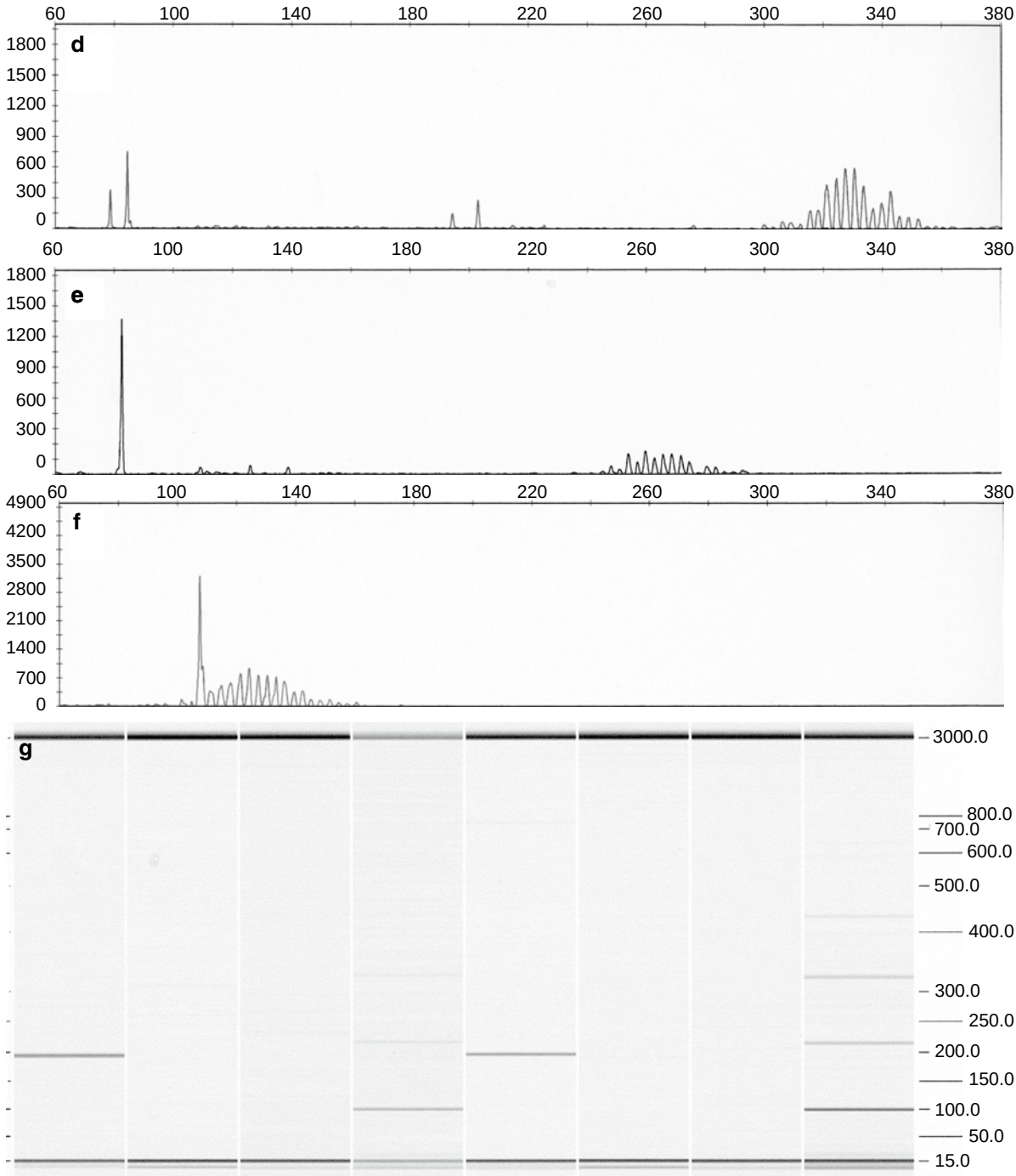

Fig. 11.2 (continued)

metaphases with t(14;18) as well as deletion of the long arm of chromosome 7, gain of chromosome 7, and gain of 1–2 marker chromosomes. In ISCN nomenclature, the karyotype was described as 41–49,XY,+7,del(7)(q31),t(14;18)(q32;q21),+1–2mar[cp3]/46,XY [5].

The final diagnosis in this case based on all of the various testing was Follicular lymphoma, Grade 3A (greater than 15 centroblasts per high power field and centrocytes are present), Follicular Pattern (100%).

Table 11.3 Result interpretation for our patient

Patient sample	IGH rearrangement results peak sizes (bp)	IGH-BCL2 results
Lymph node, left neck	FR1: No clonal rearrangement detected	MBR: Translocation detected
	FR2: No clonal rearrangement detected	3′-MBR: No translocation detected
	FR3: 108 bp	mcr: No translocation detected
	DH1-6: No clonal rearrangement detected	
	DH7: No clonal rearrangement detected	
Bone marrow aspirate	FR1: No clonal rearrangement detected	MBR: Translocation detected
	FR2: No clonal rearrangement detected	3′-MBR: No translocation detected
	FR3: 107 bp	mcr: No translocation detected
	DH1–6: No clonal rearrangement detected	
	DH7: No clonal rearrangement detected	

Although it was not necessary in this case, testing for rearrangement of the kappa light chain locus may be helpful under certain circumstances. Somatic hypermutation may lead to poor primer binding in the *IGH* test. If the index of suspicion is high for a clonal proliferation and the *IGH* assay is negative, kappa light chain gene rearrangement testing may help establish clonality.

Other Considerations

Neither of the two molecular tests presented is sufficient to diagnose follicular lymphoma. Both tests may be positive in other processes and therefore other information must also be integrated to arrive at a diagnosis of follicular lymphoma. While the molecular results on the lymph node sample were supportive of the diagnosis, correlation with the flow cytometric immunophenotyping results and morphologic assessment were necessary for a final diagnosis. Testing of the bone marrow established that the same clonal process present in the lymph node was also present in the bone marrow. Although bone marrow involvement is not part of the grading scheme, the extent of disease is important for assessing prognosis.

Immunoglobulin and T-cell receptor gene rearrangement testing are protected under patent in the USA and other jurisdictions (US Patents 5296351 and 5418134, accessed at www.uspto.gov, 07-01-10). These tests currently may not be performed in jurisdictions that have awarded protected intellectual property rights without appropriate licensing. In the USA, Invivoscribe Technologies, Inc. (San Diego, CA) is the holder of the property rights to immunoglobulin and T-cell receptor gene rearrangement testing and offers commercial reagents.

Background and Molecular Pathology

Follicular lymphoma is a low-grade B-cell neoplasm of germinal center B cells [6]. The neoplastic cells proliferate predominantly in lymph nodes leading to lymphadenopathy, but other sites may be involved and primary extranodal follicular lymphoma may occur. Secondary sites of involvement include the spleen, bone marrow, peripheral blood, gastrointestinal tract, and soft tissues. These proliferations typically have back to back follicles that efface the normal nodal architecture. Most patients are relatively asymptomatic and therefore tend to present with widespread disease. Grading relies on quantification of the number of centroblasts (larger germinal center B cells). The immunoglobulin heavy and light chain genes are rearranged, but such rearrangements may not always be detected due to a variety of factors including loss of primer binding as a result of somatic hypermutation. By testing both the *IGH* and kappa light chain loci for clonality, the majority of these rearrangements can be detected, approaching a 100% detection rate [7].

The characteristic molecular change in follicular lymphoma is the chromosomal translocation t(14;18), which results in overexpression of the *BCL2* gene. BCL2 function has been extensively studied and antagonizes apoptosis [5]. However, the overexpression of BCL2 can also be identified in other hematologic neoplasms and does not lead to the development of follicular lymphoma in the absence of other changes [8]. Other cytogenetic abnormalities are common and most confer an unfavorable prognosis. The more common secondary abnormalities include gain of chromosomes 7, 12q, and 18q, as well as losses of 1p, 6q, 9p, and 17p [9].

Through genome-wide association studies, a locus on chromosome 6p has been associated with increased risk of developing follicular lymphoma [10]. Specific HLA haplotypes may increase the susceptibility for developing follicular lymphoma. In addition, methylation may be an important factor in its pathogenesis. Several tumor suppressor genes have altered methylation patterns in follicular lymphoma [11]. Methylation profiling with more global, array-based approaches has demonstrated widespread hypermethylation in this disease [12]. By further characterizing the biology of follicular lymphoma, it may be possible to stratify patients into more refined prognostic and treatment groups.

Multiple Choice Questions

1. Which of the following statements about follicular lymphoma is correct? Follicular lymphoma:
   A. Cannot be diagnosed without the t(14;18)
   B. Is characterized by proliferation of immature T cells
   C. Is diagnosed using a combination of morphology and laboratory testing
   D. Is highly aggressive and leads to rapid demise
   E. Is never associated with clonal rearrangement of the *IGH* locus
2. The *IGH-BCL2* translocation:
   A. Alters the DNA-binding domain of *BCL2*
   B. Can be detected only by PCR-based methods
   C. Increases the rate of apoptosis in B cells
   D. Most frequently involves the 3′ portion of exon 3 in *BCL2*
   E. Results in a fusion protein with novel function
3. DNA from formalin-fixed, paraffin-embedded tissue:
   A. Cannot be used to test for t(14;18)
   B. Is frequently fragmented due to protein cross-linking
   C. Must be chemically modified prior to PCR
   D. Requires significantly less time to extract compared to DNA from peripheral blood
   E. Should be cloned prior to further testing
4. Follicular lymphoma with gain of chromosome 7:
   A. Always carries a V617F mutation of the *JAK2* gene
   B. Has a diffuse architecture
   C. Is associated with a worse prognosis
   D. Is limited to lymphoma of the head and neck
   E. Is more likely to respond to chemotherapy than lymphoma lacking this gain
5. Testing for rearrangements of the *IGH* locus:
   A. Allows for distinction of malignant and benign proliferations
   B. Can be easily performed with a single PCR reaction
   C. Is useful only for research purposes
   D. May be used to determine whether two tissues contain the same clonal cells
   E. Should be performed on all patients with myeloid neoplsams

Answers to Multiple Choice Questions

1. *The correct answer is C.*
2. *The correct answer is D.*
3. *The correct answer is B.*
4. *The correct answer is C.*
5. *The correct answer is D.*

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T-Cell Lymphoma

12

Marian H. Harris and Janina A. Longtine

Clinical Background

A 51-year-old female came to the dermatology clinic with a 20 year history of a rash on her trunk and inner arms that had previously been diagnosed as chronic dermatitis. Physical exam revealed that approximately 10% of her body surface was involved by red to brown scaly patches. No lymphadenopathy or hepatosplenomegaly was present. A biopsy of the involved skin was obtained. Complete blood count and peripheral blood flow cytometry were within normal limits.

The biopsy (Fig.12.1a) showed a mild lymphocytic infiltrate, with some tagging along the dermal–epidermal junction, composed predominantly of CD3-positive, CD4-positive T-cells (not shown). There was no significant epidermotropism or cytologic atypia. The histologic features were not diagnostic, but in the appropriate clinical context suggest early cutaneous T-cell lymphoma (CTCL). To further clarify the diagnosis, molecular analysis of T-cell receptor (TCR) gene rearrangements was performed.

Question 1: What is the differential diagnosis?

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Reason for Molecular Testing

CTCL represents a spectrum of mature T-cell neoplasms arising in the skin. The prototype of CTCL is mycosis fungoides (MF), a usually indolent neoplasm that comprises approximately 50% of all primary cutaneous lymphomas [1]. Early clinical findings in the skin can include scaly eruptions, and patches and/or plaques, particularly on the trunk. These findings may persist for many years before a definitive diagnosis is made. Eventually, the disease may progress to more generalized plaques and then to tumors. Late in the course of MF, lymph node, visceral organ, and blood involvement may be present.

The typical histologic features of MF are a band-like infiltrate of lymphocytes along the dermal–epidermal junction with atypical highly convoluted (cerebriform) nuclei within the epidermis (i.e., epidermotropic). Intraepidermal clusters of atypical lymphocytes (Pautrier's microabscesses) are the most specific histologic feature, but present in only a small percentage of cases. The lymphocytes are CD4-positive T-cells that frequently show loss of expression of T-cell antigens such as CD7 or CD5 by immunohistochemistry. In early MF, the histologic features are subtle and resemble chronic inflammatory processes, often precluding a definitive diagnosis. Furthermore, the immunophenotype of MF can be shared by reactive dermatitides. Thus, evaluating the clonality of T-lymphocytes by TCR gene rearrangement analysis can be helpful in establishing the diagnosis of MF. In fact, molecular testing for T-cell clonality has become a critical tool in the evaluation of CTCL. Guidelines from the International Society

I. Schrijver (ed.), *Diagnostic Molecular Pathology in Practice*,
DOI: 10.1007/978-3-642-19677-5_12,

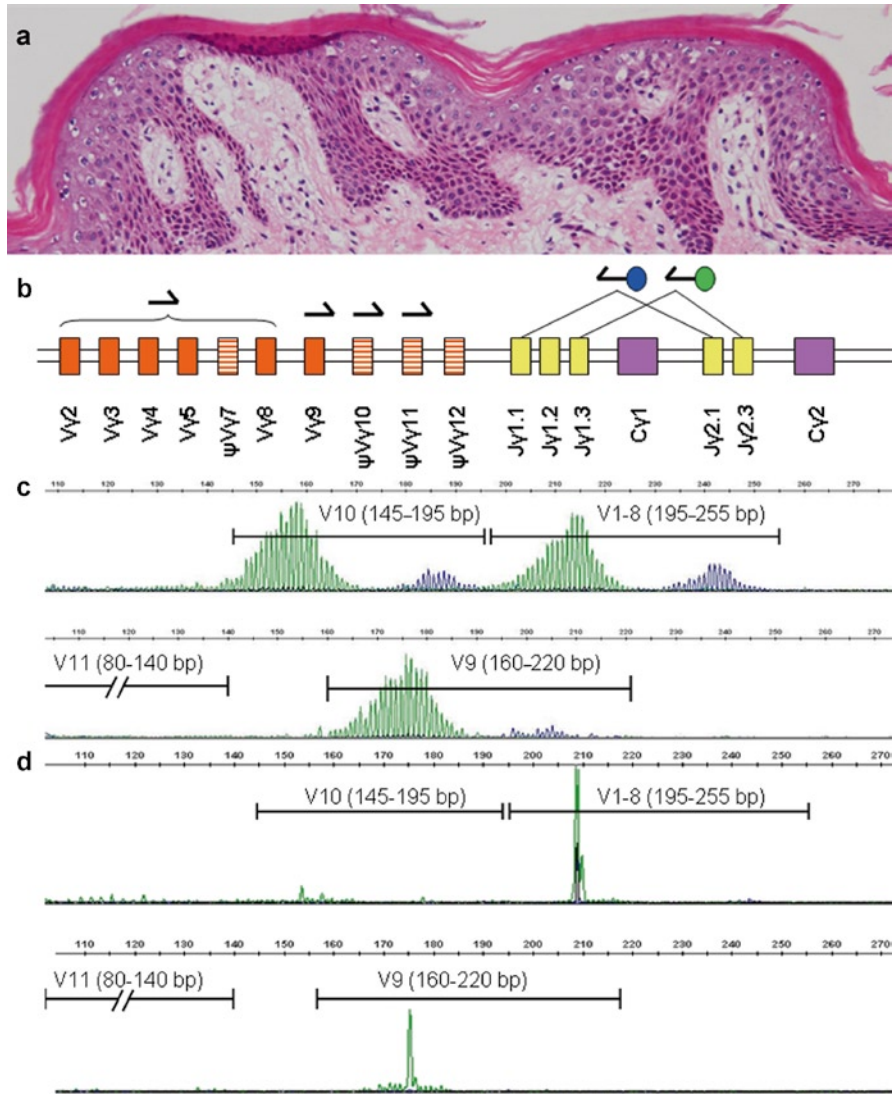

Fig. 12.1 Patient's biopsy, primer design, and test results. (**a**) The patient's biopsy, stained with hematoxylin and eosin. Note the sparse lymphocytic infiltrate in the dermis and the slight tagging of lymphocytes at the dermal–epidermal junction. (**b**) The *TCRG* locus, with V segments in orange (rearranged but nonfunctional segments are striped), J segments in yellow, and C segments in purple. The primers used in the BIOMED-2 protocol are shown by *arrows*, with the two J primers differentially labeled (*blue* and *green*) to facilitate gene scanning analysis. (**c**) A typical polyclonal pattern following CE-GS. Each V primer can generate two normal curves, one with each J primer. Normal Vγ11 curves are frequently absent (see text). (**d**) A typical clonal pattern following CE-GS. There are two prominent peaks: one in the Vγ1–8/Jγ1.3/2.3 region and one in the Vγ9/Jγ1.3/2.3 region

for Cutaneous Lymphomas (ISCL) and the European Organization of Research and Treatment of Cancer (EORTC) support a diagnostic algorithm for early MF which includes TCR gene rearrangement analysis [2].

Question 2: How would TCR gene rearrangement analysis distinguish between neoplastic and reactive T-cell populations?

Test Ordered

TCR gene rearrangement analysis was ordered. Most neoplasms are thought to arise from one original transformed cell, and thus to be clonal. While the identification of a clonal population is difficult in most tissue types absent specific genetic knowledge about the tumor in question, lymphocytes have unique receptors that differ from cell to cell in order to be able to recognize a diversity of antigens. There are four TCR chains: α, β, γ, and δ, encoded at four separate loci: *TCRA*, *TCRB*, *TCRG*, and *TCRD*. Each locus is comprised of V (variable), (±)D (diversity), and J (joining) segments, which recombine during T-cell development to form the mature V(D)J sequence of these genes. Testing for TCR rearrangements takes advantage of this clonal diversity in T-cells. In a normal, nonneoplastic population of T-cells, a wide variety of T-lymphocytes, and therefore TCR rearrangements, is expected, while in a

neoplastic population of T-cells, a single clone of T-lymphocytes, with a unique TCR rearrangement, is expected.

Question 3: What laboratory techniques might be useful in evaluating TCR gene rearrangements?

Laboratory Test Performed

For many years, Southern blots were the gold standard for assessing lymphocyte clonality. However, Southern blots are technically complex to perform, time consuming, and, perhaps most significantly, because they require large quantities of high quality DNA, they are incompatible with small biopsies and with formalin-fixed, paraffin-embedded tissue. PCR for lymphocyte gene rearrangement analysis was first proposed in the late 1980s and is now the preferred test in most labs. Compared to Southern blots, PCR-based assays for clonality are easier, faster, compatible with formalin-fixed, paraffin-embedded tissue, require less DNA, and have the potential to offer more accurate information about the rearrangement(s) present in a clonal or oligoclonal population.

In 2003, the BIOMED-2 consortium published multiplex PCR protocols with consensus primers for the amplification of the δ, γ, and β genes of the TCR (*TCRD*, *TCRG*, and *TCRB*) [3]. These assays are now commercially available (InVivoScribe Technologies, San Diego, CA) and are used by many labs across the country and internationally which allows for standardization of testing. In addition, several other strategies have also been described and are in current use [4–9].

The *TCRG* locus is particularly attractive for PCR-based clonality studies both because virtually all T-cells have rearranged *TCRG* and because the locus is significantly less complex than *TCRB*. There are a total of 14 Vγ segments, of which only 10 undergo rearrangement, and 5 Jγ segments. No D segments are present at the gamma locus, and the N region nucleotides added during rearrangement are limited in number. Therefore, it is possible to amplify all of the major possible Vγ–Jγ combinations with a relatively small set of primers. *TCRB* is considerably more complex than *TCRG*, with 52 Vβ segments, 2 Dβ segments, and 13 Jβ segments. Because of these factors, many labs use *TCRG* analysis as their sole or first-line clonality assay.

The BIOMED-2 protocol for *TCRG* analysis consists of six primers, one each for Vγ1–8, Vγ9, Vγ10, and Vγ11, as well as one for Jγ1.1/2.1, and one for Jγ1.3/2.3. Primers for Vγ12 and Jγ1.2 were not included due to the general rarity of their usage in rearrangements. These primers are used in two multiplexed reaction tubes: one with Vγ1–8, Vγ10, and the two J primers and one with Vγ9, Vγ11, and the two J primers (Fig.12.1b). In a normal, nonneoplastic population of T-cells, multiple different rearrangements are present, yielding a normal distribution of peak or band sizes. Each V–J primer combination yields its own curve, so that in theory eight different curves are generated, four with each tube. In practice, the Vγ11 primer often does not yield robust polyclonal distribution with the BIOMED-2 primer set (Fig. 12.1c). In a clonal population of T-cells, only one or two rearrangements are present, yielding a single peak or band, or two peaks, such as in the case of biallelic rearrangements (Fig. 12.1d).

Following PCR, there are multiple ways to analyze the results, including simple agarose or polyacrylamide gels, heteroduplex analysis [10], denaturing gradient gel electrophoresis [11], and capillary electrophoresis with fragment length analysis (gene scanning, CE-GS) [12]. CE-GS obviates the need for ethidium bromide and polyacrylamide while providing precise sizing data and V family usage information that can be easily used to compare samples, but requires fluorescently labeled primers. In the BIOMED-2 protocol, the two J primers are differentially labeled for CE-GS. The detection threshold for a neoplastic population with CE-GS has been estimated at 0.5–5% but depends on a number of variables such as V region utilized, DNA quality, primer sets, and the proportion of reactive lymphoid cells in the sample [13, 14].

TCRG-PCR can be performed on DNA extracted from almost any source, including fresh or frozen tissue, formalin-fixed paraffin-embedded tissue, blood, and body fluids. The DNA should be free of inhibitors of PCR (such as heavy metals), and proper controls should be part of each assay, including a water (blank) control, a negative (known polyclonal) control, and a positive (known clonal) control. In addition, the BIOMED-2 protocol suggests running a size control on the sample DNA, consisting of a series of multiplexed primers that generate amplicons of 100, 200, 300, 400, 500, and 600 basepairs to assess the quality, quantity, and fragment size of the sample DNA. The *TCRG* BIOMED-2 protocol needs fragments of at least 255 bp (the size of the largest amplicon).

While the primers in the BIOMED-2 assay are estimated to cover almost all *TCRG* rearrangements, the sensitivity of the assay for detecting CTCL has ranged from approximately 64% to 84% [6, 13–16] in cases with a confirmed histopathologic/clinical diagnosis. Not surprisingly, the sensitivity in early MF, with fewer tumor cells in the sample, is somewhat lower than the sensitivity in late MF. Some labs use lab-developed PCR assays for *TCRG* analysis and have demonstrated similar overall sensitivity (although some may be more efficient at detecting rearrangements using Vγ11) ([6] and references therein).

Question 4: What might sources of false-positive or false-negative results be in this assay?

Results with Interpretation Guideline

The CE-GS results for *TCRG*-PCR with Vγ1–8, Vγ10, Jγ1.1/2.1, and Jγ1.3/2.3 primers are shown in Fig. 12.2a. The Vγ9, Vγ11, Jγ1.1/2.1, and Jγ1.3/2.3 primers gave a polyclonal, Gaussian distribution (not shown).

One challenge of using CE-GS for gene rearrangement analyses is the interpretation of the results. While a normal distribution of peaks clearly represents a polyclonal population, and a single sharp peak clearly represents a clonal population, many results fall somewhere between these extremes, presenting an interpretive challenge. This is particularly true in the analysis of *TCRG*. Because the baseline diversity of rearrangements is lower, more than one prominent peak of varying heights is commonly present in *TCRG* rearrangement studies. For example, in a typical polyclonal population, the number of T-cells using one of the six V regions covered by the Vγ1–8 primer is much higher than the number of T-cells using either Vγ9, Vγ10, or Vγ11. Therefore a small reactive clone that uses Vγ9, Vγ10, or Vγ11 has a greater chance of creating a visible peak than a clone using Vγ1–8 [17].

Numerous methodologies have been proposed for interpretation, but a consensus has not yet been reached. The BIOMED-2 group discourages the use of a strictly algorithmic approach to interpretation because of case-to-case variation in expected results. This variability is due to both differences in the ratio of malignant cells to reactive lymphocytes in each case and to the fact that the PCR reactions in the BIOMED-2 protocol are not strictly quantitative [18]. Nevertheless, multiple algorithms have been proposed and may be helpful in interpretation if used judiciously. One method is to consider a peak clonal when it is greater than two times the height of the polyclonal background [19]; however, more complex algorithms can also be helpful, especially in cases with more than one prominent peak or without a recognizable background. Some algorithms include relative peak height (RPH), peak height ratio (PHR), and normal distribution (ND) analysis. Relative peak height is the ratio of the height of the peak in question above the polyclonal background to the maximum height of the polyclonal background $((h_p - h_b)/h_b$, where h_p is the height of the peak and h_b is the height of the background) [20]. A relative peak height greater than 3 is considered consistent with clonality, while relative peak heights between 1.5 and 3 are considered indeterminate. Peak height ratio is the ratio between the peak in question and the average of the two immediately flanking peaks [21]. In normal distribution analysis, a computer program uses the data from gene scanning to fit a normal distribution curve and then to identify peaks that deviate significantly from the curve [17]. ND values greater than 1.0 are consistent with clonality, while values between 0.1 and 1.0 are indeterminate. The ND method has the advantage of being fully automated and is highly concordant with relative peak height calculations. Regardless of which method of peak interpretation is used, it is of critical importance to interpret peaks within the unique clinicopathologic context of each case. Incorporation into the algorithm of an assay for the *TCRB* locus may also be helpful [18].

Question 5: How would you interpret this patient's results?

Result Interpretation

The initial *TCRG*-PCR for this patient was interpreted as oligoclonal, with one definitive clonal peak (RPH = 11.8), one indeterminate peak (RPH = 1.58), and one peak that does not meet RPH clonality criteria (RPH = 0.81) but is worrisome nonetheless in this context (Fig. 12.2a). Oligoclonality has recently been reported to be common in early-stage MF [16].

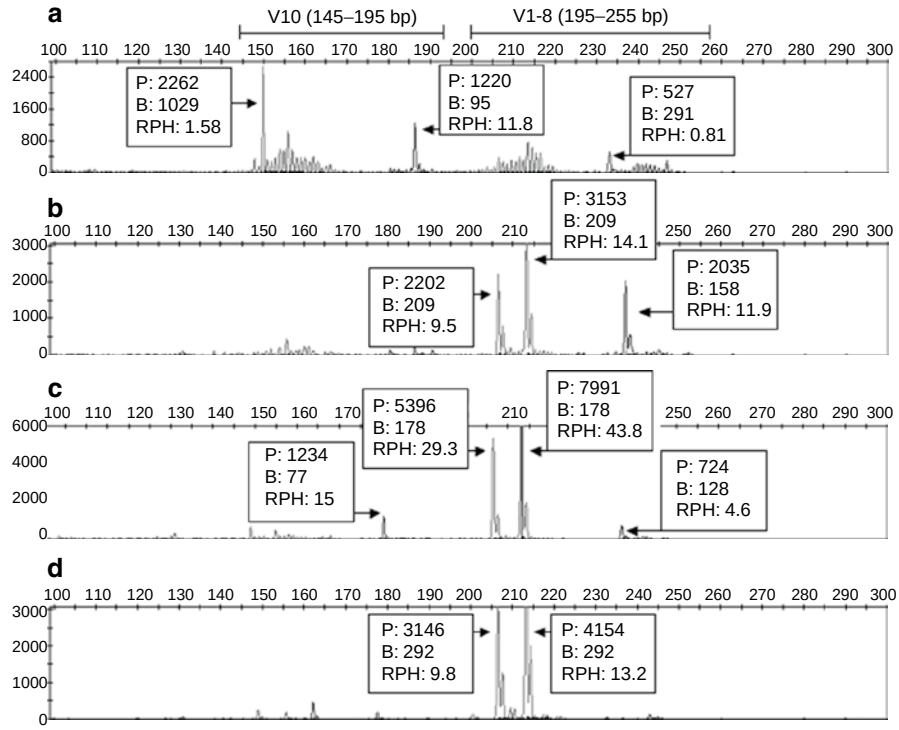

Fig. 12.2 CE-GS results for the patient following *TCRG*-PCR. Only the results from the first tube are shown (primers Vγ1–8, Vγ10, Jγ1.1/2.1, and Jγ1.3/2.3). With each peak, the peak height (*P*), the background height (*B*), and the calculated relative peak height (*RPH*) are shown. (**a**) The first biopsy, which is oligoclonal, with three peaks (*arrows*). (**b**) The second biopsy, which is also oligoclonal, with three peaks (*arrows*). None of the peaks is the same as the peaks in the first biopsy. (**c**) The third biopsy, which is oligoclonal, with four peaks (*arrows*). Three of the four peaks are the same as those seen previously. This pattern is concerning for malignancy. (**d**) The fourth biopsy, which is definitively clonal, with two peaks that are the same as the dominant peaks in the second and third biopsies (*arrows*)

Further Testing

This patient was followed over the course of the next six years, with a gradual progression of her rash, including some plaques, to cover up to 25% of her body surface, including both her upper and lower extremities as well as anterior trunk and breasts. Subsequent biopsies showed a more pronounced lymphocytic infiltrate with tagging of the dermal–epidermal junction and epidermotropism. *TCRG*-PCR results show the evolution from a polyclonal pattern to two predominant peaks, consistent with a biallelic *TCRG* clone (Fig. 12.2b–d). The final *TCRG*-PCR shows a definitive clonal pattern, with the same two dominant peaks seen in the two prior studies, without the additional smaller peaks. Interestingly, the one definitive peak in the first biopsy is not present in the subsequent three biopsies. As MF is a systemic disease, the presence of identical clones in biopsies separated by time and/or site is expected and is helpful in distinguishing MF from benign dermatitides [22].

Other Considerations

Clinicopathologic correlation is essential to avoid overinterpretation of positive or negative results. It is important to be aware that reactive populations of lymphocytes may be clonal upon TCR rearrangement analysis; therefore the presence of a T-cell clone is not always equivalent to the presence of a T-cell neoplasm. For example, a recent study of 157 cases found that 14% of cutaneous specimens with a benign inflammatory infiltrate demonstrated clonality with TCRγ gene rearrangement analysis [13]. The clonal populations in these specimens may represent the local expansion of a reactive

clone. Another reason for false-positive results is a paucity of T-cells in the sample. In such cases, the PCR reactions may reflect the amplification of DNA from one or two individual T-cells. Repeat PCR will generate a different set of peaks, confirming the absence of clonality. Some "clonal dermatitis" may progress to overt CTCL [23], and in these instances the ability to compare the clonal *TCRG*-PCR peaks in successive biopsies can be very helpful in establishing the diagnosis.

False-negative results may occur in cases where the percentage of neoplastic cells is below the level of detection, in cases where the primers are not directed to the V or J segment involved (i.e., Vγ12 or Jγ1.2), or in cases where a chromosomal translocation involves the TCR locus being tested. The method of analysis can also affect sensitivity. CE-GS is more sensitive than heteroduplex analysis or DGGE. Poor-quality DNA from paraffin-embedded tissue can also lead to false-negatives. The most common V segments used in *TCRG* rearrangement are Vγ1–8, which generate the largest PCR products with the BIOMED-2 primers (195–255 bp). This highlights the importance of including DNA quality controls in the assay and interpretation. In cases where the index of suspicion is high, yet *TCRG*-PCR is negative, Southern blot analysis for *TCRB* or *TCRB*-PCR provides additional sensitivity. Some studies have suggested that the combination of *TCRG*-PCR with *TCRB*-PCR can improve overall sensitivity to between 90 and 94% [6, 16, 24, 25].

Background and Molecular Pathology

A mature T-cell can display one of two different forms of the TCR on its surface: a receptor comprised of an α chain paired with a β chain (α/β T-cells, ~95% of T-cells) or a receptor comprised of a γ chain paired with a δ chain (γ/δ T-cells, enriched at epithelial sites). The somatic rearrangement of these chains during T-cell ontogeny is developmentally programmed such that the δ chain rearranges first, followed by γ, β, and α. In general, the δ, γ, and β chains rearrange early, followed by the expression of both a γ/δ receptor and a pre-TCR comprised of a β chain paired with a pre-Tα chain. Depending on which receptor (if any) generates survival signals, the T-cell will go on to be a γ/δ T-cell (without rearrangement of the α locus) or an α/β T-cell (with destruction of the δ locus during rearrangement of the α locus). Thus virtually all α/β T-cells have at least one rearranged γ locus and most γ/δ T-cells have a rearranged β locus, making the γ and β loci the most attractive targets for clonality studies. Recombination at these loci is mediated by the proteins encoded by the recombination-activating genes *RAG1* and *RAG2*. These recombinases act specifically at recombination signal sequences (RSSs) that flank V, D, and J segments. The RAG recombinases create double-strand breaks that are subsequently repaired by ubiquitously expressed DNA repair machinery, with the addition of *N*-nucleotides by the enzyme TdT. The repaired joints are unique and add to the ultimate variability of the T-cell repertoire.

Multiple Choice Questions

1. Which of the following is NOT an advantage of TCR-PCR over Southern blot?
   A. TCR-PCR evaluates all possible TCR rearrangements
   B. TCR-PCR has a lower threshold of detection
   C. TCR-PCR is compatible with formalin-fixed paraffin-embedded tissue
   D. TCR-PCR is faster
   E. TCR-PCR requires fewer potentially harmful reagents
2. In what order do TCR loci undergo rearrangement?
   A. α then β then γ then δ
   B. β then δ then γ then α
   C. γ then α then β then δ
   D. δ then β then α then γ
   E. δ then γ then β then α
3. Which TCR loci are best suited for gene rearrangement studies?
   A. α and β
   B. α and δ
   C. β and δ
   D. γ and β
   E. γ and δ
4. A skin biopsy with a sparse lymphoid infiltrate shows two low-amplitude peaks following TCR-PCR with CE-GS. Repeat analysis shows two different low-amplitude peaks. Size control primers amplified the sample DNA up to 400 bp. What is the best interpretation?
   A. Consistent with a clonal population of T-cells
   B. Consistent with an inadequate sample

C. Consistent with a paucity of T-cells in the sample (pseudoclonality)
D. Consistent with a polyclonal population of T-cells
E. Consistent with a technical failure

5. A skin biopsy with a moderately dense lymphoid infiltrate shows two prominent peaks following TCR- PCR with CE-GS; however, both the submitting clinician and the pathologist interpreting the slides have a low index of suspicion for mycosis fungoides. What is the best interpretation?
A. Clonal population present, definitive diagnosis of CTCL
B. Clonal population present, reactive clone
C. Clonal population present, uncertain significance
D. Pseudoclonality
E. Technical failure

Answers to Multiple Choice Questions

1. *The correct answer is A.*

TCR-PCR is faster and easier than Southern blotting. It does not require polyacrylamide, ethidium bromide, or radioactivity, is compatible with formalin-fixed, paraffin-embedded tissue, and has a lower threshold of detection. Although TCR-PCR can assess most possible rearrangements, no primer set can detect all possible rearrangements. Southern blotting can theoretically detect all possible rearrangements.

2. *The correct answer is E.*

TCR loci typically rearrange in the order: δ then γ then β then α.

3. *The correct answer is D.*

The γ and β loci are best suited for TCR-PCR. Both loci are rearranged in most T-cells. The δ locus is destroyed during α rearrangement, while the α locus is the most complex and is not rearranged in γ/δ T-cells.

4. *The correct answer is C.*

Different low-amplitude peaks on repeat analysis (pseudoclonality) can indicate a paucity of T-cells in a sample. This is caused by the DNA from only a few cells being amplified in each run. The fact that the sample DNA amplified up to 400 bp with size control primers suggests that the DNA is of sufficient quality and quantity. Such results should not be overinterpreted as clonal.

5. *The correct answer is C.*

A prominent peak represents a clonal population of T-cells but does not give information about whether the population is neoplastic or reactive. In a case where the clinical index of suspicion is low, it is important not to render a definitive diagnosis of malignancy. On the other hand, such results should not be entirely ignored. Clinical followup with rebiopsy at a different site would be a reasonable approach.

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Posttransplant Lymphoproliferative Disorder

13

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Clinical Background

A 32-year-old white male with cystic fibrosis underwent a double lung transplant three months ago. Prior genotyping of the patient's cystic fibrosis transmembrane conductance regulator (*CFTR*) gene indicated that the patient is homozygous for the c.1521_1523delCTT [p.Phe508del] mutation. One brother died of cystic fibrosis at the age of two years. Since the time of transplantation, the patient has been on immunosuppressive therapy (azathioprine and cyclosporine) to prevent graft rejection. Then the patient presented with a fever unresponsive to antibiotics. Physical examination revealed abdominal lymphadenopathy.

Among the initial diagnostic test results was a chest X-ray with multiple lung nodules. An Epstein–Barr virus (EBV) serology panel was negative for viral capsid antigen (VCA) IgG and IgM, early antigen (EA) IgG, and EBV nuclear antigen (EBNA) IgG. Similar serologic results were obtained pretransplant. Histopathology of an excised right upper lobe pulmonary nodule demonstrated nodular angiocentric infiltrates of atypical small to large lymphocytes and plasma cells. Immunohistochemistry revealed CD20 (B-cell antigen) expression in the atypical large cells, CD3 (T-cell antigen) expression in scattered small lymphocytes, and anti-light chain antibodies staining plasma cells at a 4:1 kappa: lambda ratio.

Question 1: What is your differential diagnosis for this patient?

Reason for Molecular Testing

Biopsy of a lung nodule was performed to sort out the differential diagnosis list that includes posttransplant lymphoproliferative disorder (PTLD), infection, graft-versus-host disease, and rejection, each of which is treated quite differently. CD20 expression implied that the atypical cells were of B-lymphocyte lineage. PTLD was suspected based on clinical and histologic grounds. To establish a diagnosis of PTLD and to classify the type of PTLD, further testing for EBV and for lymphoid clonality was indicated.

This patient was EBV seronegative prior to transplant, indicating lack of prior immunity. This placed the patient at increased risk of EBV-related PTLD. Unlike in immunocompetent individuals, serology is not reliable in immunocompromised hosts. Therefore, although posttransplant EBV serology suggested no exposure to EBV, followup molecular testing was indicated to more reliably detect and characterize EBV infection in the setting of iatrogenic immunosuppression.

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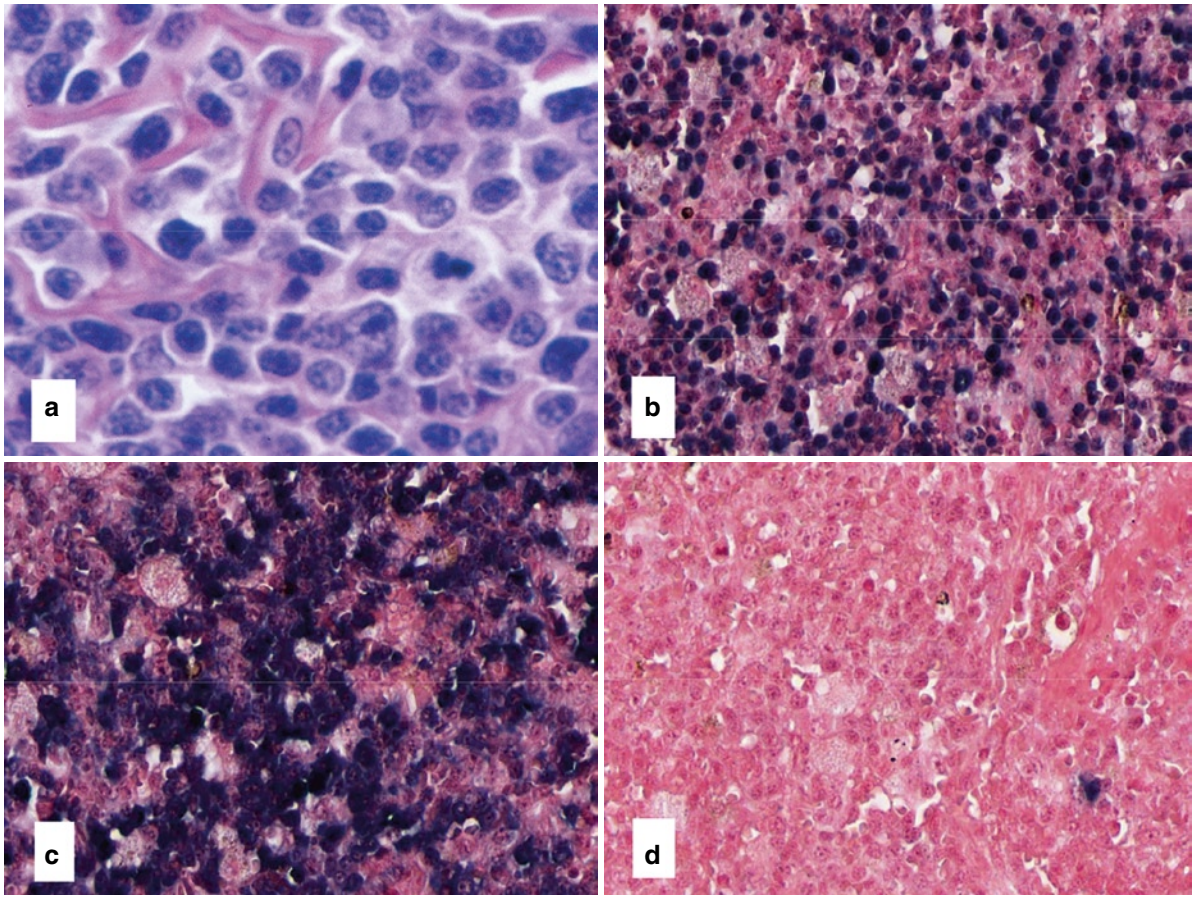

Fig. 13.1 Histochemical stain results. (**a**) H&E (hematoxylin and eosin) stain. (**b**) *EBER* in situ hybridization. (**c**) *Kappa* in situ hybridization. (**d**) *Lambda* in situ hybridization

Immunostains revealed a 4:1 ratio of cells expressing kappa versus lambda immunoglobulin light chain proteins, suggesting the presence of polyclonal plasma cells. However, immunoglobulin in interstitial fluid makes it notoriously difficult to interpret clonality results using immunohistochemical stains. Molecular testing was indicated to more fully explore whether a clonal B-cell population is present.

Test Ordered

Detection of EBV was indicated to assist with the diagnostic and classification process of PTLD and, if a PTLD diagnosis were confirmed, to explore virus-directed therapy and potential for monitoring using a viral load assay. The most informative and practical assays to detect EBV include in situ hybridization for EBV-encoded RNA (*EBER*) on paraffin sections of the biopsy, and EBV viral load testing in blood or plasma.

Molecular testing was indicated to determine whether a clonal population of B lymphocytes was present. The most informative and practical assays to detect B-cell clonality include in situ hybridization to localize and count cells expressing kappa and lambda RNA within lesional cells in paraffin sections, and PCR or Southern blot analysis of the *IGH* and *IGK* genes in frozen or paraffin-embedded tissue.

Question 2: Are these appropriately ordered tests?

Laboratory Test Performed

Molecular assays were performed to detect EBV and to evaluate B-cell clonality. *EBER*, *kappa*, and *lambda* RNA were evaluated by in situ hybridization on paraffin-embedded sections of the lung nodule (Fig. 13.1). EBV viral load was measured by real-time PCR in plasma (Fig. 13.2). Finally, B-cell clonality

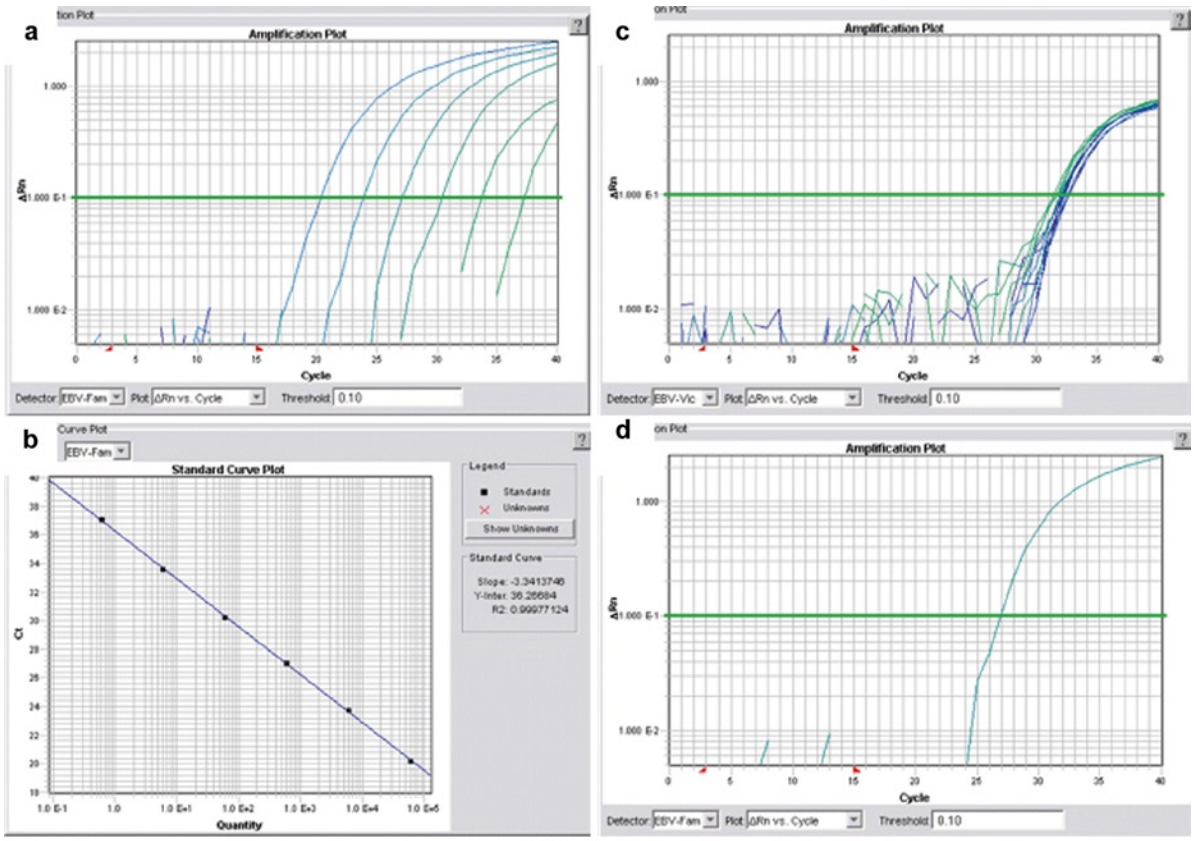

Fig. 13.2 Raw data from the EBV viral load assay. (**a**) The amplification plot reveals accumulation of EBV-targeted PCR products in real time for each of six standards representing serial tenfold dilutions of EBV DNA. In this and other amplification plots, the cycle number is on the *x*-axis and the measured fluorescence is on the *y*-axis, with a horizontal green line representing the threshold. (**b**) The standard curve plots the known starting EBV template amount of each of the six standards on the *x*-axis and the cycle number at which each standard crosses the threshold (*Ct*) on the *y*-axis. (**c**) The amplification plot reveals the recovery of spiked DNA across all specimens in the run, including our patient, by real time accumulation of PCR products. (**d**) The amplification plot for our patient demonstrates the accumulation of EBV PCR products in real time

testing by PCR across the rearranged *IGH* gene was performed on the paraffin-embedded lung nodule (Fig. 13.3). Test methods and quality control procedures are described below.

In Situ Hybridization Test Methods and Controls

EBER in situ hybridization is considered the gold standard assay for detecting and localizing latent EBV infection. Likewise, in situ hybridization to kappa and lambda transcripts permits localization of kappa-versus-lambda-expressing cells. Plasma cells and late-stage B lymphocytes naturally express abundant kappa or lambda transcripts, whereas less-mature B lineage cells may have inadequate light chain RNA expression for interpretation of clonality. These assays are performed on paraffin sections of formalin-fixed biopsy tissue. Riboprobes, oligonucleotide probes, or peptide nucleic acid probes can be used [1]. Interpretation by microscopy, taking into account cytopathologic features and tissue architecture, permits an EBV-related tumor diagnosis and helps establish tumor clonality.

Many commercial products are available to facilitate *EBER*, *kappa*, and *lambda* in situ hybridization [1, 2]. Commercial instruments or reagents from vendors such as Ventana (Tucson, AZ), Leica Microsystems (Bannockburn, IL), and Dako (Glostrup, Denmark) are typically used in clinical laboratories. Briefly, paraffin-embedded sections on coated glass slides are dewaxed and hydrated. Protein and lipid degradation

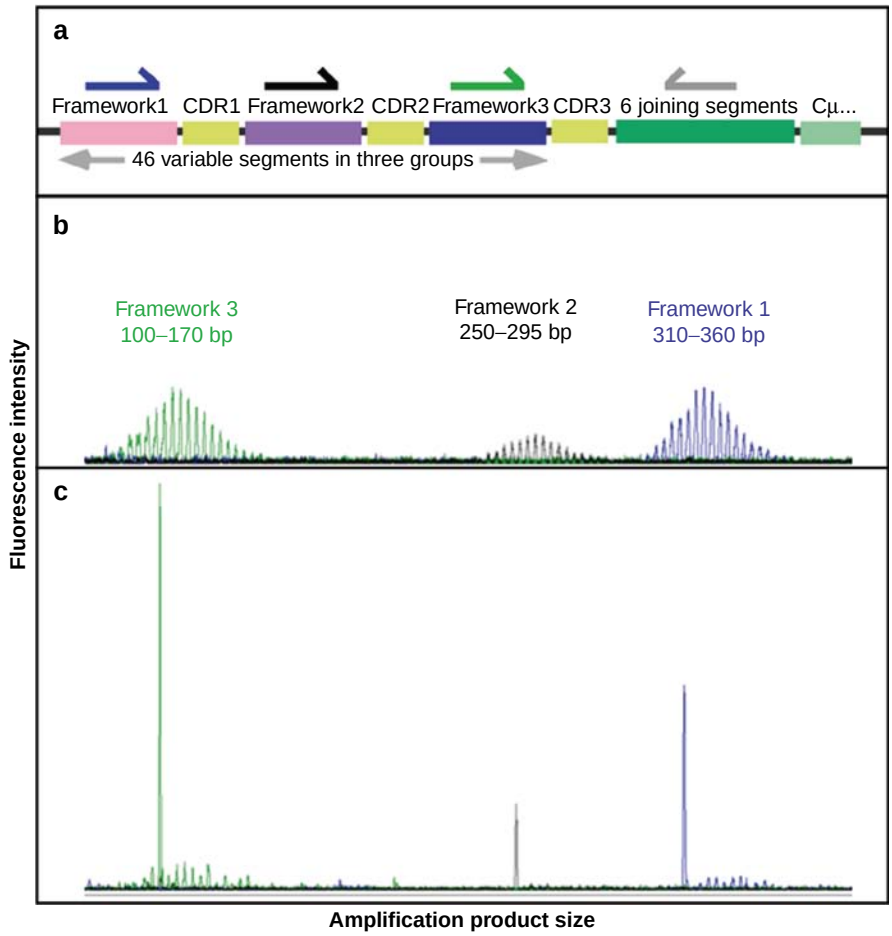

Fig. 13.3 Raw data from the B-cell clonality assay for your interpretation. (**a**) Map of the *IGH* gene depicting primers that amplify across the rearranged *IGH* gene, including a consensus joining region primer and any one of three fluorescence-labeled consensus variable region primers. (**b**) Size distribution of PCR products in reactive tonsil tissue. (**c**) Size distribution of PCR products in our patient's lung nodule

(e.g., proteinase K, detergent) helps make target nucleic acid accessible for probing, and probe hybridization is followed by washing away unbound probe and then detecting bound probe using reagents to identify the probe label (e.g., fluorescein). Subsequently, stained slides are dehydrated, counterstained, and examined by microscopy.

Even though *EBER*, *kappa*, and *lambda* RNA is typically abundant in PTLD tissues, these and other RNA transcripts are subject to degradation by ubiquitous RNase enzymes, potentially causing loss of signal in all or part of the tissue. To avoid false-negative results, and to properly judge the morphologic and cytologic distribution of target RNA in human tissues, it is imperative that controls be evaluated. A control assay targeting a ubiquitous RNA (e.g., U6 RNA) or the poly-A tails of mRNA is typically included to evaluate RNA preservation in each patient tissue. External controls assure that the test system performs as expected. For example, an EBV-related Hodgkin lymphoma might be chosen to assure that the *EBER* assay works as intended, while a lymphoplasmacytic lymphoma or plasma cell dyscrasia might be chosen as a control for kappa and lambda light chain RNA. Endogenous latently EBV infected cells and scattered plasma cells present in many human tissues provide further assurance that the assays work as intended in patient specimens.

Viral Load Test Methods and Controls

EBV viral load measurement is a noninvasive way to detect and monitor levels of EBV DNA in blood or body fluids [2–4]. For our patient, a laboratory-developed EBV viral load assay was performed that relies on quantitative PCR (Q-PCR) on an ABI 7900HT Sequence Detection System (Applied

Biosystems). Briefly, total nucleic acid is extracted from EDTA plasma on a BioRobot EZ1 Workstation (Qiagen) using a commercial kit (EZ 1 Virus Mini Kit, Qiagen) after spiking the plasma with an exogenous DNA sequence (ExoIPC DNA, Applied Biosystems). Accumulation of PCR products is measured in real time based on hydrolysis probe chemistry [5]. Each 30 uL reaction contains TaqMan Universal PCR Master Mix (Applied Biosystems), forward and reverse primers, and FAM-labeled hydrolysis probe targeting the EBV BamH1W segment, TaqMan Exogenous Internal Positive Control Reagents (primers and VIC-labeled probe, Applied Biosystems) to detect the spiked sequence in duplex PCR, and 5 uL of template nucleic acid. Each 96-well plate includes six standards ranging from 1 to 100,000 copies of the EBV genome, as well as positive and negative controls, and no-template control wells. Viral load is calculated by extrapolation from a standard curve with further adjustment for dilution factors. Amplification levels of the spiked control reflect the efficacy of extraction and amplification in individual patient wells. Results of patient and control assays are reviewed by a technologist and a pathologist. EBV viral load is reported in EBV copies/mL of plasma. The assay is precise and linear with a wide dynamic range for measuring the EBV genome [3].

The College of American Pathologists Checklists provides helpful guidance on the many aspects of laboratory practice promoting high quality outcomes. Standards and multiple controls are included in each real-time PCR run to assure that the assay performs as intended. Acceptable ranges are preestablished for assay performance (sensitivity, linearity) as reflected by results in standards and controls, and trends are plotted to detect drift over time. Contamination by extraneous DNA is evaluated in the no-template controls. The system is calibrated at least every six months or whenever a new lot of reagents is introduced or an instrument is serviced.

B-Cell Clonality Test Methods and Controls

Immunoglobulin heavy chain (*IGH*) gene rearrangement testing by PCR evaluates clonality of B-lineage populations regardless of their stage of differentiation. All B-lineage tumors harbor a clonal *IGH* gene rearrangement. In contrast, benign B lymphocytes, as found in normal tissue or inflammatory infiltrates, harbor polyclonal B cells having a variety of *IGH* gene rearrangements.

Although *IGH* gene rearrangement can be analyzed by Southern blot or PCR methods, there is a trend toward multiplex PCR replacing Southern blots in clinical laboratory practice. For our patient, *IGH* gene variable and joining region (VH-JH) primers were utilized to amplify rearranged segments by multiplex PCR and to visualize them by capillary gel electrophoresis.

Briefly, DNA extracted from fresh, frozen, or formalin-fixed paraffin-embedded (FFPE) tissues serves as the template for multiplex PCR using consensus primers targeting conserved framework 1, 2, and 3 variable segments and any of six joining regions in *IGH* (Fig. 13.3a). The primers were originally developed by the Europe Against Cancer Program (BIOMED-2 protocol) and are manufactured by a commercial vendor (InVivoScribe Technologies). In this case, PCR products were sized by capillary gel electrophoresis using an ABI 3130xl Genetic Analyzer (Applied Biosystems).

Clonality is interpreted by evaluating peak patterns, ideally in concert with knowledge of other clinicopathologic findings such as the proportion of B cells and atypical lymphoid cells that are present. Patient specimens are also checked to assure that amplifiable DNA is present at sizes equivalent to or exceeding those expected in the *IGH* amplification assay. Endogenous *IGH* rearrangements may serve this purpose although other gene sequences are typically amplified in a separate reaction to assure that the test system is functional even when little or no *IGH* gene amplification is visible. Reactive lymphoid tissue (tonsil) is run in parallel to demonstrate that the assay performs as intended and to define the typical range of amplicon sizes in polyclonal cells. A no-template control is used to check for contamination of reagents by extraneous DNA. Duplicate PCR facilitates interpretation of test results in patient specimens, as a true clone should have the same dominant peak(s) in both replicates while a paucity of *IGH* templates can yield a pseudoclonal pattern that fails to replicate.

Question 3: What are the advantages and limitations of EBER in situ hybridization compared with an EBV viral load assay?

Results with Interpretation Guideline

EBER, Kappa, Lambda In Situ Hybridization Assay Interpretation Guideline

Histochemical stain results are interpreted by a pathologist who evaluates the external control hybridization to assure that the assay worked as intended on a specimen of known outcome, and who interprets the RNA preservation control which demonstrates which parts of the patient tissue contain hybridizable RNA. Results of *EBER*, *kappa*, and *lambda* hybridization in each patient are then evaluated to estimate the number of cells staining, their distribution, and their cell type based on cytologic features in the context of any lesion that was seen on H&E stain (Fig. 13.1).

Rare *EBER*-positive lymphoid cells are expected in healthy viral carriers, with an estimated frequency of one in a million nucleated blood cells, or a few cells per section in solid tissues. High numbers of infected cells, together with their location, can help sort out whether a given lesion is EBV related [6]. If both *EBER* and RNA preservation control hybridizations reveal no staining, one should consider repeating the assays using a stringent RNase-free technique, or using alternative methods of histochemical localization of latent EBV in paraffin-embedded tissue such as EBNA1 or LMP1 immunohistochemistry, or by using PCR amplification of the viral genome on extracted DNA [2].

Either *kappa* or *lambda* RNA is expressed in the late-stage B cells, including plasmacytoid lymphocytes and plasma cells. Most forms of PTLD harbor differentiated B-lineage cells within the neoplastic clone. A kappa:lambda ratio of >10 or <0.2 is considered evidence of monoclonality [7].

Question 4: Are the in situ hybridization results interpretable, or must controls be evaluated before interpreting the findings in Fig. 13.1?

EBV Viral Load Assay Interpretation Guideline

Analytic interpretation is performed to obtain a reportable result, and clinical interpretation is performed to judge its clinical significance. Analytic interpretation involves checking the standard curve to assure that results are within preestablished limits, for example, sensitive for detecting low level EBV and linear across the dynamic range. No-template controls included in every run, and an EBV-negative specimen control, should be negative for EBV DNA, and any amplification signal should be investigated for possible contamination by extraneous DNA (e.g., amplicons from prior assays). High and low EBV-positive specimen control results should be within expected ranges.

In each patient specimen, an amplification control is used to assure that extraction and amplification were efficient based on sufficient amplification of a spiked control sequence. Next, any viral DNA amplification in the patient specimen is visualized on the amplification plot (Fig. 13.2d), and the curve location and appearance are evaluated for consistency with the extrapolated value calculated by the software using the standard curve.

Clinical interpretation is done in the context of the patient presentation, the specimen type, and the performance characteristics of the assay. The assay's analytic variation (coefficient of variation), as established by replicate extraction and analysis of the same specimen during assay validation, helps interpret whether serial values are significantly different from each other. In this particular assay, serial EBV loads differing by more than threefold are considered a significant change whereas smaller changes lie within the analytic variability of the assay. Levels above 500 copies/mL of plasma are considered abnormal in allogeneic transplant recipients, thus triggering a search for PTLD or preemptive therapy to prevent incipient PTLD, whereas lower values can be found in healthy recipients. For both screening a high risk patient and for monitoring PTLD during therapy, trends in viral load tend to be more informative than a value at a single time point.

IGH Gene Rearrangement Assay Interpretation Guideline

Antigen receptor gene rearrangement assays are among the most challenging laboratory tests done in clinical settings. Here, too, analytic interpretation is performed to obtain a reportable result, and clinical interpretation is performed to judge its clinical significance. For analytic interpretation, controls are evaluated to show that the assay performed as intended, and that adequate amplifiable DNA is present in the patient sample. Lack of large amplicons would indicate DNA degradation

and is especially prevalent in FFPE samples. The no-template (blank) control should display no peaks in any of the amplification reactions, and detection of any peaks triggers an investigation of possible contamination by extraneous DNA. The polyclonal control is a bell-shaped curve of peaks differing by three bases, and the range of amplicon sizes expected for each reaction is defined by upper and lower size limits of the Gaussian distribution. Each of three Gaussian curves is colored differently depending on which fluorochrome was present on the V region primer, thus permitting each peak to be assigned to a given framework. The patient's peak pattern is examined for evidence of polyclonal (Gaussian distribution) and/or monoclonal (dominant) *IGH* rearrangements. Replicate PCRs should produce similar *IGH* gene rearrangement patterns if the result is truly representative of multiple B cells in the patient specimen. If a clonal population of cells is present, replicate testing should produce a clonal peak, of identical size, color (framework), and similar relative peak height as the original assay. Some laboratories use one tenth of the original amount of template DNA in the replicate test because occasionally, especially in FFPE, a smaller template amount improves productivity by diluting PCR inhibitors. Although semiquantitative methods are a subject of controversy, a rough guideline is that a clonal peak should have a height ≥2.5 times that of the region on the bell-shaped curve in which the peak is located. Note that there may be two dominant peaks per clone if *IGH* rearrangements are biallelic. The same clonal *IGH* rearrangement may be visible as a dominant peak in one, two, or all three frameworks, depending on what part of the variable region was spliced and the degree of somatic hypermutation. In a worst case scenario, estimated to occur in about 9% of mature B-cell neoplasms, false-negative results are obtained when no amplification of the rearrangement occurs in any framework [8].

The size of a given peak may be estimated by comparing it to a size ladder. Followup specimens from the same patient may be interpreted as persistent tumor if the same sized amplicon still dominates.

The clinical significance of a clonal *IGH* gene rearrangement varies by clinical situation. In the setting of suspected PTLD, a clonal *IGH* gene rearrangement demonstrates presence of neoplastic lymphocytes, which helps confirm the diagnosis. Negative results do not rule out a diagnosis because some PTLDs are polyclonal (especially "early lesions"), are of T-cell lineage, or otherwise lack demonstrable dominant peaks by the *IGH* clonality assay. Results of *IGH* gene rearrangement assays should always be interpreted in the context of available clinicopathologic information, such as morphology, and other laboratory tests including T-cell receptor gene rearrangement assays when appropriate. Questionable B-cell clonality results can be evaluated using complementary methods: *IGK* PCR, Southern blot analysis of *IGH* or of the EBV genome structure, DNA sequencing of the *IGH* amplicons, FISH for translocations, and nonmolecular methods (flow cytometry, immunohistochemistry, karyotype). While clonality is suggestive of malignancy, clonality should not be used as the sole criterion for diagnosis of lymphoid malignancy. Clonal *IGH* rearrangements are not exclusively detected in B-cell malignancies. Some B-cell clones are transient or are proposed to be preneoplastic, and some T lineage or myeloid malignancies exhibit *IGH* rearrangement.

Result Interpretation

Tissue-Based Molecular Testing

The number of *EBER*-expressing cells far exceeds that found in normal tissue. The ratio of *kappa* RNA compared to *lambda* RNA-expressing cells is far above the 10:1 cutoff for demonstrating clonal light chain restriction. These results, combined with histopathologic evidence of CD20-expressing atypical large cells in the setting of a lung transplant recipient with fever, lymphadenopathy, and multiple lung nodules, are diagnostic of EBV-related polymorphous PTLD.

Immunohistochemistry failed to detect light chain restriction whereas in situ hybridization clearly demonstrated clonality as evidenced by *kappa* and not *lambda* RNA in the vast majority of lesional cells. It is likely that the immunostain results were misleading as a consequence of extracellular immunoglobulin confounding localization of light chain antigens to the lesional cells. This problem has led many laboratories to abandon protein-based histochemistry in favor of RNA-based histochemical localization of light chain products. If the tumor had been less well differentiated, then both protein and RNA-based histochemical stains might have failed to detect clonality by virtue of insufficient light chain gene expression. If such were the case, *IGH* gene rearrangement testing would have

been quite useful since it demonstrated monoclonality and therefore supported the neoplastic nature of the lymphoid infiltrate.

A few polyclonal *IGH* genes are present in the tissue, as shown by the spectra of short peaks below the single dominant clonal peak generated by framework 1, 2, and 3 primer sets (Fig. 13.3c). The presence of rare non-neoplastic plasmacytic cells was confirmed by *lambda* RNA in situ hybridization.

Blood-Based Molecular Testing

An EBV viral load result of 5,000 copies/mL of plasma exceeds the threshold of 500 copies/mL that was established during validation studies as being specific for PTLD. The standards produce a linear standard curve with approximately 3.3 cycles between each serial tenfold dilution of EBV DNA, as expected. (If PCR efficiency is 100%, the amount of product should double with each cycle, and the slope is 3.3 since $2^{3.3} = 10$). Spiked DNA shows equivalent recovery in all specimens in the run, implying good extraction and amplification efficiency (lack of inhibitors) for all specimens. This high EBV load would trigger a search for possible EBV-related disease, even in the absence of signs and symptoms of PTLD.

Question 5: The patient was treated with reduction of immunosuppression (imuran was discontinued; cyclosporine was decreased) and addition of an antiviral agent (acyclovir). Which molecular test(s) is/are most appropriate for monitoring the efficacy of therapy?

Further Testing

In PTLD patients, EBV viral load assays are used not only to facilitate diagnosis but also to monitor the efficacy of treatment. Our patient was treated by cutting back on immunosuppressive drugs to enhance tumor recognition and destruction by the patient's own immune system. This is an effective way of reversing the propagation of infected lymphocytes. An antiviral agent (acyclovir) was used in our patient to minimize viral replication, although the evidence for utility of antiviral therapy is uncertain given that much of the infection is latent rather than replicative. The EBV viral load fell in our patient from 5,000 to 500 copies/mL of plasma within a few days of intervention, and the clinical signs and symptoms resolved over the ensuing days.

Because PTLD is such an aggressive and potentially fatal complication of allogeneic transplantation, preventive measures are taken to minimize risk. Recipients are tested for prior exposure to EBV by serology just prior to transplant, and active primary infection is considered a contraindication to transplantation. Patients who are judged to be at high risk of PTLD are monitored for EBV viral load at regular intervals after transplantation [4]. This permits early intervention to thwart frank neoplasia or helps prevent progression of PTLD.

Background and Molecular Pathology

Molecular tests complement morphology and immunohistochemistry in the diagnosis and classification of PTLD. Suspicion of PTLD is based on clinical grounds, such as fever, malaise, lymphadenopathy, and extranodal masses that often involve the grafted organ. EBV viral load on blood is a fairly sensitive and specific approach for narrowing the differential diagnosis since most PTLDs are EBV-related and are associated with high levels of circulating EBV DNA in whole blood and plasma.

High EBV loads are not specific for PTLD. Primary infection causes transient inflammatory lesions in healthy individuals (with or without a clinical diagnosis of infectious mononucleosis). The reactive lymphocytosis that is characteristic of infectious mononucleosis is comprised in small part by infected B lymphocytes and in larger part by EBV-negative T lymphocytes that are so important in immune recognition and control of the virus. High EBV loads are also characteristic of a wide spectrum of EBV-related malignancies, such as some cases of non-Hodgkin lymphoma (selected B, T, or NK cell types), Hodgkin lymphoma, nasopharyngeal carcinoma, gastric adenocarcinoma, and immunodeficiency-related neoplasms.

The diagnosis of PTLD requires pathologist evaluation of biopsy tissue as described in the World Health Organization (WHO) classification system. Further subclassification into four major types (early lesion, polymorphic PTLD, monomorphic PTLD, or classic Hodgkin lymphoma-type) is based, in part, on

molecular features. An early lesion tends to be polyclonal and does not efface tissue architecture. The more advanced subtypes of PTLD tend to be monoclonal and effacing. Polymorphic PTLD is characterized by an infiltrate of small to large lymphocytes and immunoblasts as seen in our patient, while monomorphic PTLD contains sheets of atypical large lymphocytes. Classic Hodgkin lymphoma-type PTLD is less common and exhibits Reed–Sternberg cells of the usual Hodgkin lymphoma immunophenotype [9].

Even though PTLD biopsy may resemble various benign or malignant lymphoproliferations, ranging from infectious mononucleosis to non-Hodgkin or Hodgkin lymphoma, these lesions are classified as PTLD when they occur in a transplant setting because of their unique biology, distinctive therapeutic strategies, and urgent need for clinical response. Restoring immunity by reducing immunosuppression is a cornerstone of PTLD therapy that can be effective even in monoclonal cases. Other therapies include anti-CD20 antibody, infusing donor T cells, and infusing ex vivo generated cytotoxic T cells that are EBV-specific and HLA-matched, and antiviral therapy. Radiation and multidrug chemotherapy are used for aggressive or nonresponsive tumors. Vaccination and novel therapies are being explored. Practice guidelines recommend routine monitoring of high risk solid organ or stem cell recipients so that preemptive therapy is possible [4].

Immunosuppression, particularly T-cell dysfunction, seems to be the critical factor leading to active EBV infection and heightened risk of EBV-related neoplasia. Certain drugs used to prevent graft rejection or to stifle autoimmune disease (e.g., methotrexate) are associated with EBV-related lymphoma, as are certain inherited immunologic deficiencies (e.g., Wiskott–Aldrich syndrome) as well as the diminished immunity of old age [9].

EBV is a double stranded DNA virus whose genome lies latent within the nucleus of a small fraction of B lymphocytes in all humans who have been previously infected. Periodic reactivation with shedding of virions in saliva promotes transmission to nearly every human before adulthood. Primary infection is characterized by high levels of EBV DNA in whole blood or plasma, followed within a couple of months by immune control of the infection and disappearance of the virus from plasma. Subsequent EBV-associated neoplasia (e.g., PTLD) is accompanied by elevated levels of EBV DNA in blood specimens of affected patients, including extracellular EBV DNA measurable in plasma. EBV viral load, as measured by quantitative molecular analysis of the viral genome, serves as a biomarker for predicting and monitoring the course of PTLD.

Immunosuppression at the time of primary infection, as occurred in our patient as evidenced by negative EBV serology pretransplant, may limit the humoral and cell-mediated responses that normally keep viral infection in check, thus increasing the likelihood of viral reactivation and neoplastic transformation. Secondary genetic defects are thought to drive progression to frank neoplasia. Gross chromosomal changes and/or mutation and epigenetic silencing of a wide range of genes have been described [4]. It is speculated that the process of somatic hypermutation in B lymphocytes contributes to acquired genetic defects driving neoplasia.

Regardless of any lymphomagenic mutation that a given PTLD might contain, nearly all PTLDs, with the notable exception of some early lesions, harbor clonal immunoglobulin (*IGH* and *IGK*) gene rearrangement with light chain restriction, indicating that they are comprised of neoplastic B cells. The cell of origin is typically a donor-derived B lymphocyte for marrow or stem cell transplant patients, although it is a recipient-derived B lymphocyte in solid organ transplant patients. Rarely PTLD arises from another cell type such as a T cell or NK cell, and these unusual cases are also less likely to be EBV related.

IGH gene rearrangement is a sign of commitment to the B-cell lineage. *IGK* rearrangement occurs later but before the mature B-lymphocyte stage of differentiation. All lymphoid malignancies arise when a single lymphocyte, harboring a particular set of gene rearrangements, goes awry and proliferates out of control. The particular set of gene rearrangements is inherited by all tumor cell progeny. Therefore, malignant lymphomas and lymphoid leukemias are characterized by clonal gene rearrangement, while benign reactive lymphoid hyperplasias are not. The particular rearrangement is specific to a given tumor, and when the tumor recurs it generally has the identical rearrangement that was identified in the parental tumor. In patient biopsies where diagnostic uncertainty remains after microscopy, gene rearrangement studies are often helpful in resolving whether a lesion is polyclonal (reactive) or monoclonal (more likely neoplastic). Kappa and lambda light chain restriction, as shown by in situ hybridization to RNA or immunologic detection of protein, is quite useful in demonstrating clonality in

the more mature lymphoid and plasmacytic neoplasms. Because clonality is not synonymous with malignancy, clinicopathologic correlation is required.

Interestingly, EBV-infected tumors may be evaluated for clonality with respect to the structure of the EBV genome, because each infected cell has a relatively unique fused terminal repeat structure within the viral genome, once it circularizes inside the cell. While Southern blot analysis of the viral terminal repeats can be used to assess clonality, its clinical utility is limited because there are many ways to evaluate clonality in lymphoid lesions [2].

PTLDs tend to be monoclonal and aggressive, except for early lesions that are often polyclonal and relatively benign [10, 11]. Oligoclonal PTLDs are rare and are thought to represent independent tumors arising synchronously in a host who is exquisitely susceptible.

Multiple Choice Questions

1. When EBV viral load testing is done in a patient suspected of having PTLD, which sample type is most informative?
   A. Biopsy of the grafted organ
   B. Buccal cells or saliva
   C. Cultured lymphocytes
   D. Lymph node biopsy
   E. Peripheral blood or plasma
2. If the *EBER* in situ hybridization in our patient's lung nodule revealed almost no *EBER*-positive nuclei, while the control hybridization showed adequate RNA preservation, then which of the following interpretations would be most appropriate:
   A. A completely negative *EBER* in situ hybridization result
   B. An indeterminate result
   C. A weak positive *EBER* in situ hybridization result
   D. *EBER*-positive cells have lost their nuclear localization during mitosis
   E. The lesion is not EBV related but this does not exclude a diagnosis of PTLD
3. To detect EBV in lesional cells of a paraffin-embedded biopsy tissue, the most informative assay is
   A. In situ hybridization targeting EBV-encoded RNA (*EBER*)
   B. In situ hybridization targeting messenger RNA using an oligo dT probe
   C. Qualitative PCR targeting the EBV genome
   D. Southern blot analysis of immunoglobulin (*IGH* and/or *IGK*) gene rearrangement
   E. Southern blot analysis of the EBV genome
4. To establish B-cell clonality in a paraffin-embedded biopsy tissue suspected of having PTLD, the most informative assay is
   A. Immunohistochemistry targeting kappa and lambda light chains
   B. In situ hybridization targeting of kappa and lambda genes
   C. In situ hybridization targeting of kappa and lambda gene transcripts
   D. In situ hybridization targeting of kappa and lambda protein
   E. Southern blot analysis of immunoglobulin (*IGH* and/or *IGK*) gene rearrangement
5. Which of the following scenarios places a recipient at highest risk of developing PTLD after stem cell transplantation?
   A. A history of infectious mononucleosis pretransplant
   B. Being an autologous donor
   C. Being EBV seronegative at the time of transplant
   D. Having a cord blood donor
   E. Having an EBV seropositive donor

Answers to Questions Embedded in the Text

Question 1: What is your differential diagnosis for this patient?

The clinical and histopathologic findings are suspicious for PTLD. Key clinical features are a history of allogeneic transplant with iatrogenic immunosuppression, fever, lymphadenopathy, and multiple lung nodules. The key histopathologic finding is the presence of atypical large B lymphocytes in the lung biopsy. A 4:1 ratio of light chain protein expression suggests polyclonal plasma cells, although this assay is notoriously difficult to interpret due to extracellular light chain interference. EBV serology suggests no prior exposure to EBV, although immunosuppressive therapy might render serology false-negative. The differential diagnosis includes: (1) PTLD, either a polytypic early lesion or polymorphic subtype, (2) infection, (3) graft-versus-host disease, and (4) organ rejection.

Question 2: Are these appropriately ordered tests?

The tests are appropriate. *EBER* in situ hybridization helps establish whether a lesion contains latent EBV infection, or whether a tumor is EBV related. The EBV viral load assay is a noninvasive way to assess elevated EBV DNA levels associated with a PTLD diagnosis. Serial monitoring of EBV load assists in monitoring the efficacy of treatment for EBV-related PTLD.

B-cell clonality detection by *kappa* and *lambda* RNA in situ hybridization is the most appropriate assay for evaluating clonality of late-stage B cells or plasma cells in paraffin sections. PCR or Southern blot analysis of immunoglobulin heavy or light chain gene (*IGH* and *IGK*) rearrangements can help to establish clonality of a B-cell infiltrate regardless of the differentiation stage.

Question 3: What are the advantages and limitations of EBER in situ hybridization compared with an EBV viral load assay?

The *EBER* in situ hybridization assay is useful when microscopy can be used to evaluate results in the context of cell type and tissue architecture. Because *EBER* RNA is so abundant, the assay performs well in paraffin-embedded tissues and can be used to define a lesion as being EBV related. Efficacy is limited when RNA quality is poor.

The EBV viral load assay is typically applied to body fluids that are less invasively collected, such as whole blood or plasma. Because viral load typically reflects disease burden, the assay is convenient to determine presence or absence of disease in high risk individuals and to serially monitor disease status during therapy. Elevated viral load cannot be used to differentiate the type of EBV-related disorder.

Question 4: Are the in situ hybridization assays valid, or must controls be evaluated before interpreting the findings in Fig. 13.1?

The RNA preservation control stain was not shown, and the positive control slides for each of the three hybridization assays were not shown in Fig. 13.1. Technically, these controls should be evaluated prior to evaluating patient results. Nevertheless, the patient specimen contains numerous cells positive for *EBER* and *kappa* RNA, suggesting that RNA was well preserved. The pattern of staining (nuclear for *EBER*, cytoplasmic and nuclear for *kappa* and *lambda*) and its localization to lymphoid cells combined with lack of staining in some stromal cells suggest legitimate localization of each analyte. Rare cells expressing *lambda* RNA serve as an endogenous indicator that the lambda hybridization assay was operational.

When multiple molecular assays are being done simultaneously on a given patient specimen, it is reasonable to consider whether the results of one assay could serve as control for another assay. While it might seem superfluous to do an RNA preservation control in this case, or to run this RNA preservation control only when the assays of interest are negative, a delayed diagnosis is not tolerable in the case of PTLD workups, as patients can rapidly progress to a fatal conclusion when therapy is delayed. In addition, each assay is often treated as a self-contained quality unit.

Question 5: The patient was treated with reduction of immunosuppression (imuran was discontinued; cyclosporine was decreased) and addition of an antiviral agent (acyclovir). Which molecular test(s) is/are most appropriate for monitoring the efficacy of therapy?

EBV DNA is present within all of the neoplastic cells and serves as a tumor marker. EBV viral load measurement is a noninvasive laboratory assay that can be followed in serial blood collections. The level of 5,000 EBV copies/mL at diagnosis establishes a baseline for serial monitoring of plasma EBV loads during therapy.

Answers to Multiple Choice Questions

1. *The correct answer is E.*

   High levels of EBV DNA are found in the circulation of patients with PTLD, while uninvolved sites are expected to have little or no detectable EBV DNA.

2. *The correct answer is E.*

   This scenario differs from the case described in this chapter. If rare cells express *EBER* then choice A is incorrect for technical reasons. Because the RNA preservation control assay shows that hybridizable RNA is present, the *EBER* result is interpreted as clinically negative with respect to localization of EBV to lesional cells, and EBV-negative PTLD remains in the differential diagnosis. Results should be correlated with EBV viral load results in blood or plasma. A low to negative viral load confirms that the patient's signs and symptoms are unrelated to EBV. Rarely, cases of PTLD are EBV negative. As an aside, if the RNA preservation control assay and the *EBER* hybridization are both negative, then results should report as indeterminate with respect to EBV status, and repeat testing or alternative strategies should be used to test for EBV.

3. *The correct answer is A.*

Although EBV viral load assays can be adapted to work on paraffin-embedded tissue, *EBER* in situ hybridization has the advantage of localizing the latent EBV infection to particular lesional cells. Southern blot analysis is usually not interpretable on paraffin-embedded tissue samples due to insufficient high molecular weight DNA.

4. *The correct answer is C.*

In situ hybridization targeting gene transcripts is easier to interpret and is more informative for showing clonality than is immunohistochemistry targeting protein produced from light chain genes. In situ hybridization targeting kappa or lambda *genes* should show approximately two copies/cell of each human gene, but this information would not be informative with respect to clonality. Southern blot analysis is usually not interpretable on paraffin-embedded tissue samples due to insufficient high molecular weight DNA.

5. *The correct answer is C.*

Lack of prior immunity against EBV is a major risk factor for PTLD, as the infection occurs once the patient is already immunosuppressed.

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Recommended Reading

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HIV-Associated Hodgkin Lymphoma

14

George Fedoriw and Margaret L. Gulley

Clinical Background

A 43-year-old man presented with a two month history of intermittent fevers and diarrhea. He was diagnosed with human immunodeficiency virus (HIV) infection and over the ensuing seven years had not been regularly compliant with his highly active antiretroviral therapy (HAART). Past medical history was significant for hypertension, type 2 diabetes mellitus, and a poorly defined seizure disorder.

Besides fatigue and perceived weight loss, a thorough review of systems was unremarkable. On physical examination, the patient was pale and febrile with mild abdominal discomfort upon palpation. Widespread lymphadenopathy was noted, with the largest lymph node measuring 2 cm, located in the right axilla. Complete blood count with differential revealed pancytopenia (Table 14.1).

The absolute CD4 T-cell count was low at 148 cells/uL (reference range 510–2,320). Blood samples were sent to the microbiology lab for bacterial and fungal cultures, and empiric treatment with broad spectrum antibiotics and antifungals was initiated. All cultures eventually proved negative, and both the fevers and cytopenias persisted. A lymph node biopsy was performed to investigate the cause of the lymphadenopathy, and a bone marrow biopsy was done for persistent cytopenias.

Biopsy of the largest axillary lymph node revealed histopathologic changes associated with HIV infection, namely involution of germinal centers and increased collections of plasma cells and perivascular monocytoid lymphocytes. There was no morphologic evidence of malignant lymphoma, which was further supported by negative flow cytometric and karyotype analyses.

Question 1: What molecular tests should be ordered?

Reason for Molecular Testing

HIV patients are at significant risk of developing comorbid conditions related to progressive immune dysfunction [1]. Opportunistic infection by bacteria, fungi, and viruses represents the greatest threat. The likelihood of developing neoplastic disorders, including vascular, hematopoietic, and epithelial malignancies, is also markedly increased. Lymphadenopathy is a common and nonspecific feature of HIV-positive individuals in both the acute and chronic stages of infection. Although lymphadenopathy may remain

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Table 14.1 Laboratory data on admission

Complete blood count	White blood cell differential	
WBC 3.2×10^9/L (4.5–11)	Neutrophils	2.5×10^9/L
RBC 2.9×10^{12}/L (4.5–5.9)	Lymphocytes	0.2×10^9/L
HGB 8.5 g/dL (13.5–17.5)	Monocytes	0.1×10^9/L
HCT 24.7% (41.0–53.0)	Eosinophils	0.1×10^9/L
MCV 85fL (80–100)		
PLT 134×10^9/L (150–440)		

unchanged for many years in an otherwise stable clinical setting, enlarging lymph nodes and other rapid clinical changes may herald a dire pathologic process associated with progressive immune dysregulation. Identifying the underlying etiology of lymphadenopathy and related symptoms relies heavily on an understanding of HIV progression to acquired immune deficiency syndrome (AIDS), as the relative likelihood of specific etiologies is differentially related to the degree of ongoing HIV replication and the state of host immune competence. Rapid and sensitive molecular identification of pertinent viral pathogens can direct further diagnostic procedures or therapeutic intervention.

Test Ordered

The following molecular tests were ordered for this patient:

- HIV viral load on plasma
- Epstein–Barr virus (EBV) viral load on plasma
- Cytomegalovirus (CMV) viral load on plasma
- Human herpesvirus 8 (HHV8) PCR on whole blood

Laboratory Test Performed

HIV viral load testing was done on plasma by quantitative reverse transcription polymerase chain reaction (Qrt-PCR) to directly assess infectious burden of this retrovirus. The results reflect the efficacy of antiretroviral therapy and the immunodeficiency state, which in turn impacts the risk of opportunistic infection and neoplasia [2]. Preanalytic variables are crucial to accurate HIV measurement, so careful specimen collection and handling are essential to preserving RNA integrity for downstream reverse transcription and cDNA amplification [3]. Amplicon contamination issues are largely surmounted by automation and limited sample manipulation, but appropriate controls are essential to demonstrate lack of false-positive results in matrix-appropriate negative control(s). Additional matrix-appropriate controls include low and high positives to demonstrate assay sensitivity and linearity, as well as efficacy of extraction.

Similarly useful are assays to detect and quantify other viral agents commonly implicated in HIV-associated disease. EBV and CMV viral loads were measured in plasma by Q-PCR targeting the DNA genome of each virus. Quantification is important because these viruses may be present at a low level in healthy individuals, while high levels imply virus-related disease [4]. The controls are similar to those described for HIV viral load assays. HHV8, a virus that is partly homologous to EBV and CMV and consequently is classified as member of the herpesvirus family, is not as ubiquitous as EBV or CMV in the general population. EBV, CMV, and HHV8 are frequently pathogenic in immunosuppressed hosts.

Results with Interpretation Guideline

Viral Load Results

- HIV viral load: Undetectable to a sensitivity of 50 copies/mL of plasma (normal range: Undetectable)
- EBV viral load: 6,098 copies/mL of plasma (normal range: Undetectable to a sensitivity of 250 copies/mL of plasma)
- CMV viral load: Undetectable (normal range: Undetectable to a sensitivity of 500 copies/mL of plasma)
- HHV8 viral DNA: Not Detected in whole blood (normal result: Not detected to a sensitivity of 50 copies/PCR)

See Chapter 13 for an example of viral load measurement and further guidance on analytic interpretation of real-time PCR assays including exogenous and endogenous controls.

Question 2: What is your clinical interpretation of the viral load results, and how should these results guide patient management?

Result Interpretation

Viral Load Clinical Interpretation

Generally undetectable HIV in plasma suggests effective viral control, but such an interpretation is likely to be misleading in this case. The reported spotty compliance with his HAART regimen and the low CD4 level implies that recent HAART use reduced the viral load to undetectable levels, but immune dysregulation had already been realized [5].

Some of the common viral mediators of HIV-related disease, including EBV, CMV, and HHV8, were measured by sensitive molecular means to assess their possible contribution to fevers and lymphadenopathy. While CMV and HHV8 were not detected, the high copy number of EBV DNA implied active EBV-related disease. Despite the high prevalence of EBV infection in the general adult population, EBV DNA is not typically detectable in the plasma of healthy individuals. In patients whose immune system is compromised by active HIV infection, EBV DNA may be detected in plasma where it could reflect viral replication (virion production) or could be a sign of EBV-related neoplasia [6]. The likelihood of lymphoma is already increased in the immunosuppressed HIV-infected population, and a high copy number of circulating EBV, as seen in this patient, demands further investigation of EBV-related disease [2, 6].

Further Testing

In light of the reactive lymph node evaluation, continued pancytopenia, and evidence of EBV-related disease, a bone marrow biopsy was performed.

Question 3: What are the morphologic findings in Fig. 14.1?

Marrow Histopathology

Assessment of H&E stained sections revealed a hypercellular bone marrow with preserved trilineage hematopoiesis. Scattered large abnormal cells were identified among pale-staining zones of marrow space associated with increased eosinophils, neutrophils, and plasma cells. Rare abnormal cells were binucleated with prominent eosinophilic nucleoli, pathognomonic for the Reed–Sternberg (R–S) cells of classical Hodgkin lymphoma (HL). The R–S/Hodgkin cells expressed the B-cell specific antigen, PAX5, and other traditional markers of HL, including CD30 and CD15 by immunohistochemical staining. The malignant cells were negative for the lymphocyte marker CD45, the B-cell antigen CD20, and the T-cell antigen CD3, a pattern further supporting the diagnosis of classical HL.

Question 4: What molecular assays are indicated given the findings in the marrow?

Molecular Analysis of the Bone Marrow and Interpretation Guideline

Epstein–Barr encoded RNA (*EBER*) in situ hybridization was performed on a paraffin section of the marrow (Fig. 14.2). In this assay, an *EBER*-specific digoxigenin-labeled nucleic acid probe was incubated on deparaffinized sections where it hybridized to target RNA. Unbound probe was removed and a secondary reporter was used for signal amplification, in this case a polymer linked to antidigoxigenin antibody and to multiple horseradish peroxidase molecules. Enzymatic activity of the horseradish peroxidase causes 3,3′-diaminobenzidine (DAB) to form an insoluble blue precipitate that marks the location of *EBER*. The tissue is then counterstained with eosin to allow histopathologic evaluation of *EBER* stain results in the context of cytology and tissue architecture. *EBER* is localized to the nucleus of latently infected cells and is visible in the cytoplasm only during mitosis. Microscopic evaluation of the types and distribution of *EBER*-expressing cells helps a pathologist interpret the histopathologic findings, in concert with the H&E stain and other clinicopathologic data. Controls include an external control to demonstrate that the assay performed as expected on a known EBV-related lesion, and an endogenous control to demonstrate that RNA was adequately preserved in the patient specimen.

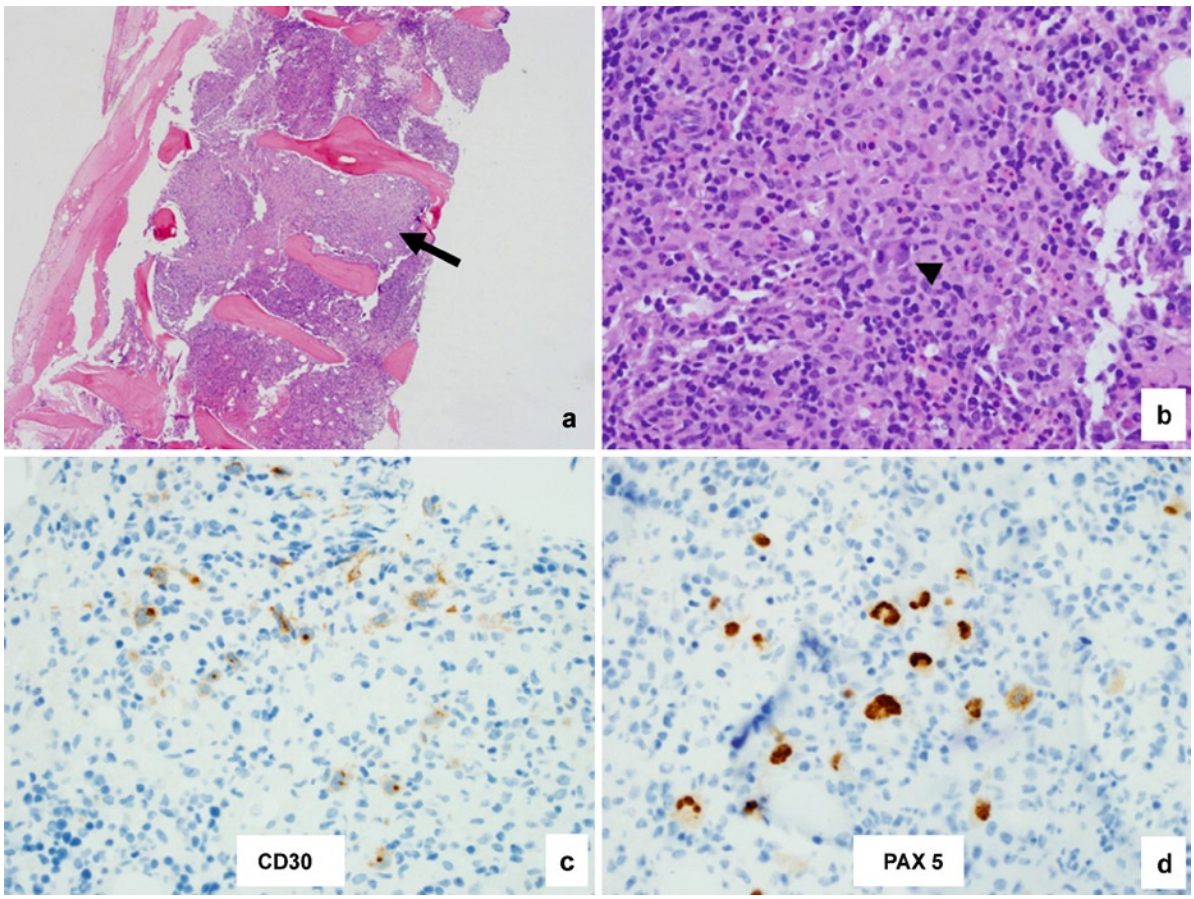

Fig. 14.1 Bone marrow biopsy findings. (**a**) H&E stained biopsy section with focal areas of pale staining (*arrow*). (**b**) High power view of one of the focal areas reveals an atypical large cell (arrowhead). (**c**) CD30 and (**d**) PAX5

Question 5: In Fig. 14.2, what is the EBER in situ *hybridization result, and how should the marrow findings guide patient management?*

Molecular Histochemical Results and Histopathologic Diagnosis of the Marrow Biopsy

In situ hybridization demonstrated that the external control behaved as expected and that there was adequate RNA preservation in the patient's marrow specimen. *EBER* signal was localized to the nuclei of atypical large cells in the patient's marrow, confirming that the marrow lesion was EBV associated. The *EBER*-expressing cells had cytologic features of R–S/Hodgkin cells. In light of the clinical history and histopathologic findings, the marrow was diagnostic of EBV-related classic HL.

Other Considerations

In this case, it was clear that *EBER* was localized to the R–S/Hodgkin cells, confirming that this neoplasm was EBV associated. An alternative strategy for demonstrating EBV is to PCR amplify a segment of the EBV genome in DNA extracted from the paraffin-embedded tissue; however, this "grind and find" strategy fails to distinguish whether the EBV signal is localized to neoplastic R–S/Hodgkin cells or to reactive-appearing "background" lymphocytes, a distinction that is critical for determining whether this is an EBV-related malignancy.

The role of viral infection in tumorigenesis remains uncertain. Nevertheless, consistent presence of EBV in HIV-related HLs not only improves diagnostic capabilities, but also demonstrates therapeutic promise. EBV antigens expressed in infected cells can serve as

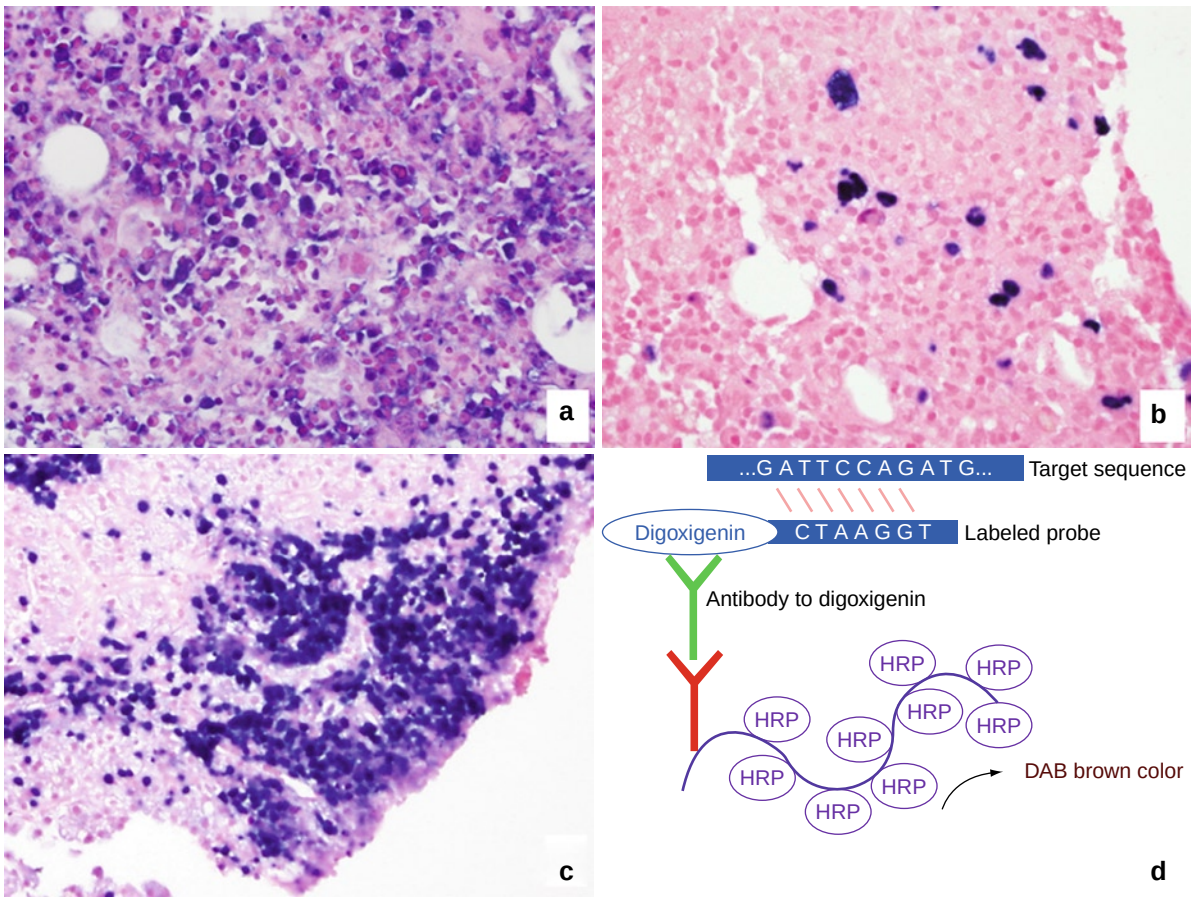

Fig. 14.2 In situ hybridization for *EBER* on paraffin sections. (**a**) The RNA preservation control in the patient marrow. (**b**) *EBER* hybridization in the patient marrow. (**c**) External positive control: *EBER* hybridization of a liver tumor in a child with posttransplant lymphoproliferative disorder. (**d**) Example of probe hybridization chemistry

targets for immune-based therapies [7, 8]. Cytotoxic T cells raised against EBV infected cells ex vivo can be infused into an affected patient to mount a physiologic response against infected tumor cells [7]. This method appears to be both well tolerated by patients and capable of significant disease response [8]. Further research with respect to both lymphoma and EBV-associated malignancies will certainly contribute to progress in patient management.

Cytogenetic analysis is frequently done on suspected lymphomas to assist in identifying a clone and to help classify a tumor. Routine karyotype analysis is a powerful genome-wide screening tool for numerical and structural chromosomal aberrations. In many subtypes of malignant lymphoma, specific cytogenetic changes inform diagnosis and/or prognosis. In the case of HL, however, no particular karyotypic findings are diagnostic of this class of malignancy, and a false-negative karyotype can occur because malignant R–S/Hodgkin cells are far outnumbered by background nonneoplastic cells.

Background and Molecular Pathology

HIV infection is associated with a markedly increased risk of developing lymphoma. The vast majority of these lymphomas are of B-cell origin and are considered to be an AIDS-defining illness. HIV-associated lymphomas appear to represent a malignant consequence of the complex interplay of ongoing immune dysregulation, viral replication, and environmental pressures inducing secondary genetic events required for frank neoplasia. In addition to the obvious loss of T cells, innate and microbial stimuli promote chronic inflammation, which contributes to risk of B-cell

transformation. The degree of HIV-related immunosuppression, as measured by CD4 count and HIV load, typically predicts lymphoma risk. Interestingly, this prediction does not hold true for HL which arises across a broad range of CD4 counts and HIV viral loads [9–11]. Although the morphologically diagnostic features are similar between HIV-infected and noninfected patients, HL typically presents with more widespread and aggressive disease in the HIV-positive cohort [12]. Moreover, HAART has reduced the incidence of most malignant lymphomas and prolonged survival, but such improvement is not clearly demonstrable for the HL subgroup [13]. While not categorized as an AIDS-defining illness, HL represents a significant cause of morbidity and mortality in the HIV-positive population [12, 14].

EBV is nearly universally associated with HL arising in the HIV setting [15]. In contrast, EBV is found only in about half of HLs occurring in immunocompetent hosts [16]. Only about half of non-Hodgkin lymphomas arising in the HIV setting are EBV related, as defined by localization of EBV to the malignant cells. Of note, *EBER* in situ hybridization is considered the gold standard assay for identifying latent EBV infection and for defining a tumor as EBV related. Other histochemical assays may be misleading. For example, latent membrane proteins (LMP1 and LMP2) are not generally expressed in *EBER*-positive Burkitt lymphoma, although they are reliably expressed in infected HL by immunohistochemistry. Identification of EBV within the malignant lymphoma cells supports a role for EBV in lymphomagenesis or tumor maintenance.

A variety of viral proteins and noncoding RNAs have been implicated in the transforming potential of EBV infection [17]. We now understand, at least in part, the mechanisms by which EBV LMP1 and LMP2 contribute to constitutive activation of NFKB1 and B-cell receptor signaling pathways, respectively, promoting cell survival and proliferation [18]. LMP1 activates NFKB1 without the need for CD40 ligand–receptor interaction, and LMP2 likewise acts without antigen stimulation of the immunoglobulin (Ig) surface receptor, thus bypassing the normal regulatory apparati [19]. In conjunction with other effects of ongoing immunosuppression by HIV infection, EBV infection appears to produce an environment that favors lymphomagenesis.

EBV viral load testing in the high-risk HIV-positive population may contribute to early diagnosis of EBV-related neoplasia by triggering a search for the cause of high circulating EBV levels [20]. Once diagnosed with EBV-related neoplasia, serial EBV viral loads may serve as a marker of tumor burden that assists in evaluating therapeutic response. Resurgence of high EBV viral load may herald relapse and provide an opportunity for early intervention.

Multiple Choice Questions

1. HIV-positive patients with poorly controlled infection are at risk for developing:
   A. Coinfection with a variety of bacteria, viruses, and fungi
   B. Epithelial malignancies
   C. Lymphoma
   D. Vascular tumors
   E. All of the above
2. A unique advantage of *EBER* in situ hybridization studies on paraffin-embedded tissue is:
   A. Direct evaluation of EBV infection in the cells of interest
   B. Does not require preserved RNA
   C. Precise viral load quantification
   D. Rapid turnaround time
   E. Specificity for lymphoma
3. HIV viral load testing alone is sufficient to determine stage of HIV infection and thus predict risk for the development of comorbid conditions
   A. True
   B. False
4. Which EBV gene product is thought to promote survival and proliferation of B lymphocytes through the NFKB1 pathway:
   A. Epstein–Barr nuclear antigen 1 (EBNA1)
   B. Epstein–Barr virus encoded RNA *(EBER*)
   C. Glycoprotein-41 (gp41)
   D. Glycoprotein-350 (gp350)
   E. Latent membrane protein 1 (LMP1)
5. In an HIV-positive individual, an increased EBV viral load is sufficient for a diagnosis of lymphoma
   A. True
   B. False

Answers to Multiple Choice Questions

1. *The correct answer is E.*

Many of the sequelae of HIV infection are explained by the ongoing dysregulation and compromise of the immune system with associated permissive microbiological infection. The risk of developing fungal, bacterial, and viral infections is increased compared to the immunocompetent population. Coinfections in this clinical setting are also associated with developing any of several types of malignancy. Dysregulation of the cell cycle induced by human papilloma virus (HPV), for example, is thought to promote malignant transformation of infected cells. Transforming mechanisms have been elucidated for several herpes family viruses.

2. *The correct answer is A.*

EBER in situ hybridization allows for direct detection of abundant viral gene products in specific cells or cellular subsets. This technology incorporates nucleic acid probes with conventional light microscopy, permitting definitive localization of virus to particular cells. The visualization of EBV within tissue, however, does not provide a precise quantitative measure of viral load, nor does it definitively imply malignancy. At least partially preserved target sequence is necessary for sensitive testing and interpretation, and RNA preservation controls are essential for test validity.

3. *The correct answer is B.*

Valuable information can be gleaned from the determination of HIV viral load. However, the prognostic and predictive quality of these data is dramatically increased when correlated with clinical presentation and immune status. Decreasing absolute CD4 T-cell counts are associated with increased risk of coinfection and malignancy. Additional correlation with other viral load assays and serologic studies may aid in the diagnosis and/or monitoring of HIV-associated diseases.

4. *The correct answer is E.*

Similar to other viruses, EBV can manipulate the molecular machinery of host cells. The viral life cycle is driven by differential expression of viral proteins over time, and patterns of gene expression are similarly linked to disease states. The expression of LMP1 is thought to contribute to EBV-mediated transformation through NFKB1 activation. EBNA1 and *EBER* expression are also relevant to the life cycle of EBV; however, these viral gene products operate primarily through other pathways. Gp350 and gp110 are expressed in lytic phase of EBV infection.

5. *The correct answer is B.*

High EBV viral load alone is insufficient to warrant a diagnosis of lymphoma. However, the degree to which the viral load is elevated may predict neoplasia. Importantly, the viral load can be used to monitor effectiveness of lymphoma therapy, once the diagnosis has been established.

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Recommended Reading

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Chronic Myelogenous Leukemia

15

Michael J. Kluk and Janina A. Longtine

Clinical Background

The patient was a 50-year-old man with a history of hypertension and hyperlipidemia who presented to his primary care physician for malaise, fatigue, and pain in the left upper quadrant. An enlarged spleen was identified on physical exam and initial laboratory analysis of the peripheral blood revealed a marked leukocytosis consisting of increased granulocytic precursors at various stages of maturation. The complete blood count values at the time of presentation are shown in Table 15.1. The patient underwent a bone marrow biopsy which also showed increased granulocytic precursors with maturation (Fig. 15.1a). The patient's family history was negative for any hematologic disorders.

Reason for Molecular Testing

Molecular testing in this case is important because some reactive conditions (e.g., leukemoid reaction) and various neoplastic myeloproliferative disorders (such as chronic myelogenous leukemia (CML), chronic neutrophilic leukemia, and chronic myelomonocytic leukemia) can have overlapping clinical and pathological features. Thus, in this case, the molecular testing has diagnostic significance. In addition, molecular testing is used in CML to monitor the patient's response to therapy. Therefore, molecular testing at this point is also important because it will provide baseline values that can be used to compare to future studies.

Question 1: What is the differential diagnosis in this case at presentation?

Test Ordered

Initial testing for a suspected case of CML may include a combination of qualitative and/or quantitative RT-PCR (reverse transcription, polymerase chain reaction) assays which target the most common *BCR-ABL1* fusion transcripts associated with CML. The qualitative RT-PCR allows one to simply detect the presence or absence of the *BCR-ABL1* fusion transcripts, while the quantitative RT-PCR assay allows one to not only detect the presence of a *BCR-ABL1* fusion transcript, but also quantitate its level relative to an internal control transcript. This quantitative aspect is advantageous because it allows one to follow changes in transcript level associated with a patient's response to therapy. If there is a very high index of suspicion for CML, or when other evidence of a *BCR-ABL1* rearrangement is already available such as a positive FISH (fluorescence in situ hybridization) or metaphase karyotype analysis, then only the quantitative assay may be ordered. In some cases,

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Table 15.1 Complete blood count at presentation

Complete blood count
WBC (white blood cells): 215,000/μL
Differential:
21% segmented neutrophils
23% band neutrophils
15% metamyelocytes
22% myelocytes
8% promyelocytes
4% myeloblasts
1% nucleated red blood cells
3% lymphocytes
2% monocytes
1% basophils
Hematocrit: 33%
Platelets: 268,000/μL

however, a combination of the qualitative and the quantitative test may be helpful because the type of *BCR-ABL1* transcripts detected by each assay may be different depending on the laboratory.

Question 2: What tests are typically ordered when a clinician suspects a diagnosis of CML?
Question 3: What are the advantages and disadvantages of these tests?

Laboratory Test Performed

The initial testing in this case included the qualitative RT-PCR assay for the *BCR-ABL1* fusion transcript (Fig. 15.1b). This is a rapid, simple assay whereby total RNA (including messenger RNA) is isolated from leukocytes in the peripheral blood. Alternatively, bone marrow aspirate specimens may be used. The next step involves targeted reverse transcription whereby the messenger RNA is converted to complementary DNA (cDNA) which, in turn, is used in the final PCR amplification. The primers (Fig. 15.1c) used in this last amplification step are designed to yield PCR products of different sizes and, thereby, indicate the underlying *BCR-ABL1* gene rearrangement. Note that RNA is used in this assay (as well as in the quantitative RT-PCR assay described below) because the variability in the breakpoints of the *BCR* and *ABL1* genes and the large intervening introns complicate direct PCR detection of the *BCR-ABL1* gene rearrangement at the level of genomic DNA.

In addition to the qualitative assay, initial testing often includes a quantitative RT-PCR assay in order to document the baseline levels of the *BCR-ABL1* fusion transcript, which can then be compared to subsequent specimens from the same patient in order to monitor response to therapy. The quantitative RT-PCR assay uses RNA from patient and control specimens in a one-step procedure that includes targeted reverse transcription and subsequent PCR amplification. This "one step" RT-PCR reaction mix minimizes the need for additional pipetting and, therefore, reduces the risk of sample cross-contamination. Three separate amplification reactions are performed for each patient sample using primers for the e14a2 (formerly known as b3a2) and e13a2 (formerly known as b2a2) fusion transcripts and for the internal control transcript beta glucuronidase (*GUSB*), which serves to assess RNA integrity of the sample. This quantitative real-time RT-PCR technique is based on hydrolysis chemistry and during each PCR cycle a fluorescent signal is generated when the polymerase encounters the reporter probe and, via its 5′ to 3′ exonuclease activity, liberates the fluorescent signal at one end of the reporter from the quencher moiety at the other end of the reporter probe. The fluorescent signal at each cycle increases proportionally to the amount of target template present. Quantitation is performed by first determining the cycle number at which the fluorescent signal reaches a point above the background during the linear phase of amplification; this is referred to as the CT (cycle threshold) value. The second step in quantitation involves comparing the CT value for each sample to a standard curve generated with the CT values of different dilutions of a control sample with a known amount of mRNA target. Lastly, in order to compensate for variation in RNA quality between samples, the e14a2 (b3a2) and e13a2 (b2a2) *BCR-ABL1* fusion transcript levels are normalized by the *GUSB* transcript level. Instead of *GUSB*, a variety of other internal control transcripts (e.g., endogenous *ABL1*, *BCR*, *B2M* encoding beta-2-microglobulin, *ACTB* encoding beta actin and *GAPDH*) may be used in different laboratories to normalize *BCR-ABL1* fusion transcript levels. Among those listed, *GUSB*, *ABL1*, and *B2M* are most advantageous, because they have no pseudogenes, have a medium to high level of expression, there are no significant differences in expression between normal and leukemic samples, and there are no significant differences in expression in peripheral blood and bone marrow

samples [1]. Using similar criteria, an international consensus meeting held to harmonize methodologies for measuring *BCR-ABL1* transcripts recommended *GUSB*, *ABL1*, and *BCR* as control transcripts [2]. The quantitative real-time RT-PCR assay was performed in our patient (Fig. 15.1d).

Results with Interpretation Guideline

The qualitative RT-PCR (Fig. 15.1b) performed on this patient revealed an e14a2 (b3a2) *BCR-ABL1* fusion transcript in both of the replicate samples (upper panel). The nature of the fusion transcript is

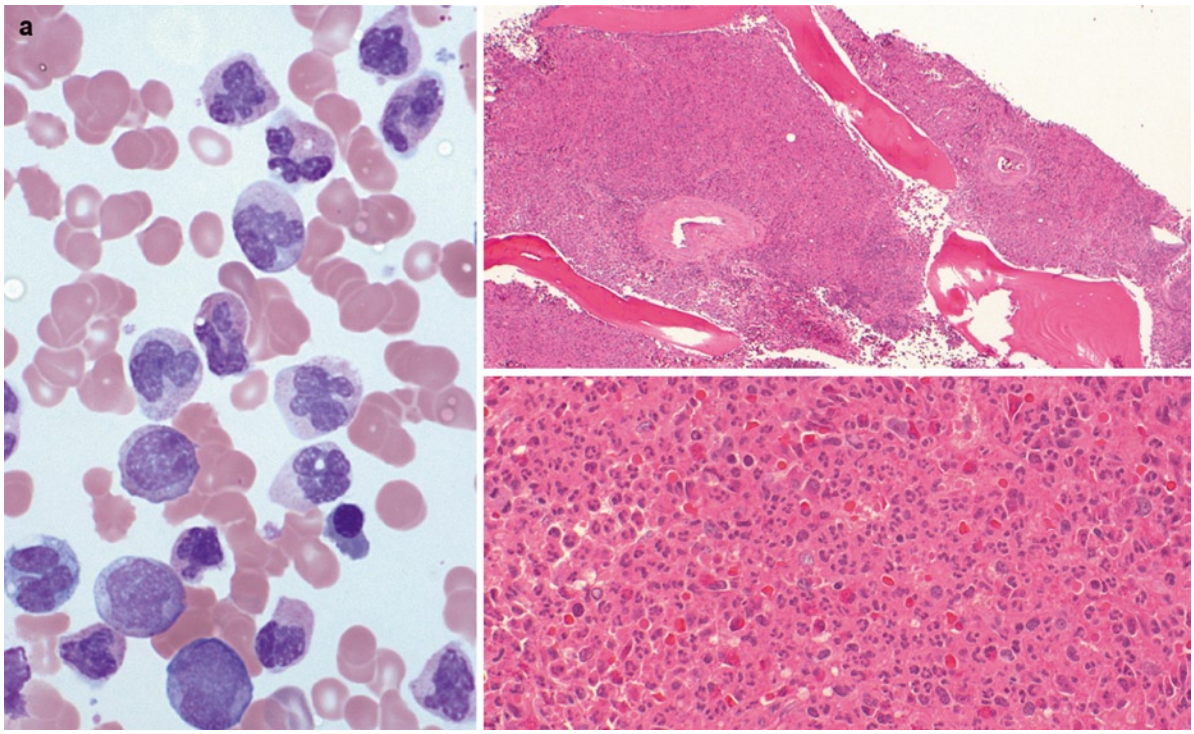

Fig. 15.1 Histopathology and initial molecular analysis. (**a**) Histopathology of CML. *Left panel*: The peripheral blood demonstrated marked leukocytosis consisting predominantly of granulocytic precursors at different stages of maturation. *Right panel*: The bone marrow core biopsy (shown at two levels of magnification) demonstrated marked hypercellularity, increased myeloid:erythroid ratio, and a predominance of granulocytic precursors at different stages of maturation. (**b**) Qualitative RT-PCR for *BCR-ABL1* fusion transcripts. *Upper panel*: PCR result for the patient (in duplicate) compared to the different control specimens. *Lower panel*: The RT-PCR result for the internal control transcript (*ABL1*). The patient specimen is clearly positive for the e14a2 (b3a2) *BCR-ABL1* fusion transcript in both replicate samples. All controls performed as expected. (**c**) Exon map of *BCR*, *ABL1*, and *BCR-ABL1* transcripts. The *BCR* transcript is shown in blue, with the relative positions of the minor, major, and micro breakpoint regions indicated. The *ABL1* transcript is shown in yellow. The e1a2 (*upper*), e13a2 (b2a2) (*middle*), and e14a2 (b3a2) (lower) *BCR-ABL1* fusion transcripts are shown. For the qualitative RT-PCR assay, the different forward primers (*orange arrow and green arrows*) are used in combination with a common reverse primer (*black arrows*). For the quantitative RT-PCR assay, the different forward primers (*light blue arrow* and *purple arrow*) are used in combination with the common reverse primer (*red arrows*); the intervening fluorescent reporter probe is also shown (*gray*). Note that for the quantitative RT-PCR assay, the reverse primers and intervening probe are complementary to exon 2 of *ABL1*; the forward primer to detect the e13a2 (b2a2) fusion transcript was specifically designed to overlap with part of exon 2 of *ABL1* (this makes this primer specific for the e13a2 (b2a2) *BCR-ABL1* gene rearrangement); this primer design provides an additional quality control measure because the nature of the rearrangement (i.e., e13a2 (b2a2) or e14a2 (b3a2)) for each patient should be the same in future specimens and, therefore, can be used to identify mislabeling or cross-contamination with other patient samples. (**d**) Quantitative real-time RT-PCR for the *BCR-ABL1* fusion transcript. The e14a2 (b3a2) *BCR-ABL1* fusion transcript was detected (*red*) as well as the internal control transcript, *GUSB* (*green*). These pretreatment baseline studies demonstrate a high level of e14a2 (b3a2) fusion transcript. Note that the e14a2 (b3a2) *BCR-ABL1* fusion transcript is even more abundant than the *GUSB* internal control transcript as it was detected at a lower cycle number (further explanation is provided in the "Results with Interpretation Guideline" section of the text). *CT* cycle threshold value

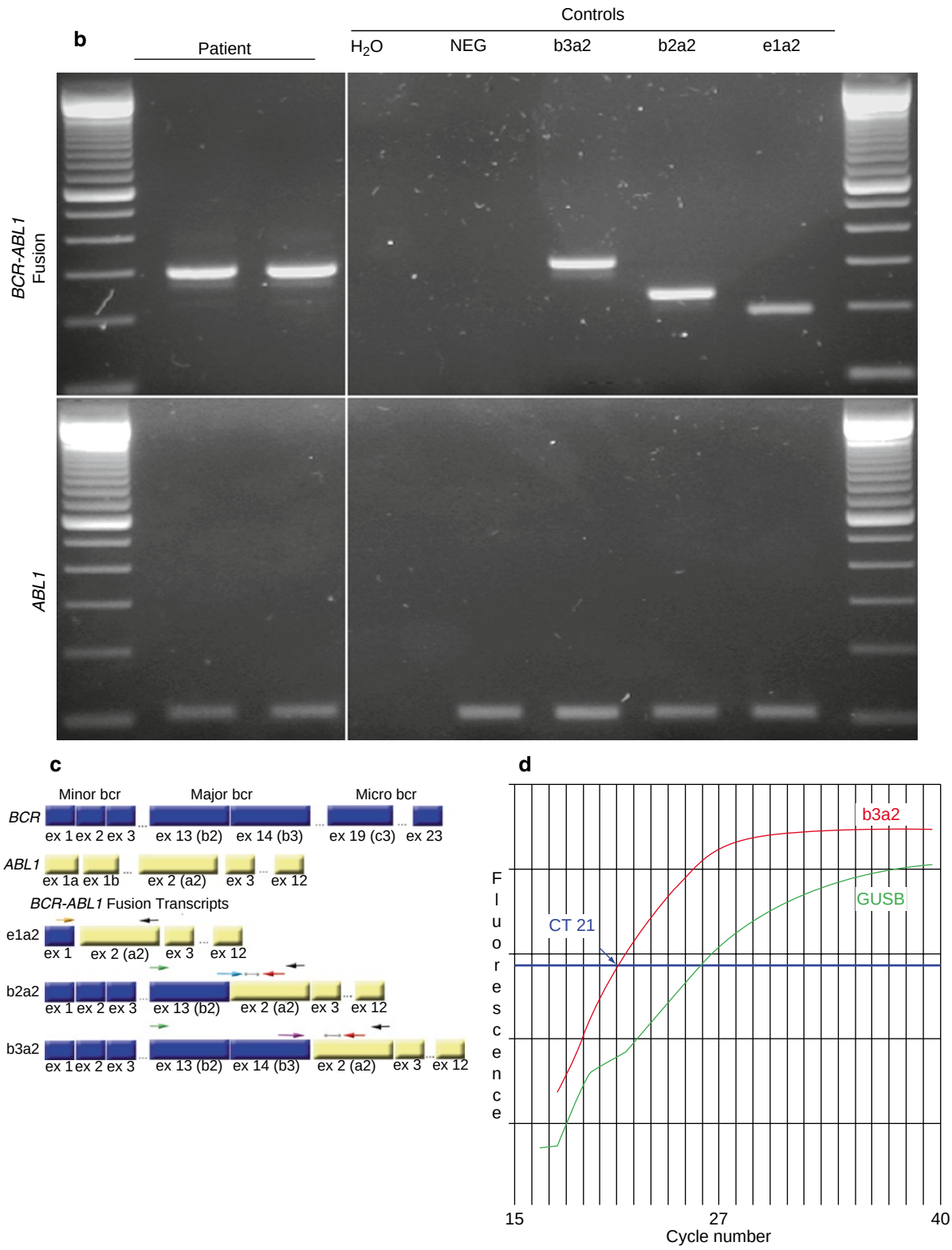

Fig. 15.1 (continued)

determined by comparing it to the known positive control bands: 305 bp for e14a2 (b3a2), 230 bp for e13a2 (b2a2), and 196 bp for e1a2 *BCR-ABL1* fusion transcripts. The *ABL1* internal control (lower panel) indicates the adequacy of RNA for all specimens and is important in patients who are negative for all three *BCR-ABL1* fusion transcripts in order to rule out false-negative results due to poor RNA quality. Note that the negative control specimen showed a positive result for the *ABL1* internal control and that the blank specimen is negative for both *BCR-ABL1* fusion transcripts and the *ABL1* internal control, as expected.

The initial quantitative RT-PCR result (Fig. 15.1d) demonstrated a high level of e14a2 (b3a2) *BCR-ABL1* fusion transcript (e14a2 (b3a2):GUSB ratio >100%). In fact, the e14a2 (b3a2) fusion transcript is present in such high levels that its CT value is less than that of the internal control *GUSB*. In other words, the e14a2 (b3a2) curve is shifted to the left of the *GUSB* curve. The CT value is inversely proportional to the amount of target mRNA present in the specimen and a low CT value indicates a high level of target transcript.

Result Interpretation

The patient was positive for a high level of e14a2 (b3a2) *BCR-ABL1* fusion transcript with an e14a2 (b3a2):*GUSB* ratio of >100%. This result, together with the clinical findings, is consistent with a diagnosis of CML.

Further Testing

The patient was initially treated with hydration, allopurinol, and hydroxyurea with close monitoring to prevent tumor lysis syndrome. He was subsequently started on the ABL1 tyrosine kinase inhibitor, imatinib (400 mg/day). After three weeks of therapy, his WBC count decreased to 5,500 cells/μL and he continued to be in hematologic remission after three months of therapy. However, at six months of therapy, although he was still in hematologic remission, a bone marrow biopsy revealed only partial cytogenetic response and the quantitative RT-PCR showed an elevated e14a2 (b3a2):*GUSB* ratio (approximately 8%, not shown). Therefore, his imatinib dose was increased to 400 mg twice a day, which led to a complete cytogenetic response by one year after initial diagnosis. At that time, the quantitative RT-PCR showed a e14a2 (b3a2):*GUSB* ratio of 2% (Fig. 15.2a, left panel). However, two years after initial diagnosis, the patient was found to have a very high e14a2 (b3a2):*GUSB* ratio >100% (Fig. 15.2a, middle panel) and his WBC count increased to 48,000 cells/μL with no evidence of progression to accelerated phase or blast phase. Therefore, the patient's therapy was switched to a different tyrosine kinase inhibitor, nilotinib, at 400 mg twice per day, since it was felt that his disease had developed resistance to imatinib. Despite this change in therapy, there was no good hematologic response and sequencing analysis of the *ABL1* kinase domain of the *BCR-ABL1* fusion transcript was ordered to determine whether the patient had developed a mutation known to be associated with resistance to imatinib and nilotinib. Indeed, the patient was found to carry a c.1075T>G (p.Phe359Val) mutation (Fig. 15.2b). In the *ABL1* sequencing assay, there is an initial RT-PCR step to amplify the region encoding the *ABL1* kinase domain from *BCR-ABL1* fusion transcript. This is accomplished using a forward primer in the *BCR* exon 13 (b2) and a reverse primer in exon 9 of *ABL1*. This combination of primers can be used for cases with the e13a2 (b2a2) or e14a2 (b3a2) *BCR-ABL1* rearrangements and serves to focus the subsequent sequencing reaction on the *ABL1* kinase domain of the *BCR-ABL1* fusion transcript rather than the endogenous normal *ABL1* transcript. Next, exons 4–9, which encode the *ABL1* kinase domain, are sequenced. Interestingly, the specific mutation identified for our patient is known to be associated with decreased sensitivity to imatinib and nilotinib and increased sensitivity to the tyrosine kinase inhibitor, dasatinib. Therefore, the patient was given targeted therapy with dasatinib (50 mg, twice a day) which resulted in complete hematologic remission and an excellent molecular response consisting of a three-log reduction of e14a2 (b3a2) *BCR-ABL1* fusion transcript level within 12 months. The e14a2 (b3a2):*GUSB* ratio which was >100% at recurrence (Fig. 15.2a, middle panel) was reduced to 0.1% (Fig. 15.2a, right panel).

Question 4: What are the possible reasons why a patient who previously responded well to imatinib would show a recurrence of CML?

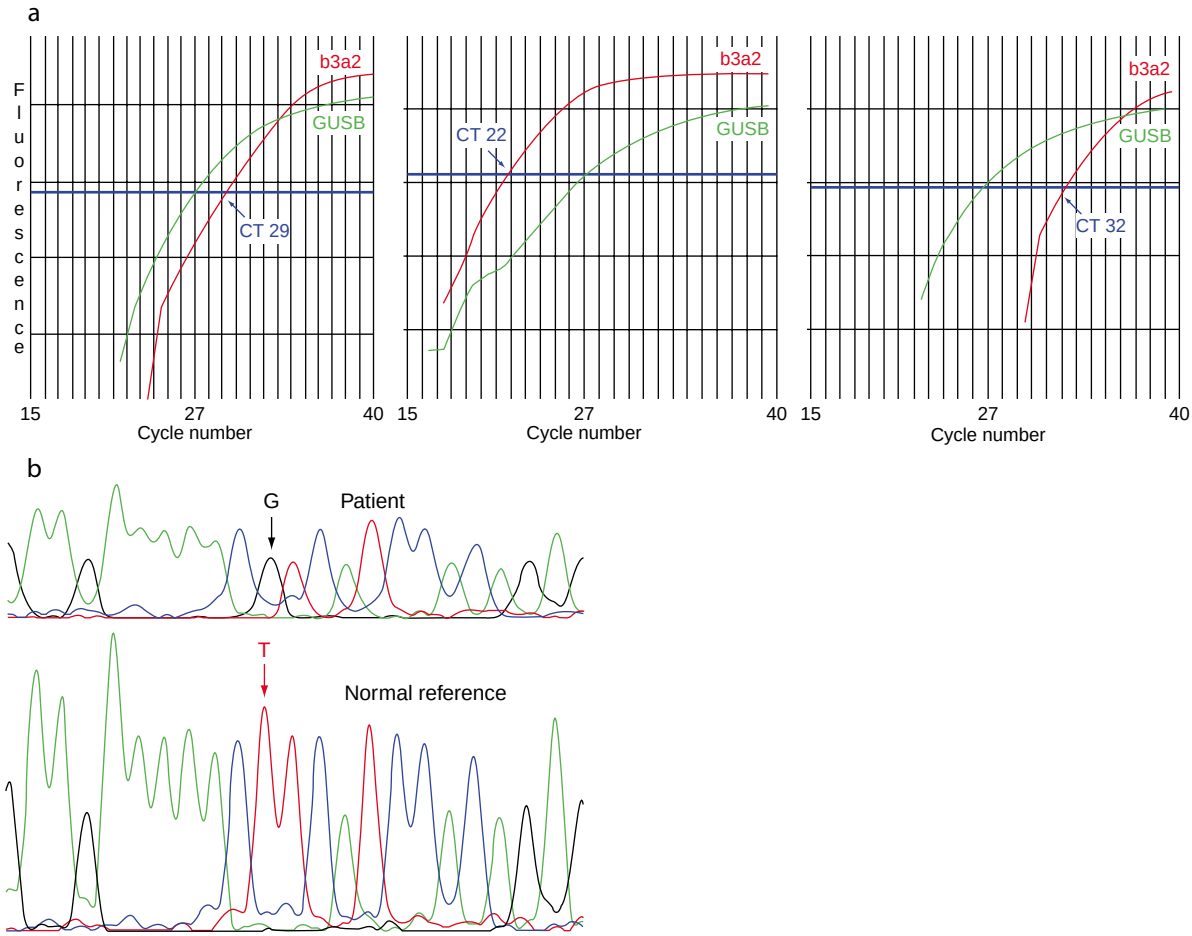

Fig. 15.2 Molecular monitoring during treatment. (**a**) Quantitative real-time RT-PCR for *BCR-ABL1* fusion transcripts. In each panel, the amplification of the e14a2 (b3a2) *BCR-ABL1* fusion transcript is depicted in red. The internal control transcript (*GUSB*) is green. *Left panel*: The levels of e14a2 (b3a2) after initiation of tyrosine kinase inhibitor therapy (e14a2 (b3a2):*GUSB* ratio of 2%). The *BCR-ABL1* fusion transcript levels are decreased from pretreatment levels (Fig. 15.1d) but are still detectable. *Middle panel*: The subsequent marked increase in e14a2 (b3a2) fusion transcript despite therapy, indicating resistance to therapy (e14a2 (b3a2):*GUSB* ratio >100%), resulting from a resistance causing mutation found in the *ABL1* kinase domain of the *BCR-ABL1* fusion transcript (Fig. 15.2b). *Right panel*: Response to new therapy, started after the resistance mutation was discovered. Note that the e14a2 (b3a2) fusion transcript levels (*right panel*) are much lower than the level seen at recurrence (*middle panel*) indicating a good response to the new therapy. More specifically, there is a three-log reduction in e14a2 (b3a2) transcript levels from the high levels seen at recurrence. Because there is an approximate doubling of PCR product with each PCR cycle during the linear phase of PCR amplification, a three-log (1,000-fold) reduction corresponds to a difference of approximately ten cycles. *CT* cycle threshold values. (**b**) Sanger sequencing of the *BCR-ABL1* kinase domain. *Upper panel*: The DNA sequence obtained from the patient. *Lower panel*: The relevant normal reference sequence. Note that there is a mutation corresponding to a single nucleotide substitution (c.1075T>G (p.Phe359Val)) identified in the patient specimen. Nucleotide color code: *A*=Green, *C*=Blue, *G*=Black, *T*=Red

Other Considerations

Currently, for the routine testing performed in the majority of clinical laboratories, the results of quantitative RT-PCR for *BCR-ABL1* fusion transcript levels are not standardized and it is usually not advisable to compare *BCR-ABL1* fusion transcript percentages performed in different laboratories. As a result, the monitoring of changes in *BCR-ABL1* fusion transcript levels in response to therapy must be done using results obtained in the same laboratory by the same technique

for accurate assessment of changes in fusion transcript levels over time. In addition, the variation in internal control transcripts used to normalize e14a2 (b3a2) fusion transcript levels complicates the comparison of quantitative RT-PCR results for *BCR-ABL1* fusion transcript levels from different laboratories. Recently, however, an international standard for interlaboratory comparison of *BCR-ABL1* fusion transcript levels has been developed [3]. In addition, the first World Health Organization International Genetic Reference Panel for quantitation of *BCR-ABL1* has been established for use as a primary standard to calibrate secondary standards [4]. This may also lead to more standardization in the field.

Background and Molecular Pathology

The Philadelphia chromosome was one of the first chromosomal abnormalities known to be associated with a neoplastic disease in humans [5] and represents the derivative chromosome 22 resulting from the reciprocal translocation between chromosomes 9 and 22 known as t(9;22)(q34;q11) [6]. The Philadelphia chromosome harbors the *BCR-ABL1* fusion gene which has tyrosine kinase activity that is thought to be important in the pathogenesis of CML [7–9]. The Philadelphia chromosome is not specific to CML because it is also found in acute lymphoblastic leukemia (ALL) [10–12]. As illustrated in Fig. 15.1c, there is variation in the breakpoints on chromosome 22 (*BCR* locus) which can result in three different *BCR-ABL1* fusion transcripts. For the sake of completeness, one should note that variation in the breakpoint on chromosome 9 (*ABL1* locus) may also exist; however, this is very rare and, therefore, it is not shown.

In CML, the most common breakpoints in *BCR* involve the major breakpoint cluster region (downstream of exons 13 (b2) or 14 (b3)). In *ABL1*, the most common breakpoint involves the sequence just upstream of exon 2. These rearrangements result in two different possible fusion transcripts designated as e13a2 (b2a2) and e14a2 (b3a2) (reviewed in [13]). Both the e13a2 (b2a2) and the e14a2 (b3a2) fusion transcripts are associated with the p210 BCR-ABL1 fusion protein and these are, by far, the most common rearrangements in CML, representing at least 95% of cases [14]. Approximately 1% of CML cases are associated with the e1a2 *BCR-ABL1* rearrangement [15] which may be associated with a worse prognosis. In addition, rare cases of CML may be associated with the p230 BCR-ABL1fusion protein (e19a2 *BCR-ABL1* gene rearrangement, formerly known as c3a2) [16]. It is interesting to note that the e13a2 (b2a2) and e14a2 (b3a2) transcripts may undergo alternative splicing which can lead to the detection of the e1a2 transcript in cases with an e13a2 (b2a2) or e14a2 (b3a2) *BCR-ABL1* gene rearrangement [17]. Also, approximately 7% of CML cases may show both e14a2 (b3a2) and e13a2 (b2a2) transcripts, which is also thought to be due to alternative splicing [18, 19].

In ALL, the Philadelphia chromosome is present in approximately 20–30% of cases of adult ALL and 5% of cases of pediatric ALL [10–12]. The e1a2 *BCR-ABL1* fusion transcript is the most frequent *BCR-ABL1* fusion transcript in adult patients with Philadelphia chromosome positive ALL (present in approximately two thirds of cases) [10–12]. Furthermore, the e13a2 (b2a2) and e14a2 (b3a2) *BCR-ABL1* fusion transcripts which, as described above, represent the vast majority of cases of CML, are found in only approximately one third of adult patients with Philadelphia chromosome positive ALL [10–12]. Interestingly, it is possible that the e13a2 (b2a2) or e14a2 (b3a2) positive cases of Philadelphia chromosome positive ALL in adults may correspond to cases of CML that were previously asymptomatic and undiagnosed and were, therefore, diagnosed during lymphoid blast crisis. In children, the e1a2 *BCR-ABL1* fusion transcript is found in almost all cases of pediatric Philadelphia chromosome positive ALL while the e13a2 (b2a2) and e14a2 (b3a2) fusion transcripts are rare.

The treatment of CML has changed significantly over the past 15 years (reviewed in [13]). Stem cell transplantation can cure CML in some patients, but it is associated with significant risk of morbidity and mortality. Therefore, other treatment modalities have been pursued to manage CML. For example, interferon alpha, with or without cytarabine, was once considered the best chemotherapy for the treatment of CML. This changed when the tyrosine kinase inhibitor imatinib mesylate proved successful for treatment in patients with CML resistant to interferon alpha. The International Randomised Study of Interferon versus STI571 (IRIS) demonstrated that the rate of complete cytogenetic remission was much higher in the imatinib group (76%) than in the interferon plus

cytarabine group (14%) [20]. Subsequently, Hughes et al. demonstrated that for patients in complete cytogenetic remission, imatinib, after 12 months of therapy, was more effective than interferon plus cytarabine at inducing a three-log reduction in *BCR-ABL1* fusion transcript levels from a median standardized baseline pretreatment value [21]. Furthermore, the three-log reduction in *BCR-ABL1* fusion transcript levels was associated with a lower risk of disease progression [21]. Recently, a six year followup from these initial studies revealed that imatinib led to a six year event free survival of 83% with an overall survival rate of 88% [22]. One of the factors complicating management of CML with imatinib has been the development of resistance to the drug (reviewed in [23]). One of the most common mechanisms for acquired imatinib resistance is the development of malignant cell clones which have acquired point mutations in the *BCR-ABL1* kinase domain; these mutations reduce the effectiveness of imatinib by impairing its ability to bind to its target. Over time, it has become clear that different mutations lead to different sensitivities to the various tyrosine kinase inhibitors (e.g., imatinib, dasatinib, and nilotinib) providing therapeutic options for patients who acquire resistance mutations (reviewed in [24]).

Question 5: What are the roles of quantitative RT-PCR and ABL1 kinase domain sequencing in monitoring response to CML therapy?

Multiple Choice Questions

1. All of the following are consistent with the clinical and pathologic findings in CML, EXCEPT
   A. Bone marrow hypercellularity
   B. Myelodysplasia
   C. Peripheral leukocytosis
   D. Presence of granulocyte precursors at various stages of maturation
   E. Splenomegaly
2. Which material is most commonly used in the PCR-based detection of *BCR-ABL1* gene rearrangements?
   A. DNA
   B. Protein
   C. RNA
   D. snRNPs (small nuclear ribonucleoproteins)
   E. tRNA (transfer RNA)
3. Which of the following statements is INCORRECT?
   A. CT values are inversely proportional to the amount of target mRNA present (i.e., a lower CT value indicates a greater amount of the target mRNA)
   B. During quantitative real-time PCR, the fluorescent signal is generated by the exonuclease activity of the polymerase, which separates the fluorescent molecule from the quencher on the reporter probe
   C. Endogenous *ABL1* transcript levels may be used to normalize *BCR-ABL1* fusion transcript levels
   D. The CT value of a sample is compared to the CT values of various standards in order to determine the amount of target fusion transcript present in a sample
   E. The final plateau phase of the PCR amplification is used to determine the CT value of a sample
4. When interpreting test results, all of the following indicate a valid run EXCEPT
   A. The blank sample (H_2O) has a band of the expected size
   B. The internal control target shows a band of the expected size for all specimens
   C. The negative control shows a band only for the internal control target
   D. The positive control specimen shows a band of the expected size
   E. The patient sample shows a band only for the internal control target
5. In CML, the most common *BCR-ABL1* fusion transcript is
   A. e1a2
   B. e6a3
   C. e6a4
   D. e13a2 (b2a2) or e14a2 (b3a2)
   E. e19a2

Answers to Questions Embedded in the Text

Question 1: What is the differential diagnosis in this case at presentation?

The differential diagnosis in this case is relatively narrow and CML (chronic phase) is the leading diagnosis until proven otherwise, given the patient's splenomegaly and marked leukocytosis consisting of granulocytic precursors at various stages of maturation with only 4% myeloblasts and 3% monocytes. Accelerated phase CML would be a diagnostic consideration if the patient

had higher levels of myeloblasts (>10%), marked basophilia (>20%), severe thrombocytopenia (<100,000/μL), or severe thrombocytosis (>1,000,000/μL). Acute leukemia would have >20% myeloblasts in the peripheral blood. Reactive conditions (e.g., leukemoid reaction) and different myeloproliferative neoplasms (e.g., chronic neutrophilic leukemia, chronic myelomonocytic leukemia) could mimic chronic phase CML in some aspects; therefore, molecular testing for the *BCR-ABL1* fusion transcript (identified at the chromosomal level as t(9;22)(q34;q11)) is important, because these other conditions will be negative for *BCR-ABL1*, while CML will be positive.

Question 2: What tests are typically ordered when a clinician suspects a diagnosis of CML?

The tests typically ordered when a clinician suspects the diagnosis of CML are a qualitative and/or a quantitative RT-PCR assay to detect any of the *BCR-ABL1* fusion transcripts most commonly associated with CML, which are described in detail in the text.

Question 3: What are the advantages and disadvantages of these tests?

The qualitative RT-PCR assay is a rapid, simple, and inexpensive assay that can detect *BCR-ABL1* fusion transcripts in CML. However, it does not allow for quantitative monitoring of the response to therapy. The quantitative RT-PCR assay is helpful because it can be used to monitor response to therapy over time. However, some disadvantages of this technique include (1) expense and (2) lack of standardized quantitation which prohibits the comparison of results obtained in different laboratories. Nevertheless, the patient's results from specimens tested in the same laboratory over time can be compared to each other as long as the same quantitative technique was used for the various specimens. A disadvantage of both of these assays is that RNA, which is prone to degradation and less stable than DNA, is used as the test substrate.

Question 4: What are the possible reasons why a patient who previously responded well to imatinib would show a recurrence of CML?

Possible reasons for recurrence of CML during imatinib therapy include (1) progression to accelerated phase or blast phase CML, (2) noncompliance with medication dosage, and (3) development of drug resistance, which is most often due to a subclone within the malignant stem cell population that acquires a point mutation in the *ABL1* kinase domain, thereby impairing the ability of imatinib (or another tyrosine kinase inhibitor) to bind.

Question 5: What are the roles of quantitative RT-PCR and ABL1 kinase domain sequencing in monitoring response to CML therapy?

Quantitative RT-PCR can be used to monitor therapy-induced changes in the level of *BCR-ABL1* fusion transcript (relative to an internal control transcript such as *GUSB* or endogenous *ABL1*). One should only compare results from a patient's samples that were tested with the same technique in the same laboratory, because variation in assay platforms and normalization procedures will affect the absolute quantitation values. Patients who have a major molecular response to therapy, defined as a more than three-log reduction in *BCR-ABL1* fusion transcript levels from a standardized baseline, have improved survival. The National Comprehensive Cancer Network has issued guidelines for laboratory testing in CML that include recommendations for quantitative RT-PCR testing at initial diagnosis and then every three months, if the patient responds well to therapy. If there is a one-log increase between successive measurements (which could suggest possible ineffectiveness of therapy) then quantitative RT-PCR is recommended at shorter intervals. In addition, in such cases, it is also recommended to consider *ABL1* kinase domain sequencing to detect resistance mutations [25].

Answers to Multiple Choice Questions

1. *The correct answer is B.*
2. *The correct answer is C.*
3. *The correct answer is E.*
4. *The correct answer is A.*
5. *The correct answer is D.*

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Myeloproliferative Neoplasms

16

John A. Thorson and Huan-You Wang

Clinical Background

A 59-year-old female was referred to the hematology department for evaluation of persistent erythrocytosis. Two years prior to this visit, she was seen by her primary care physician with complaints of intermittent mild night sweats, frequent vasomotor symptoms such as hot flashes, and early satiety with abdominal fullness and pain if she laid on her right side. Laboratory evaluation at that time revealed a hematocrit of 57.6% (reference interval 38.5–49%), hemoglobin of 18.3 g/dL (reference interval 13.2–16.9 g/dL), and red blood cell count of $7.0 \times 10^6/\mu L$ (reference interval $4.5–5.1 \times 10^6/\mu L$). She was treated with phlebotomy alone for approximately two years. However, she continued to have persistent erythrocytosis and during her last checkup was found to have an elevated platelet count of 915,000/μL (reference interval 150,000–450,000/μL).

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Upon evaluation by the hematologist, a physical examination revealed that her spleen was 13 cm below the left costal margin and her liver was percussed at 7 cm in the right midclavicular line. Laboratory evaluation revealed a white blood cell count (WBC) of 85,400/μL (reference interval 4,000–11,000/μL), red blood cell count of 4,950,000/μL, a hemoglobin of 18.3 g/dL, a hematocrit of 52%, and a platelet count of 700,000/μL. The bone marrow biopsy showed a hypercellular marrow for age with trilineage hematopoiesis and granulocytic hyperplasia. The blast count was less than 5%. A reticulin stain of the bone marrow revealed grade 2 (on a 0–3 scale) fibrosis. Based upon the patient's presentation and initial laboratory evaluation, a working diagnosis of polycythemia vera was considered probable.

Reason for Molecular Testing

Polycythemia vera (PV) is in a group of hematologic diseases referred to as myeloproliferative neoplasms (MPN), all of which are clonal disorders thought to result from a lesion at the level of the hematopoietic stem cell [1]. PV is characterized by excess red blood cell production, which occurs independently of the normal regulatory mechanisms of erythropoiesis, and typically also presents with increased numbers of circulating cells from the granulocyte and megakaryocyte lineages.

PV has long been recognized as being closely related to two other members of the MPN category: essential thrombocythemia (ET) and primary myelofibrosis

I. Schrijver (ed.), *Diagnostic Molecular Pathology in Practice*,
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(PMF) [2]. The laboratory diagnosis of these diseases was revolutionized in 2005, when it was discovered by several groups independently that a specific, somatic gain of function mutation in the Janus kinase 2 (*JAK2*) gene characterizes >95% of PV cases and approximately 50% of ET and PMF cases [3–6]. The mutation results in the substitution of phenylalanine for valine at amino acid position 617 (p.Val617Phe, c.1849G > T) and leads to constitutive activation of the kinase. The active kinase stimulates multiple signal transduction cascades and leads to cellular proliferation in the absence of normal cytokine stimulation, thus leading to expanded cell numbers.

Because the *JAK2* V617F allele is found in the vast majority of PV cases and in approximately one half of all cases of PMF and ET, *JAK2* mutation testing has been incorporated into the World Health Organization (WHO) classification scheme for MPN. In the current WHO classification, major diagnostic criteria for PV include (1) a hemoglobin level >18.5 g/dL in men or >16.5 g/dL in women and (2) the presence of either the *JAK2* V617F mutation or other functionally similar *JAK2* mutations [7]. In regard to this last point, the initial observations that approximately 5% of PV patients are negative for the *JAK2* V617F mutation suggested that other kinase activating alleles may be present in this subset. Subsequent studies of *JAK2* V617F negative cases have identified a series of missense, deletion, and insertion mutations within exon 12 of the *JAK2* gene, which also lead to a gain of function [8–10]. Interestingly, unlike the V617F mutation, the *JAK2* exon 12 mutations have only been observed in cases of PV, and not in ET or PMF.

In view of the finding that expression of the protein encoded by the *JAK2* V617F allele confers cytokine independent growth in various cell lines [3, 6], there is optimism that small molecule inhibitors may offer benefit to MPN patients. Clinical trials of specific JAK2 inhibitors in patients with MPN have suggested that such targeted therapy may be useful for reducing many of the symptoms of these diseases, including splenomegaly, night sweats, fatigue, and pruritis. However, the ability of these drugs to reduce or eliminate the neoplastic cells is unclear [11].

In the present case, a molecular test for the *JAK2* V617F mutation was requested in order to firmly establish the diagnosis of PV, and to confirm the presence of this mutation for the potential inclusion of the patient in a clinical trial of targeted therapy.

Test Ordered

The test ordered was an evaluation for the presence of the mutation in codon 617 of the *JAK2* gene, which results in the V617F amino acid substitution.

Laboratory Test Performed

The most common mutation responsible for the V617F mutation in the JAK2 protein is a single base change at nucleotide position 1849 in exon 14 of the *JAK2* gene, in which a guanine is replaced by a thymidine (1849G > T). This results in a change at codon 617 from GTC, which encodes valine, to TTC, which encodes phenylalanine. Several techniques can be used for the detection of this mutation. Methods in widespread use include mutation-specific assays such as allele specific PCR or real-time PCR, sequence amplification followed by restriction endonuclease digestion to detect altered fragment lengths, as well as direct DNA sequencing techniques that do not solely target the point mutation site [12]. Currently, the choice of technique is primarily dependent upon user familiarity and platform availability. However, there are differences in the achievable detection limit between the various formats and this may have implications for the ability to accurately classify the disease [13]. An additional caveat related to technique is that, although extremely rare, a double mutation within codon 617 can also result in the V617F mutation, that is, 1849G > T combined with 1851C > T. In this case, codon 617 is changed from GTC to TTT, which also encodes phenylalanine. The occurrence of this double mutation has been reported to produce a false negative result in an assay, which specifically targets the 1849G > T mutation [14].

Finally, it should be noted that several studies have examined the use of quantitative assays to measure the allele burden of *JAK2* V617F. In general, two potential uses of a quantitative *JAK2* V617F assay have been suggested. The first is as an aid to evaluating disease progression or therapeutic efficacy, with the assumption that the measureable burden of the V617F allele will wax or wane in proportion to the level of disease [15]. The second potential use is in evaluating prognosis, that is, identifying MPN that may be more aggressive in their course or result in greater risk of

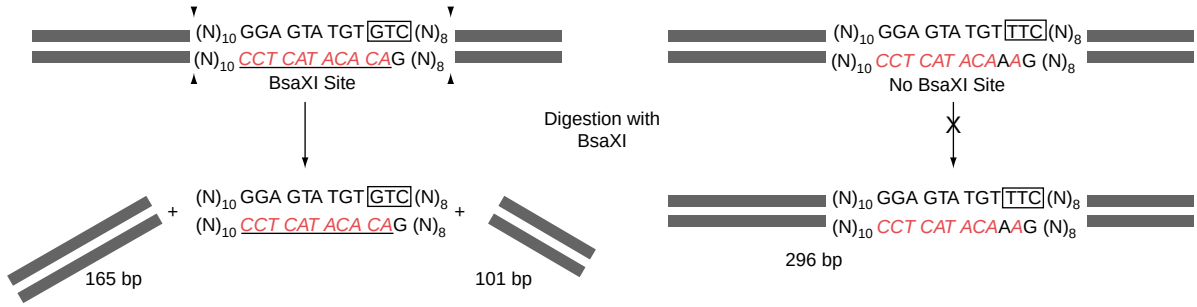

Fig. 16.1 Diagrammatic representation of a restriction endonuclease digestion strategy for detecting the *JAK2* V617F mutation. PCR is used to amplify a 296 bp fragment of the *JAK2* gene which comprises exon 14 and a portion of the flanking introns. Codon 617 is located within this fragment (indicated by the *rectangular box*). In the presence of the wild-type codon 617 sequence (shown on the *left* side of the figure), a BsaXI recognition site is formed (indicated by underscored sequence). Digestion of the wild-type PCR product with BsaXI results in three fragments of 165, 101, and 30 bp in size. The 1849G > T mutation converts codon 617 from GTC to TTC and eliminates the BsaXI recognition site (*right* side of the figure). No digestion of the mutant PCR product occurs with BsaXI, leaving the 296 bp product intact

complications such as thrombotic or cardiovascular events [16]. Currently, there is no generally accepted consensus regarding the value of this type of assay for either of these uses, and additional studies will be needed to clearly define what role a quantitative assay may play in patient care [13, 17].

In this case, the presence of the *JAK2* V617F mutation was assessed via an assay utilizing restriction endonuclease digestion of an amplified product followed by electrophoretic separation of the digestion fragments. PCR was used to amplify a 296 bp fragment comprising exon 14 of the *JAK2* gene and a portion of the introns flanking it. This fragment was digested with the enzyme BsaXI, which recognizes the restriction sequence 5′– AC NNNNN CTCC– 3′, present in the sequence of the noncoding strand encompassing codon 617. In the absence of any mutation, digestion of the PCR product with this enzyme results in three fragments. As BsaXI is a type IIb restriction endonuclease [18], it cuts the DNA strand on both sides of its recognition site, releasing a small fragment which contains the recognition site (30 bp) as well as the flanking sequences which are 165 bp and 101 bp in size (Fig. 16.1). In the presence of the codon 617 mutation (1849G > T), the restriction recognition site is destroyed, and the PCR product remains intact following digestion with BsaXI.

To perform the assay, DNA was extracted from 300 μL of a bone marrow sample using a standard technique. An aliquot of the patient DNA as well as separate aliquots of DNA from previously tested *JAK2* V617F-positive and *JAK2* V617F-negative patients (utilized as positive and negative controls, respectively) were subjected to PCR amplification using a pair of oligonucleotide primers annealing to regions flanking exon 14 of the *JAK2* gene. A separate reaction containing no DNA template was also performed as a control for reagent contamination. A standard PCR protocol was employed using 30 cycles of denaturing at 94°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 1 min followed by a final extension at 72°C for 5 min. The PCR products were then separated from residual oligonucleotide primers, unincorporated nucleotides, polymerase, and salts by binding and elution from a column-based silica membrane (Qiaquick PCR Purification Kit, Qiagen, Inc., Valencia, CA). Twenty-five microliter aliquots of the purified PCR products were each digested for 16 hours at 37°C with two units of BsaXI. The digestion products were analyzed by electrophoretic separation on a 3% agarose gel with ethidium bromide incorporated for visualization.

Results with Interpretation Guideline

The results of the electrophoretic analysis of the digested PCR products can be interpreted with Fig. 16.2. Lane contents are as follows: lane 1, weight markers (WM); lane 2, digest of no template control (NTC); lane 3, digest of negative control (NC); lane 4, digest of positive control (PC); lane 5 digest of patient specimen (Pt); lane 6, weight markers (WM). Arrows

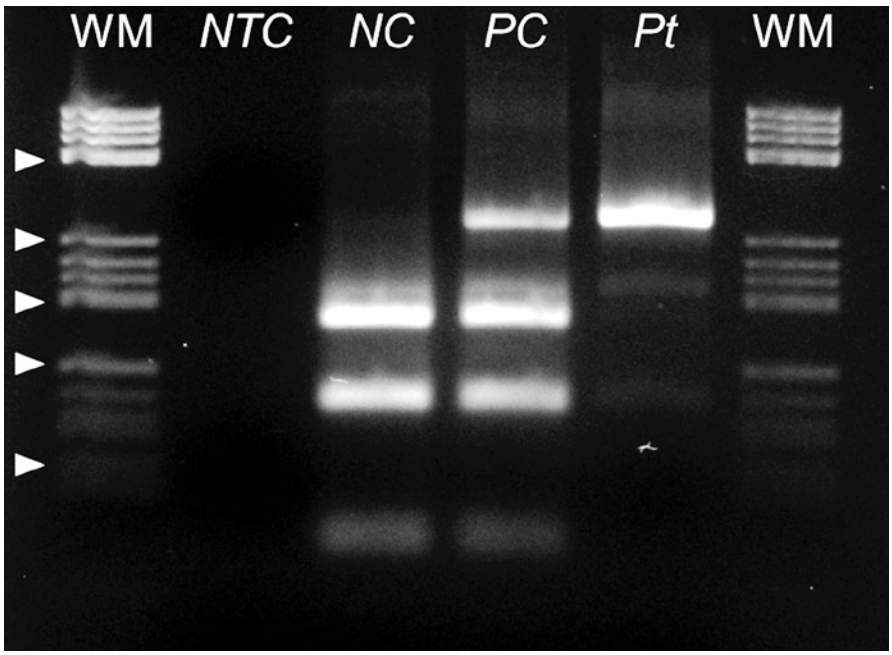

Fig. 16.2 Results from electrophoretic analysis of BsaXI digestion products

at the left side of the figure indicate molecular weight markers of 434 bp (top), 267 bp, 184 bp, 124 bp, and 80 bp (bottom).

Question 1: What is the most appropriate interpretation of these assay results?

Result Interpretation

As can be seen in Fig. 16.2, the no template control reaction (lane 2) shows no PCR or digestion products, confirming the purity of the PCR reagents. In lane 3, containing digestion products from the negative control specimen, bands at 165 bp, 101 bp, and 30 bp are present but there is no band at 296 bp, demonstrating that the PCR product was entirely digested. This is the expected result in the absence of the V617F mutation, that is, the BsaXI recognition site is intact, and confirms the activity of the enzyme. In lane 4, the digestion of the positive control specimen shows a band of 296 bp, demonstrating the presence of undigested PCR product, as well as bands at 165, 101, and 30 bp, which demonstrate digestion of a portion of the PCR product. This is the expected result for this sample, as it was derived from a previously tested sample known to be heterozygous for the V617F mutation. In lane 5, containing results from the patient specimen, the same four bands as seen with the positive control (296, 165, 101, and 30 bp) could be discerned, although at different intensities than seen with the positive control. This finding (and primarily the presence of the band at 296 bp) suggests that the patient's sample is positive for the *JAK2* V617F mutation.

A point to note about these results is that faint bands migrating slightly slower (i.e., at a higher molecular weight position) than the primary digestion product bands are visible in the control sample lanes as well as the patient sample lane. These are most apparent immediately above the band at the 165 bp position in each of the samples and most likely represent partial digestion products. As noted above, the BsaXI enzyme cuts the DNA strand in two places, resulting in the release of two large flanking fragments and a small fragment containing the restriction recognition sequence. Functionally, once the first cut in the DNA stand is made, releasing one of the large flanking fragments, the remaining recognition sequence is located very close to the end of one of the resulting DNA fragments. This proximity to the end of the strand reduces the efficiency with which the enzyme produces the second cut. Therefore, a small amount of digestion product comprising the 30 bp fragment joined to one of the larger flanking fragments remains intact and this accounts for the faint bands migrating just above the primary digestion product bands.

Finally, as alluded to above, a visual inspection of the pattern from the patient specimen (lane 5) reveals that the intensity of the bands representing digested product, that is, the bands of 165, 101, and 30 bp, are visible at much lower intensities than seen with the positive control, particularly in the case of the 30 bp band.

Question 2: What are some factors that might account for differences between specimens in the apparent intensity, or amount, of digestion products?

Several factors could account for the observed differences in band intensities between the control specimen and the patient specimen. First, it should be recognized that the apparent intensity of a band is proportional not only to the amount of DNA present in that band, but also to the molecular weight of the DNA fragment. Large fragments of DNA will bind a greater amount of the dye used for visualization relative to smaller fragments; also, for a given fragment size, a larger quantity of DNA will bind a larger amount of dye. Both of these factors contribute to the observed band intensity. Although attempts are made to equalize the amount of DNA placed into each PCR, inaccuracies in the quantitation and intrinsic variation between specimens can easily result in different amounts of product being generated in separate PCR aliquots, and this difference would then be carried over to the digestion phase of the assay. Second, a diminished intensity of the digestion product bands in the absence of an equally diminished undigested product band could indicate an incomplete digestion process. This is an important possibility to consider and is discussed further below. Finally, the zygosity with respect to *JAK2* V617F of any mutant clone population present in the sample can also have an impact on the amounts of the digestion products resulting from the analysis. For example, a specimen in which 100% of the cells in the sample are homozygous for the *JAK2* V617F mutation would be expected to yield only non-digested PCR product from this assay, that is, no wild-type DNA is present. By the same reasoning, either a sample in which 100% of the cells are heterozygous for the *JAK2* V617F mutation or a sample in which 50% of the cells are homozygous and 50% of the cells are wild-type would be expected to yield 50% digested product and 50% non-digested product. By extension, it can easily be envisioned that specimens with differing percentages of heterozygous or homozygous populations of cells admixed with wild-type cells could result in a variety of digestion product band intensities.

Question 3: Do the above results confirm the presence of the JAK2 V617F *allele?*

The result obtained in this case is highly suggestive of the presence of the *JAK2* V617F mutation based on the presence of undigested PCR product; however, it is important to note that this finding could also result from an incomplete digestion of PCR product derived from patient DNA, which is negative for the *JAK2* V617F mutation. The use of controls in restriction endonuclease digestion reactions is essential to assess whether the enzyme is effective. In this case, the results of the controls were as expected. The presence of some digestion products of the expected size in the patient's sample demonstrates that the endonuclease is functional, but it does not prove that the digestion was entirely complete. Alternatively, heterozygosity for a base change (either synonymous or non-synonymous) at one of the other specific nucleotide positions within the recognition site of the enzyme could also produce this digestion pattern but might not result in the V617F mutation at the protein level. Therefore, although it is useful as a screening tool, this assay would not be appropriately used as a confirmation for the presence of the *JAK2* V617F (1849G > T) mutation.

Question 4: What additional steps might be taken to definitively confirm the presence of the 1849G > T mutation?

Further Testing

This assay, although relatively simple and economical to perform, essentially depends upon the lack of a result, in this case the absence of restriction enzyme digestion, for the detection of a mutation. The confirmation of a positive interpretation obtained from an assay such as this could be approached in one of several ways, including a repeat analysis with the same technique, a repeat analysis using an alternative technique, or by direct DNA sequencing of the PCR product. Although repeat analysis with the same or an alternative (non-sequencing based) technique may be useful in some situations, a direct DNA sequencing approach arguably provides the most convincing confirmation. In addition, sequencing allows the identification, and avoids the potential pitfalls, of an

unexpected or novel change in the nucleotides surrounding the mutation site. In this case, the presence of the *JAK2* V617F mutation was confirmed by direct DNA sequence analysis of the PCR product.

Other Considerations

This patient was enrolled into a Phase II clinical trial of the JAK2-specific inhibitor TG101348 (TargeGen) [19]. During the course of therapy, she has been monitored with physical examinations and complete blood counts every four weeks, and bone marrow biopsies to evaluate marrow fibrosis at six month intervals. The first bone marrow biopsy, which was performed prior to the initiation of TG101348 therapy, revealed reticulin fibrosis of grade 2 on a scale of 0–3. Six months after initiation of therapy, a repeat bone marrow analysis revealed fibrosis of 3/3; however, subsequent biopsies (performed at 12 months and 18 months after initiation of therapy), were negative (grade 0 on a scale of 0–3) for fibrosis. Reversion of myelofibrosis during treatment with TG101348 has been demonstrated in animal models of PV as well [19]. The percentage of myeloid blasts was not increased in any of the biopsies.

Background and Molecular Pathology

PV, PMF, and ET along with chronic myelogenous leukemia (CML) belong to a group of disorders called chronic myeloproliferative neoplasms, which are characterized by overlapping yet distinct clinical characteristics. Because CML is characterized by the hallmark Philadelphia chromosome, this disease is readily distinguished from the remaining MPN from a molecular aspect. Among the Philadelphia chromosome-negative MPN, PV, PMF, and ET share overlapping features. The primary distinguishing feature of PV is erythrocytosis. However, three distinct phases of PV have been described which occur sequentially. The first is an initial pre-polycythemic phase in which only mild erythrocytosis may be observed, followed by a true polycythemic phase with overt erythrocytosis, and finally a post-PV or "spent" phase characterized by cytopenias, including anemia and bone marrow fibrosis. In contrast to this, PMF is characterized by a transition from expanded numbers of dysplastic megakaryocytes in the bone marrow to overt marrow fibrosis. Finally, ET is a disease characterized primarily by expanded numbers of mature, hyperlobated megakaryocytes in the bone marrow and peripheral thrombocytosis. Very uncommonly, ET may ultimately progress to a stage of bone marrow fibrosis. The presence of marrow fibrosis, however, is typically considered cause to question the diagnosis of ET. Because of the significant overlap in clinical features and laboratory findings seen with PV, PMF, and ET, accurate diagnosis of these disorders is challenging and requires adherence to strict diagnostic criteria [7]. In some situations, the diagnosis becomes one of exclusion, particularly if sufficient molecular or other laboratory data are not available to meet the required criteria. Possibly accounting for some of the clinical similarities observed between PV, PMF, and ET, these three diseases also show some overlap at the genetic level, with at least a subset of each being characterized by *JAK2* mutations.

JAK2 is a member of the Janus family of non-receptor tyrosine kinases which are involved in multiple signal transduction pathways. In general, inactive forms of the JAK kinases are recruited to specific cell surface receptors upon binding of the receptor by its cognate ligand. Subsequent dimerization leads to activation of the JAK kinase and further propagation of the signal via downstream mediators including the STAT (Signal Transducers and Activators of Transcription) family of transcription factors. JAK2 has been shown to be the major JAK family member involved in signaling by the erythropoietin and thrombopoietin receptors, and is crucial to erythropoiesis [2, 20].

At the protein level, each member of the JAK family of kinases has 7 JH (JAK homology) domains. The catalytic site is located within the carboxyl terminal JH1 domain, while cytokine receptor association domains are present in the amino terminal JH7 domain. Within JAK2, V617 is located in the JH2 domain, which is a non-active, pseudokinase domain thought to be involved in auto-inhibitory regulation of the JH1 kinase activity. The proposed mechanism of constitutive activation of JAK2 by the V617F mutation involves disruption of this auto-inhibitory function of the JH2 domain [1]. A similar mechanism of action has been proposed for *JAK2* exon 12 mutations, which characterize a small number of PV cases [21].

Although *JAK2* exon 12 mutations have been identified in the small subpopulation of PV cases which are negative for the V617F mutation, such mutations have not been observed in *JAK2* V617F negative ET and

PMF. However, unlike PV, approximately 10% of *JAK2* V617F negative PMF cases and 2% of *JAK2* V617F negative ET cases have been found to harbor mutations at codon 515 of the thrombopoietin receptor gene, *MPL*. These mutations result in a substitution of either lysine or leucine for tryptophan at amino acid 515 of the MPL protein (p.Trp515Leu, c.1544G>T or p.Trp515Lys, c.1543_1544delinsAA), and expression of MPL W515L leads to constitutive activation of JAK2 [22]. Therefore, it appears that JAK2 activation plays an important role in the development of many if not all cases of PV, PMF, and ET. The exact mechanisms by which particular disease phenotypes are driven remain to be elucidated.

Multiple Choice Questions

1. Which of the following statements are true regarding *JAK2* and its relationship to myeloproliferative neoplasms?
   A. Cases of PMF and ET which harbor mutations in exon 12 of *JAK2* are clinically more aggressive than those which harbor the *MPL* W515K mutation
   B. For both the *JAK2* V617F mutation and the *JAK2* exon 12 mutations described in PV, the mechanism of constitutive activation of the protein product has been suggested to involve elimination of autoinhibitory regulation of the JAK2 kinase domain
   C. *JAK2* encodes a cell surface receptor tyrosine kinase and is frequently mutated, resulting in a constitutively active protein, in polycythemia vera
   D. The *JAK2* V617F mutation, when present together with the *MPL* W515K mutation within the same clonal population of stem cells, has been implicated in the development of PMF
   E. Both B and C
2. A quantitative laboratory assay specific for the *JAK2* V617F allele is used to measure the allele burden of a patient specimen. The result shows 75% *JAK2* V617F (quantified as a percentage of the total *JAK2*, i.e., mutant + wild-type, in the sample). Based upon this finding, which of the following statements is most accurate?
   A. All of the cells within the sample are heterozygous for the *JAK2* V617F allele
   B. It is not possible to predict the zygosity of any cells within the sample population based upon this result
   C. There is a mixture of cells which are *JAK2* V617F heterozygous and wild-type cells within the sample
   D. There is a mixture of *JAK2* V617F heterozygous cells and *JAK2* V617F homozygous cells within the sample
   E. There is a population of cells within the sample which are *JAK2* V617F homozygous
3. Considering the frequency of *JAK2* and *MPL* mutations in the myeloproliferative neoplasms, which of the following might be appropriate additional tests for a suspected case of ET which has been found to be negative for the *JAK2* V617F mutation?
   A. Testing for additional *JAK2* exon 14 mutations
   B. Testing for *JAK2* exon 12 mutations
   C. Testing for the *MPL* codon 515 mutations
   D. Both B and C
   E. None of the above
4. Given our current understanding of PV and specific therapies for PV, which of the following statements is most correct regarding the value of a quantitative analysis for *JAK2* V617F in the initial or ongoing evaluation of a patient with PV?
   A. In patients treated with *JAK2*-specific small molecule inhibitors, quantitative measurement of *JAK2* V617F allele burden can predict the development of resistance to these drugs
   B. Quantitative analysis of the *JAK2* V617F allele burden has been suggested to be useful for monitoring therapeutic efficacy of some but not all treatment regimens
   C. Quantitative analysis of the *JAK2* V617F allele burden is reliably useful in predicting prognosis in cases of PV as well as ET and PMF
   D. Serial quantitative analyses of the *JAK2* V617F allele burden (performed every six months) is useful in guiding drug selection and dose adjustments in cases of PV treated with *JAK2*-specific inhibitors
   E. Both A and D
5. Myelofibrosis is often seen in which of the following disorders?
   A. Acute panmyelosis with myelofibrosis
   B. Essential thrombocythemia
   C. Polycythemia vera, spent phase
   D. Primary myelofibrosis
   E. A, C, and D

Answers to Multiple Choice Questions

1. *The correct answer is B.*

JAK2 is a member of a family of *non-receptor* tyrosine kinases. These are normally located within the cytosol of a cell and are recruited to the inner surface of the cellular membrane upon binding of a ligand by a cell surface receptor, such as the erythropoietin receptor. *JAK2* exon 12 mutations have, to date, only been described in cases of PV and not in cases of ET or PMF. *JAK2* mutations and *MPL* mutations have not been described together within the same cell population and appear to be mutually exclusive, that is, *MPL* mutations have only been identified in *JAK2* V617F negative cases of ET and PMF. The postulated mechanism of action of both the *JAK2* V617F and the *JAK2* exon 12 mutations involves a release of auto-inhibition of the JAK2 kinase by eliminating the interaction of the kinase domain with an adjacent pseudokinase domain.

2. *The correct answer is E.*

In the absence of a homozygous population of cells, the greatest percentage of mutant allele obtainable would be 50%, the expected result if every cell in the population was heterozygous for the mutation. Therefore, given that 75% of the total *JAK2* alleles in this sample are positive for the V617F mutation by the assay, it can be concluded that there must be a population of cells present which are homozygous for the mutation. Although it is possible that a mixture of heterozygous and homozygous cells (as suggested in answer D) could produce this finding, the data presented are not sufficient to make this determination. In view of this, the best answer is E.

3. *The correct answer is C.*

The *JAK2* V617F mutation, located in exon 14 of *JAK2*, is found in approximately 50% of ET cases. To date, no other causative exon 14 mutations have been described in PV, ET, or PMF. *JAK2* exon 12 mutations have only been described in cases of PV, not in ET or PMF. In contrast, the *MPL* W515L and W515K mutations have been described in a small subset of *JAK2* V617F negative ET and PMF cases (but not in PV). Therefore, at present, there is some rationale for testing a *JAK2* V617F negative ET case for the *MPL* mutations but not for additional *JAK2* mutation testing.

4. *The correct answer is B.*

As described in the "Laboratory test performed" section, potential uses for quantitative *JAK2* V617F analysis include monitoring disease progression or response to therapy and prognostic evaluation of specific cases. The value of a quantitative assay for prognostic evaluation remains a matter of interest but has not been fully validated, and could not be considered reliably useful for this purpose. There is interest in the use of a quantitative assay to monitor disease progression or response to therapy, similar to the use of quantitative *BCR-ABL* analysis in CML cases. At present, there is some evidence that allele burden (as measured by a *JAK2* V617F quantitative assay) may accurately reflect response to some therapies, such as interferon alpha [15]. However, there is no evidence that it could be used to predict resistance or to specifically guide therapeutic alterations.

5. *The correct answer is E.*

Myelofibrosis is a term used to describe reticulin or collagen fibrosis of bone marrow. As the name suggests, myelofibrosis is a cardinal feature of acute panmyelosis with myelofibrosis and PMF. In the spent phase, patients with PV will demonstrate overt reticulin fibrosis or collagen fibrosis. Bone marrow specimens from patients with ET usually show normal or minimal myelofibrosis. In fact, significant reticulin fibrosis or collagen fibrosis excludes the diagnosis of ET.

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Acute Myeloid Leukemia: *FLT3/NPM1*

17

Ruan T. Ramjit and Charles E. Hill

Clinical Background

A 74-year-old male was found to have pancytopenia with low vitamin B12 levels on routine physical exam with his primary care physician. He complained of increasing fatigue over the last several months, which he attributed to his advancing age, but otherwise felt well. The patient denied any other symptoms. Pertinent family history revealed that his father died of acute leukemia at the age of 66 and his mother died at age 78 with heart complications and possible chronic lymphocytic leukemia. His primary care physician attempted to treat him with vitamin B12 in an effort to address the low levels, but there was no response. For additional assessment of his pancytopenia, the patient was referred to a hematologist who ordered laboratory studies to evaluate his peripheral blood and bone marrow. A complete blood count (CBC) showed megaloblastic anemia, a critically low white blood cell count, and thrombocytopenia. Upon review of the peripheral smear, the pathologist noted moderate anisopoikilocytosis of the red cells and rare circulating blasts. On aspirate smear, an increase in blasts was identified comprising approximately 30% of total nucleated cells. Erythroid precursors were markedly decreased. The core biopsy of the bone marrow correlated with these findings and showed decreased marrow cellularity of approximately 10%. A patchy interstitial increase in blasts was noted, comprising approximately 20% of total nucleated cells. Megakaryocytes were also decreased. Flow cytometric analysis on bone marrow revealed a distinct population of cells, comprising 20% of the total sample which expressed CD13, CD33 (low density), CD34, CD38, CD117, HLA-DR, and CD 45 (low density).

Question 1: After reviewing this preliminary information, what neoplastic hematologic disease is most likely?
Question 2: What additional laboratory studies might be helpful?

Data from flow cytometry demonstrated a phenotypically distinct population of myeloblasts consistent with acute myeloid leukemia (AML). A typical workup for a patient with acute leukemia includes cytogenetic analysis on the bone marrow and molecular testing. Cytogenetic analysis on the patient's bone marrow showed a normal male karyotype and fluorescent in situ hybridization (FISH) studies, performed using a panel of DNA probes to detect abnormalities frequently involved in myeloid neoplasia, were negative for del(5q), del(7q), trisomy 8, and 11q23 abnormalities (*MLL* gene). Based on these findings, the patient was diagnosed with AML with a normal karyotype.

Question 3: Why is molecular testing useful in patients diagnosed with AML with a normal karyotype?

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Table 17.1 Prognostic significance of selected genetic alterations in AML with normal karyotype

Prognostic significance	Site of genetic abnormality	Expression
Favorable	NPM1	Mutation
	CEBPα	Mutation
Unfavorable	FLT3-ITD	Mutation
	BAALC	Overexpression
	MNI	Overexpression
	MLL-PTD	Mutation/overexpression
	ERG-1	Overexpression
	AFIq	Overexpression
Unclear	FLT3-Asp835	Mutation

Reason for Molecular Testing

AML is a complex, highly heterogeneous disorder characterized by an arrest of myeloid maturation along with uncontrolled proliferation of hematopoietic progenitor cells or blasts. This heterogeneous nature is exemplified in the French-American-British (FAB) classification that had previously subdivided AML into subgroups (M0-M7) based on its degree of differentiation and morphology. This system was limited in biologic, prognostic, and therapeutic importance. In an effort to integrate this information clinically and diagnostically, the 2001 World Health Organization (WHO) classification incorporated both cytogenetic and molecular findings while also introducing significant prognostic correlations. The 2008 WHO revision increased the number of entities with recurrent chromosomal translocations and gene mutations. Therefore, due to the broad variability of AML, there is also a variable prognosis among AML patients. Prognosis, choice of treatment regimen, and minimal residual disease (MRD) monitoring are critical in these patients and are evolving to depend on identification of gene mutations that are not observed on typical cytogenetic analysis. In an estimated 60% of cases, specific recurrent chromosomal aberrations can be identified by modern cytogenetic techniques, although there is a large subgroup of AML patients (~40%) who have no distinguishable cytogenetic abnormalities [1]. According to current literature, cytogenetic information is the single most important tool to categorize patients at their initial diagnosis into three prognostic categories: favorable, intermediate, and poor [1]. Table 17.1 illustrates some of the currently known genetic abnormalities in AML with normal karyotype. Only the mutations pertinent to this case will be further discussed.

At this time, patients who can be classified according to some of the known balanced chromosomal translocations, such as t(8;21) or inv(16), have a favorable prognosis and are generally treated with contemporary chemotherapy while those who have chromosomal aberrations associated with an unfavorable prognosis undergo allogeneic stem cell transplantation, provided suitable donors exist. The therapeutic options, however, are unclear for AML patients without cytogenetic abnormalities, making molecular risk stratification of greater significance to refine the prognostic characteristics in this group. Due to this clinical necessity, several genetic alterations have been studied and there is abundant evidence indicating that mutations in fms-like tyrosine receptor kinase 3 (*FLT3*) carry a poor prognosis and mutations in nucleophosmin (*NPM1*) are associated with a more favorable prognosis [2, 3].

Test Ordered

After reviewing the laboratory data and current literature, the hematologist ordered molecular diagnostic testing for *FLT3* and *NPM1* gene mutations. Mutations of *FLT3* may be either internal tandem duplications within the juxtamembrane domain (ITD) or activating point mutations in the tyrosine kinase domain (TKD). Tests for the ITD involve detection of the additional genetic material as an increase in size of this portion of the gene. TKD mutations may be detected by methods that interrogate the sequence of the gene. *NPM1* mutations are typically insertions or deletions ranging from four to nine base pairs (bp) and are identified by detection of the resulting increase or decrease in genetic material in the C-terminal portion of the *NPM1* gene.

Laboratory Test Performed

FLT3 and *NPM1* mutation detection can be assessed by polymerase chain reaction (PCR). This molecular technique has impressive analytic sensitivity and has become the foundation of molecular genetic pathology. Since its advent, PCR has allowed for the identification of mutations that are either missed or cannot be detected in traditional cytogenetic or FISH analysis due to probe design, specimen quality, and the number of cells that

are scored [4]. After 30 cycles of amplification, each DNA or cDNA target sequence has been copied 230 times, generating approximately a billion amplicons, which can be quantified using real-time instrumentation. The amplicons can be further analyzed by methods such as sequencing, melting curve analysis, and electrophoresis. Clinical samples with a low cell count and even partially degraded nucleic acid can often still be tested, though care should be taken to prevent degradation before analysis. To monitor specimen quality, results are typically correlated with the results of a control gene demonstrating amplifiable DNA.

The recommended sample types include fresh blood, marrow aspirate, frozen or paraffin-embedded tissue. Almost all PCR-based assays show better responses when performed with fresh or frozen cells. This differs from formalin-fixed cells due to cross-linking, making the nucleic acid less accommodating to hybridization. EDTA is the preferred anticoagulant for PCR-based reactions as heparin is known to interfere with DNA amplification.

For PCR amplification of the internal tandem duplication, primers were utilized which have been selected to amplify exons 14 and 15, targeting the juxtamembrane domain (Invivoscribe Technologies, San Diego, CA). Forward and reverse ITD PCR primers are fluorescently tagged with 6-FAM and HEX, which display as blue and green when analyzed by differential fluorescence software on a capillary electrophoresis instrument. For the results to be valid, the ITD peak must display both colors. This decreases the possibility of a false positive result. An unmutated or wild-type product consists of a peak at 330 bp and also serves as the amplification control. A peak greater than 332 bp is considered to be positive for the presence of an ITD mutation.

The TKD mutation is detected using a forward PCR primer which binds to exon 20 and has a fluorescent NED molecule added to the 5′-end. The forward primer is displayed as black by the capillary electrophoresis software. The reverse primer is unlabeled and is directed toward the reverse sequence of exon 20. A restriction enzyme (*Eco*RV) cut site is engineered into the reverse D835 (Asp835) PCR primer. Initial PCR amplification yields an undigested 150 bp product. Restriction enzyme digestion of an unmutated D835 product yields an 80 bp NED-labeled fragment. This wild-type product will be detected by capillary electrophoresis and displayed as a black peak by the differential software. If, however, there is a D835 mutation present, the *Eco*RV wild-type cut site will be eliminated, resulting in a longer NED-labeled product of 130 bp from the mutant allele. This product is also displayed as a black peak by the capillary electrophoresis software.

Question 4: Why was the EcoRV cut site engineered into the reverse primer in the manner described?

The *Eco*RV cut site was built into the reverse primer to serve as an internal control to monitor for completeness of restriction enzyme digestion. Incomplete digestion or failure of the restriction enzyme step would correlate with the undigested NED-labeled 150 bp wild-type product from initial PCR amplification. Further, the cut site allows one to easily distinguish between the digestion products which occur at either 80 bp for the wild-type or 130 bp for the D835 mutant allele. Because there are no fluorophores on the reverse primer, the opposing fragments generated by restriction enzyme digestion and formation of the 80 bp product and the 130 bp product are not displayed by the differential software.

Mutations in *NPM1* have been described as another of the most frequent genetic aberrations in normal karyotype AML typically occurring as insertions or deletions in exon 12 [1, 2, 5]. Two types of mutations have been described. The most frequent is a four bp insertion downstream from nucleotide 959 and the second most common is an insertion of nine extra nucleotides; both types result in alterations at the C-terminus of the mutant proteins [5, 6]. Detection of these mutations is performed with PCR using primers directed at exon 12. The product is detected by a 6-FAM labeled probe and is illustrated as a peak on the electropherogram. An unmutated *NPM1* product would appear as a 187 bp peak. Patients with the more common four bp insertion mutation for *NPM1* would have a 191 bp mutated product. If there are larger insertions, such as the nine bp insertion, then the product size could be larger.

Results with Interpretation Guideline

DNA isolated from the patient's leukemic cells was amplified using PCR and sized by capillary electrophoresis as described in the previous section. The results of the *FLT3* ITD analysis are displayed in Fig. 17.1a and the results of the *FLT3* D835 TKD mutational analysis

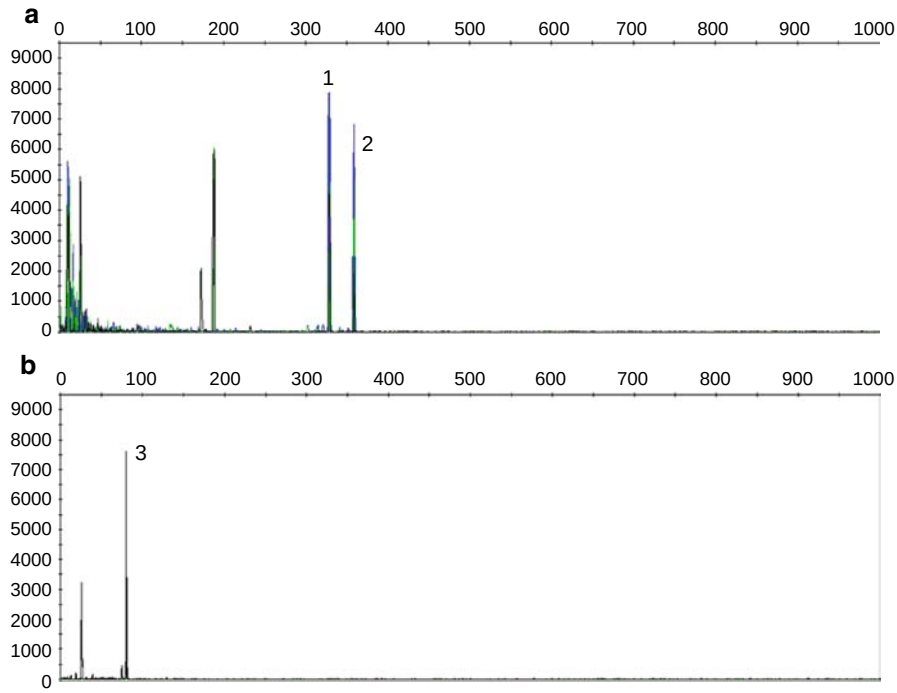

Fig. 17.1 *FLT3* testing results. (**a**) *FLT3* ITD mutation testing electropherogram. *Peak 1* =330 bp. *Peak 2* =360 bp. (**b**) *FLT3* TKD D835 mutation testing electropherogram. *Peak 3* =80 bp

Table 17.2 Patient's results for *FLT3* mutational testing

Test	Color on electropherogram	Product size in nucleotides (bp)
FLT3 ITD	Blue and green	Peak 1: 330 bp (polyclonal control)
		Peak 2: 360 bp (patient DNA)
FLT3 D835	Black	Peak 3: 80 bp (patient DNA digested)

Table 17.3 Interpretation values for *FLT3* mutational testing

Mutation	Target	Color on electropherogram	Product size in nucleotides (bp)
FLT3 ITD	ITD	Blue and green	Wild-type: 330 bp
			ITD mutation: >330 bp
FLT3 D835	TKD	Black	Undigested: 150 bp
			Wild-type digested: 80 bp
			Mutant digested: 130 bp

are displayed in Fig. 17.1b. The relevant product sizes of the peaks to be assessed for both of these tests are indicated in Table 17.2. Table 17.3 delineates the criteria to interpret the results. Additional testing for *NPM1* yielded a product of 187 bp (not shown).

Question 5: After reviewing the patient's results for FLT3 ITD, FLT3 D835 TKD, and NPM1 mutation analysis, how would you interpret the results?

Result Interpretation

Figure 17.1a displays the results for the first test evaluating for *FLT3* internal tandem duplication. According to specified criteria for the assay, PCR amplification will show a peak at approximately 330 bp for the unmutated gene (peak 1) and any peak detected at greater than 330 bp indicates the presence of the internal tandem duplication mutation which can vary from 3 to 400 bp in size. Notice that peak 2 is located at 360 bp, which is consistent with an ITD. This particular duplication is a 30 bp insertion. In addition, one can conclude that these results are valid because both peaks display both colors established for the forward and reverse primers (blue and green) when analyzed with the fluorescence analysis software. Figure 17.1b demonstrates results for restriction enzyme digestion analysis for the alteration of the aspartic acid in the TKD (codon 835 point mutation) of the *FLT3* gene. Peak 3 is located at approximately 80 bp and illustrates the absence of the D835 mutation. The PCR product is

also analyzed undigested to verify the quality of the PCR amplification and should demonstrate a 150 bp product (not shown). The patient did not have a mutated *NPM1* gene because testing also performed by PCR and capillary electrophoresis yielded a product of 187 bp. A mutated *NPM1* would consist of an insertion mutation of four bp longer than the normal sequence (191 bp). Other variants of this insertion mutation have been documented and can result in a larger bp size of the mutant peak.

Further Testing

In addition to the known balanced translocations and inversions that occur in patients afflicted with AML, specific gene mutations also occur just as illustrated in this clinical case. Patients with normal karyotype AML on standard cytogenetic examination comprise the single largest cytogenetic subset of adult AML [3]. In studies on the clinical significance of cytogenetics in AML, patients with a normal karyotype have been classified into the intermediate-risk cytogenetic group with five year survival rates between 24% and 42% [3, 7]. The disappointing cure rate is largely due to the unique biologic and clinical characteristics of the disease. Along with the aforementioned *FLT3* and *NMP1* aberrations, mutations in *CEBPA* (encoding the CCAAT/enhancer binding protein-α), *KIT, MLL, WT1, BAALC, NRAS*, and *KRAS* have also been reported [8]. It is now very clear that AML patients are heterogeneous at the molecular level and increasing importance is being placed on what these mutations mean in terms of clinical outcome. The challenge remains to integrate these genetic anomalies into innovative risk-adapted therapeutic strategies, and as it pertains to this case, it may be warranted clinically to seek additional testing for mutations other than *FLT3* or *NMP1* in patients who present with normal karyotype AML.

Other Considerations

Remission control monitoring by morphology alone is limited because residual malignant cells usually can only be detected with prognostic validity if they exceed at least 5% in the bone marrow [9]. Progress in our understanding of the biological behavior of hematologic diseases has led to the need to improve MRD monitoring in patients with AML both during and at the end of treatment. In conjunction with this clinical need, advances in PCR technology have increased the sensitivity and specificity of malignant cell detection in patients who appeared to be in remission by conventional methods. For example, cases with reciprocal gene fusions t(15;17)/*PML-RARA*, inv(16)/*CBFB-MYH11*, and t(8;21)/*AML1-ETO*, evaluated by quantitative PCR techniques provided sensitivities ranging from 10^{-4} to 10^{-6} [9–12]. Further, the ratio of aberrant gene expression following consolidation therapy and at diagnosis was demonstrated to be prognostically significant [10] with distinct thresholds of transcript copy numbers correlating with an increased risk of relapse [9]. The increase in fusion transcripts was observed three to six months before clinical manifestations of relapse [9]. From these data, one can see the importance of quantitative molecular monitoring in order to plan alternative treatment approaches. Currently, the molecular assays available to quantify MRD are limited to certain AML subtypes and none are commercially available to assess *FLT3* transcript levels. However, the allelic ratio for *FLT3* may be an important indicator of prognosis. The allelic ratio is a measurement of the mutant ITD allele compared to the normal *FLT3* allele. An allelic ratio of 0.4 or greater is associated with a poor prognosis in children [13]. For adults, an allelic ratio of 0.7 or greater appears to predict responsiveness to certain tyrosine kinase inhibitors [14]. Some clinical trials and treatment protocols, especially for children, may require measurement of the allelic ratio. The significance of similar data for the TKD mutation is unclear.

It is also important to note that a patent was issued in the USA and other countries for the association between *FLT3* mutations and prognosis in AML (US Patent 6,846,630 accessed at www.uspto.gov on 04-21-2010). Clinical testing may be limited or require the payment of royalties to patent holders or licensees in some jurisdictions. Further, the test approaches presented in this clinical case are the most commonly performed analyses for mutations of *FLT3* and *NPM1*. However, allele-specific PCR, real-time PCR, Sanger sequencing, amplicon melting analysis, and other techniques may be employed to detect these mutations [4]. The analytical properties of these testing methodologies vary widely and may or may not be appropriate for monitoring patients on therapy.

Background and Molecular Pathology

In 2008, the World Health Organization classification system of AML was revised to include a category called AML with gene mutations [8]. This group encompasses normal karyotype AML with nucleotide level changes. Among the genetic alterations discussed are the *FLT3* and *NPM1* gene mutations.

FLT3, located on 13q12, is a tyrosine kinase receptor involved in hematopoietic stem cell/progenitor cells (HSPCs) survival and proliferation [15]. It is normally expressed on HSPCs and over time expression is lost as the cells differentiate. However, in AML, alterations in *FLT3* constitute one of the most frequent somatic mutations occurring in approximately 35–45% of patients [9], causing increased proliferation and decreased apoptosis. Mutation in the *FLT3* gene causes overexpression at the level of RNA and protein, resulting in auto-phosphorylation and constitutive activation of *FLT3*. A signaling cascade then begins by inducing activation of several signaling transduction pathways such as PI-3-kinase/AKT, RAS/MAPK, and JAK/STAT, among a multitude of other channels downstream, either directly or indirectly. Two major types of mutations are observed. The first type is an internal tandem duplication (*FLT3*-ITD) in the juxtamembrane domain, varying from 3 to 400 bp in size, which always occurs in-frame to preserve the function of the protein. The second type is a point mutation that affects the aspartic acid 835 (D835) of the kinase domain (*FLT3*-TKD), likewise leading to constitutive activation of *FLT3*. Patients with *FLT3*-ITD tend to have a worse clinical outcome, whereas the prognostic impact of *FLT3*-TKD mutation alone is still unclear. There is minimal information to suggest what the outcome is for patients with combined *FLT3*-ITD and *FLT3*-TKD mutations, although preliminary data seem to indicate that the combination is associated with an unfavorable prognosis [16].

The next and most frequently mutated gene in normal karyotype AML patients is *NPM1*, mutations of which are present in about 45–55% of patients [5, 9, 17, 18]. The *NPM1* gene maps to chromosome 5q35 and encodes a nucleus-cytoplasm shuttling protein that in its unmutated state is localized to the nucleolus. These proteins are ubiquitous within the cell and are generally highly conserved playing vital roles in ribosome biogenesis, centrosome duplication, genomic stability, cell cycle progression, and apoptosis. Wild-type *NPM1* contains two nuclear export signals (NES) motifs, one within residues 94–102 and one within the N-terminus. In addition, there is a nucleolar localization signal (NLS) at the C-terminus that aids in shuttling the protein from the cytoplasm to the nucleus and then to the nucleolus via nucleolar-binding domains. Somatic mutations in *NPM1* typically consist of four bp (though sometimes up to 11 bp) insertions in exon 12. The majority of exon 12 mutations constitute a replacement of tryptophan(s) at positions 288 and/or 290, generate novel NES and motifs inserted into the C-terminus disrupting the normal NLS and causing aberrant accumulation of the protein within the cell cytoplasm. This genetic alteration has gained prognostic significance when combined with data from *FLT3* testing. The presence of *NPM1* mutation in the absence of a *FLT3*-ITD mutation correlates with a favorable prognosis. The presence of the *NPM1* mutation is associated with an intermediate prognosis when *FLT3*-ITD is also present. Younger patients with an *NPM1* mutated/*FLT3*-ITD negative genotype have a favorable prognosis similar to AML patients with t(8;21) or inv(16) and these patients may additionally have the possibility of exemption from allogeneic stem cell transplantation in first complete remission. It is not yet known whether AML with chromosomal aberration or multi-lineage dysplasia in conjunction with an *NPM1* mutated/*FLT3*-ITD negative genotype is a favorable prognostic indicator.

Multiple Choice Questions

1. Which of the following statements is correct regarding testing for the ITD mutation of *FLT3*?
   A. Cannot be performed because of a patent issued in the USA
   B. Is always performed by analysis of restriction enzyme digestion products
   C. Is commonly performed by measuring the size of the PCR amplicon
   D. Is used only for cases of AML with t(8;21)
   E. Is usually performed to monitor disease progression
2. Mutations of *NPM1*:
   A. Alter a tyrosine kinase domain and make the protein constitutively active
   B. Alter the promoter and lead to increased expression
   C. Change the DNA-binding domain

D. Change the nuclear localization signal so that the protein accumulates in the cytoplasm
E. Change the protein into an extracellular signaling ligand

3. Patients with the diagnosis of AML with *NPM1* mutated and lacking the *FLT3*-ITD:
   A. Have a better prognosis than those with a *FLT3*-ITD
   B. Have a poorer prognosis than those with unmutated *NPM1*
   C. Have an increased risk of disseminated intravascular coagulation
   D. May be treated with "watchful waiting"
   E. Need to be treated at diagnosis with allogeneic stem cell transplant
4. Clinically significant mutations of *FLT3* are typically:
   A. Activating mutations
   B. Gain of novel function mutations
   C. Loss of function mutations
   D. Silent mutations
   E. None of the above
5. Testing for *NPM1* mutations:
   A. Can be excluded if the patient has a normal karyotype
   B. Involves detection of point mutations in the N-terminal region of the gene
   C. May only be performed by Southern blot due to large insertions
   D. Must be performed on all patients with anemia
   E. Should detect insertions or deletions as small as four base pairs

Answers to Multiple Choice Questions

1. *The correct answer is C.*
2. *The correct answer is D.*
3. *The correct answer is A.*
4. *The correct answer is A.*
5. *The correct answer is E.*

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Acute Myeloid Leukemia: *CEBPA*

18

Matthew W. Anderson

Clinical Background

The patient was an 18-year-old man who presented to the emergency department complaining of a two-week history of fatigue, lightheadedness, easy bruising, and the acute onset of gross hematuria. The patient had no significant past medical history, and his other family members were reportedly healthy. Physical examination revealed a thin appearing young man with scattered petechiae and ecchymoses of the skin. There was neither hepatosplenomegaly nor enlarged lymph nodes. A complete blood count revealed a white blood cell count of 7.5 K/μL with 15% circulating blasts, hemoglobin of 9.6 g/dL, and a platelet count of 56 K/μL. Due to the suspicion for acute leukemia, a bone marrow aspirate and biopsy was performed. Morphologic examination revealed effacement of the marrow by a population of leukemic blasts with monocytic features. By flow cytometry analysis, the blasts exhibited a precursor cell immunophenotype with surface expression of CD34 and CD117. The blasts expressed myeloid lineage antigens CD13, CD33, and myeloperoxidase with partial expression of monocytic lineage markers CD11c and CD64. The blasts also aberrantly expressed the T cell antigen CD7. Cytogenetic analysis of the bone marrow aspirate revealed a normal 46,XY male karyotype, and there was no evidence by FISH of an *MLL*, *RUNX1*(*AML1*)/*RUNX1T1*(*ETO*), or *BCR/ABL1* gene rearrangement. Molecular analysis of DNA extracted from the bone marrow aspirate specimen showed no evidence of *FLT3* or *NPM1* mutations.

Question 1: What additional diagnostic testing (if any) should be performed on this patient's bone marrow specimen?

Reason for Molecular Testing

A variety of genes have been associated with the pathogenesis of acute myeloid leukemia (AML) with a normal karyotype, including those encoding fms-related tyrosine kinase 3 (*FLT3*), nucleophosmin 1 (*NPM1*), and CCAAT/enhancer-binding protein-α (*CEBPA*). Mutations in *CEBPA* were originally identified in leukemic blasts from patients with AML [1], and subsequent studies have shown that mutations in *CEBPA* are associated with a more favorable prognosis in patients with a normal karyotype AML [2–7]. Therefore, it is important to determine the mutational status of *CEBPA* for this patient, as this information will influence therapeutic decisions for this patient including eligibility for allogeneic bone marrow transplantation.

Test Ordered

CEBPA sequence analysis on DNA extracted from the bone marrow aspirate specimen.

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Laboratory Test Performed

Mutations in the *CEBPA* gene are located throughout the coding sequence, encompassing deletions, duplications, and single nucleotide substitutions. Due to the heterogeneity of *CEBPA* mutations, DNA sequencing by the Sanger dideoxynucleotide termination method is the "gold-standard" for mutation detection. Using published protocols [8], four primer sets were used to amplify the entire coding region of *CEBPA*, and then each PCR product was sequenced independently. While direct sequencing may represent the most thorough approach to mutation detection, the analysis can be laborious and expensive to perform. In addition, Sanger sequencing has a relatively low sensitivity for mutation detection (approximately 10–20% mutant DNA in wild-type DNA). Although the definition of acute leukemia by World Health Organization (WHO) criteria stipulates 20% leukemic blasts in the bone marrow, *CEBPA* sequence analysis may be requested on specimens that contain less than 20% blasts. Therefore, Sanger sequencing may fail to detect clinically relevant mutations in *CEBPA* without careful attention to specimen adequacy. To address this issue, several groups have developed multiplex PCR fragment-length assays, which offer rapid analysis with an increased sensitivity and a reduced cost [9, 10]. However, these assays can only detect changes in amplicon length due to insertions or deletions, and will not detect single nucleotide substitutions. Indeed, a study comparing both Sanger sequencing and multiplex fragment-length analysis for detecting *CEBPA* mutations found that the fragment-length analysis method failed to detect single nucleotide changes, which encompassed 40% of all *CEBPA* mutations in 33 patient specimens [11]. High resolution melting curve analysis has also been utilized as an initial screen to triage specimens for complete sequence analysis, but this approach has not yet entered routine clinical diagnostic use [12].

Results with Interpretation Guideline

The DNA sequencing results for this patient's specimen demonstrated two heterozygous sequence changes in two separate amplicons (Fig. 18.1). For clarity, only the sequence of the complementary DNA strand is shown, but the mutations were confirmed in both sequencing directions. For reference, the wild-type *CEBPA* sequence is included for comparison and interpretation of the results.

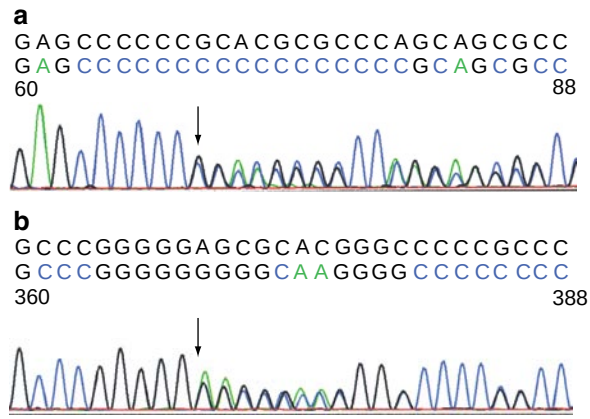

Fig. 18.1 *CEBPA* sequencing results. Two different mutations (*arrows*) in the *CEBPA* gene were identified in two different PCR amplicons (*panels* **a** and **b**). For each panel, the upper nucleotide sequence represents the wild-type *CEBPA* sequence with the DNA numbering as indicated. The lower nucleotide sequence in each panel was generated by the automated base calling software

Question 2: Using the CEBPA reference sequence NM_004364.3, what is the correct nomenclature to describe the effect of each of these sequence changes at both the DNA and protein level?

Result Interpretation

This patient's specimen shows two heterozygous mutations in the *CEBPA* gene, each of which represents a single nucleotide duplication event. The first mutation (Fig. 18.1a) is a heterozygous duplication of a cytosine nucleotide at position 68 in the nucleotide sequence (numbering based on the *CEBPA* reference sequence NM_004364.3). This duplication results in a histidine to alanine amino acid change at codon 24, a frameshift and creation of premature stop codon. The stop codon is predicted to occur 84 codons downstream from the changed amino acid (designated as the first codon in the frameshifted sequence). Using the nomenclature guidelines published by the Human Genome Variation Society (http://www.hgvs.org), the correct description of this mutation at the DNA sequence level is c.68dup. The correct nomenclature for the predicted effect on the CEBPA protein is p.His24AlafsX84. The analysis for this mutation is illustrated below with the nucleotide change in underlined italics and the amino acid change in bold.

Codon#:	20	21	22	23	24	25	26
Wild-type DNA sequence:	CAG	AGC	CCC	CCG	CAC	GCG	CCC
Wild-type amino acid sequence:	Q	S	P	P	**H**	A	P
Mutant (dupC) sequence:	CAG	AGC	CCC	CC*C*	GCA	CGC	GCC
Mutant amino acid sequence:	Q	S	P	P	**A**	R	A

The second mutation (Fig. 18.1b) is a heterozygous duplication of a guanine nucleotide at position 368 in the nucleotide sequence. This duplication results in an alanine to serine amino acid change at codon 124, a frameshift and a premature stop codon. The stop codon is predicted to occur 46 codons downstream from the changed amino acid. The correct description of this mutation at the DNA sequence level is c.368dup. The correct description for the predicted effect on the CEBPA protein is p.Ala124SerfsX46. The analysis for this mutation is illustrated below with the nucleotide change in underlined italics and the amino acid change in bold.

Codon#:	120	121	122	123	124	125
Wild-type DNA sequence:	ATG	CCC	GGG	GGA	GCG	CAC
Wild-type amino acid sequence:	M	P	G	G	**A**	H
Mutant (dupG) sequence:	ATG	CCC	GGG	GG*G*	AGC	GCA
Mutant amino acid sequence:	M	P	G	G	**S**	A

Question 3: What is the significance of accurately predicting the site of the premature stop codon?
Question 4: Can we determine from this analysis whether the two sequence changes are present on the same chromosome or on opposite chromosomes?

Further Testing

No further molecular testing was indicated for this patient.

Other Considerations

Several studies suggest that improved patient prognosis is associated only with identification of a *CEBPA* mutation on each allele of the *CEBPA* gene (biallelic) [4–7]. However, the sequencing assays routinely utilized by molecular pathology laboratories cannot discriminate whether two mutations are present on the same chromosome (in *cis*) or on opposite chromosomes (in *trans*). The presence of two sequence changes in the same amplicon would suggest that the mutations were present in *trans*, given that the presence of two mutations in close proximity on the same allele is rare in most gene regions. In this case, however, because the two mutations were identified in two different amplicons, the *cis/trans* relationship between these mutations cannot be presumed at all. One method to determine the phase of these two mutations would involve cloning the DNA products into bacterial plasmids to generate clonal templates for sequencing. However, bacterial cloning is laborious and poses too great a contamination risk for routine use in the clinical molecular pathology laboratory.

Another approach to determining phase could be through the use of next-generation sequencing technology. Most of the commercially available next-generation sequencing platforms utilize an in vitro clonal amplification step to generate multiple identical copies of an individual DNA template molecule [13]. By amplifying and sequencing each template DNA molecule independently, the *cis*/*trans* relationship of mutations can be resolved, provided that the sequencing chemistry has a sufficiently long read length to detect multiple sequence variants on one template molecule. Although pyrosequencing offers the longest read length of the currently available next-generation sequencing chemistries (~500 bases), this read length is still insufficient to cover the entire coding region (~1 kilobase) of the *CEBPA* gene. However, new high-throughput sequencing technologies are being developed at a prodigious rate, and in-phase sequencing of large genomic regions may soon be possible with the development of single-molecule sequencing instruments that can sequence kilobases (or more) of DNA from a single template molecule.

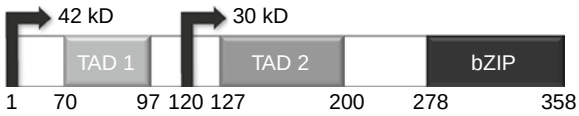

Fig. 18.2 Schematic representation of the CEBPA protein. CEBPA contains three functional domains encompassing two transactivation domains (TAD 1 and TAD 2) and a basic leucine zipper/DNA binding domain (bZIP). A second translational start site upstream of TAD2 in the *CEBPA* gene mediates expression of the 30 kD form of the protein. The numbering of the amino acid sequence is as indicated

Background and Molecular Pathology

CEBPA is an intronless gene located on chromosome 19q13.1, which encodes a transcription factor of the basic region leucine zipper family. The CEBPA protein contains three functional domains (Fig. 18.2). These include two transactivation domains (TAD) which interact with other transcription factors and a basic leucine zipper domain (bZIP) required for DNA binding and dimerization. Two forms of the protein can be expressed, a 42 kD full-length protein, and a smaller 30 kD protein which lacks the first transactivation domain. The full-length 42 kD form binds to target genes and acts to suppress cell proliferation. In contrast, the 30 kD form represses cellular differentiation by inhibiting the activity of the 42 kD form in a dominant negative manner [1]. Therefore, the biological activity of CEBPA is critically dependent on the ratio of the two CEBPA proteins within the cell.

In normal hematopoiesis, CEBPA promotes granulocyte lineage commitment. Mice conditionally lacking the *CEBPA* gene show an increase in bone marrow blasts, and fail to generate mature neutrophils [14]. Approximately, 10% of all cases of AML show *CEBPA* mutations, and *CEBPA* mutations are more frequently (15–18%) found in patients with normal karyotype AML [3]. Germline mutations in *CEBPA* have also been associated with familial cases of AML [15], suggesting that dysregulation of CEBPA expression can represent a primary initiating event in AML. The leukemic cells frequently show a myeloblastic (FAB M1 and M2) morphology and immunophenotype with expression of CD34, HLA-DR, CD13, and CD33. The blasts of our patient also showed aberrant expression of CD7, an immunophenotypic finding often observed in *CEBPA* mutated AML [16].

CEPBA mutations in AML are often biallelic, with an N-terminal mutation on one allele in combination with a C-terminal mutation on the other allele. The most common amino-terminal mutations are insertions or deletions which create a frameshift and premature stop codon before the second translational start site. This prevents translation of the 42 kD form while preserving the 30 kD form. In contrast, C-terminal mutations are most often in-frame insertions or deletions in the bZIP domain, which disrupt DNA binding and affect both the 42 and 30 kD forms. The consistent pattern of biallelic N- and C-terminal mutations in *CEBPA* strongly suggests that overproduction of the 30 kD CEBPA protein is the key molecular event which drives leukemogenesis in *CEBPA* mutated AML [1].

Although overexpression of the 30 kD CEBPA protein provides an attractive molecular mechanism for the development of AML, it is not yet known precisely why biallelic *CEBPA* mutations result in more favorable patient prognosis. Gene-expression profiling experiments have shown that the expression of homeobox gene (*HOX*) family members is consistently downregulated in leukemic blasts with biallelic *CEBPA* mutations [7, 17]. Therefore, improved patient prognosis in biallelic *CEBPA* mutated AML may be secondary to downregulation of *HOX* gene family members, and not a direct result of the activity of the 30 kD CEBPA protein.

CEBPA sequence analysis poses a unique interpretive challenge for the molecular pathologist. The description of *CEBPA* mutations in the molecular pathology report must include not only an accurate reporting of the mutation at the DNA sequence level, but a detailed description of the predicted effects of a particular mutation on the CEBPA protein. This is especially important in the context of a frameshift mutation, in which the position of the newly created stop codon will determine which (if any) of the two forms of the CEBPA protein are expressed. In this case example, the first mutation (c.68dup, p.His24AlafsX84) generates a frameshift and premature stop codon prior to the translation start site for the 30 kD form. Therefore, only the 30 kD CEBPA protein will be produced from this allele. The other *CEBPA* mutation in this patient's specimen (c.368dup, p.Ala124SerfsX46) results in a frameshift and premature stop codon past the second translational start site, preventing expression of both the 42 kD and the 30 kD CEBPA proteins from this allele (assuming a *trans* orientation of mutations).

Taken together, these sequencing results suggest that the patient's leukemic blasts express only the 30 kD CEBPA protein, leading to differentiation arrest and the development of leukemia.

Multiple Choice Questions

1. *CEBPA* sequence analysis is indicated for which of the following patients with AML?
   A. 18-year-old man with acute promyelocytic leukemia
   B. 27-year-old woman with normal karyotype AML
   C. 45-year-old woman with normal karyotype AML and a *FLT3* ITD mutation
   D. 68-year-old man with chronic lymphocytic leukemia
   E. 72-year-old man with a complex karyotype AML arising from an antecedent myelodysplastic syndrome
2. Overexpression of the 30 kD form of the CEBPA protein results in:
   A. Acute erythroblastic leukemia
   B. Decreased expression of the 42 kD CEBPA protein
   C. Dominant-negative inhibition of the activity of the 42 kD form of CEBPA
   D. Granulocytic maturation
   E. Methylation of the promoter region of *CEBPA*
3. The most frequently identified *CEBPA* mutations are:
   A. C-terminal in-frame mutations
   B. N-terminal frameshift mutations
   C. N-terminal missense mutations
   D. A and B
   E. A and C
4. The impact of *CEBPA* mutations on prognosis in patients with normal karyotype AML is best described as:
   A. Favorable prognosis in the presence of biallelic *CEBPA* mutations
   B. Favorable prognosis in the presence of monoallelic *CEBPA* mutations
   C. Poor prognosis in the presence of biallelic *CEBPA* mutations
   D. Poor prognosis in the presence of monoallelic *CEBPA* mutations
   E. A and B
5. Dysregulation of CEBPA has been identified in which of the following cancers?
   A. Acute myeloid leukemia
   B. Adenocarcinoma of the lung
   C. B lymphoblastic leukemia
   D. Squamous cell carcinoma
   E. All of the above

Answers to Multiple Choice Questions

1. *The correct answer is B.*

CEBPA sequence analysis is indicated in patients with normal karyotype AML. *CEBPA* mutations have been identified in patients with AML exhibiting more complex karyotypes (choice E) [16], but the prognostic significance of *CEBPA* mutations in this patient group is unclear. The prognostic impact of *CEBPA* mutations in the presence of *FLT3* mutations (choice C) is also unclear. One study suggests that patients with monoallelic *CEBPA* mutations have a higher rate of *FLT3* ITD mutations, suggesting a different pathway of leukemogenesis in these patients [7]. While there is evidence to suggest the PML-RARA gene fusion product in t(15;17) AML (acute promyelocytic leukemia) (choice A) can interfere with the function of the CEBPA protein [18], mutations in the *CEBPA* gene do not appear to be prevalent in t(15;17) AML [1]. Dysregulation of the *CEBPA* gene is not a known causal event in the development of chronic lymphocytic leukemia (choice D), a mature B cell neoplasm.

2. *The correct answer is C.*

Overexpression of the 30 kD form of the CEBPA protein results in dominant-negative inhibition of the activity of the 42 kD CEBPA protein. Although the precise molecular mechanism remains unclear, the dominant-negative effect may be due to the ability of the 30 kD form to bind DNA without the transactivation activity mediated by the first transactivating domain [1]. There is no evidence to suggest that the 30 kD CEBPA protein mediates methylation of the promoter region of *CEBPA* (choice E) or that it directly downregulates the expression of the 42 kD CEBPA protein (choice B). Multiple studies have shown that the 42 kD form of CEBPA, not the 30 kD form, promotes granulocytic differentiation (choice D) [19]. Overexpression of the 30 kD form of the CEBPA protein is thought to result in leukemias of the myeloid, not erythroid lineage (choice A).

3. *The correct answer is D.*

The most frequent mutations in the *CEBPA* gene are N-terminal frameshift mutations (choice A) and in-frame C-terminal mutations (choice B). In-frame N-terminal missense mutations can be identified in the *CEBPA* gene (choices C and E), but these mutations are not as frequent.

4. *The correct answer is A.*

Although initial studies suggested that both monoallelic and biallelic mutations in *CEBPA* may be associated with favorable prognosis (choice B) [2, 3], more recent studies suggest that only biallelic *CEBPA* mutations confer a better prognosis in the context of normal karyotype AML [4–7]. Monoallelic or biallelic *CEBPA* mutations are not associated with poorer prognosis (choices C and D).

5. *The correct answer is E.*

Consistent with its role as a general tumor suppressor, dysregulation of *CEBPA* has been implicated in a number of human cancers [19], including adenocarcinoma of the lung (choice B), B lymphoblastic leukemia (choice C), and squamous cell carcinoma (choice D), in addition to AML (choice A).

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Bone Marrow Engraftment Analysis

19

Hanna Rennert, Debra G.B. Leonard, and Tsiporah Shore

Clinical Background

A 66-year-old man presented with urinary retention and was having a preoperative evaluation for a transurethral prostatectomy. He was found to have pancytopenia and was referred to a hematologist. The patient had a history of eosinophilic cellulitis three years earlier, which was treated with steroids for one year with complete resolution of the problem. He also had a history of a squamous cell carcinoma of the skin, which was resected. At the time of presentation, he had no bleeding or bruising. He was fatigued but still active and had no recent infections. Medications included tamsulosin for prostatic hypertrophy and supplemental Vitamin D. The patient was a retired purchasing agent with no history of chemical exposure. His parents died of heart disease and one brother with diabetes was still alive. He had two daughters, alive and well.

A bone marrow aspirate and biopsy were diagnostic for a myelodysplastic syndrome. After several months, the patient was reevaluated. Physical examination was unremarkable apart from mild erythema around the umbilicus. There was no hepatosplenomegaly and no adenopathy. There were no petechiae. WBC was 2.8×10^9/L, hemoglobin 8.1 g/L and platelets 18×10^9/L. A repeat bone marrow aspirate and biopsy were done. At this time, the marrow biopsy was hypercellular for age. The myeloid to erythroid ratio was increased and the myeloid series was left shifted, with a decrease in neutrophils and bands. Blasts were increased and formed an interstitial infiltrate. Eosinophils were not increased. The erythroid lineage was maturing normally. Megakaryocytes were present, but overall decreased. A reticulin stain demonstrated a minimal increase in reticulin fibrosis. The bone marrow aspirate indicated that myelopoiesis was hyperplastic, left-shifted, and dysplastic. Blasts were increased, intermediate to large in size, and had a reticular chromatin pattern with occasional prominent nucleoli. The nuclear contour was often indented to convoluted. The cytoplasm was sparse to abundant, agranular and deeply basophilic, and an occasional prominent golgi area was noted. Dysgranulopoiesis was present, with hypogranulation and abnormal segmentation. Myeloblasts constituted 25% of the nucleated marrow cellularity, based on the differential count of the bone marrow aspirate smears. This increase in blasts also was demonstrated by immunohistochemistry. Cytogenetic studies demonstrated a normal 46,XY karyotype. Occasional megaloblastoid erythroid cells were seen, but megakaryocytes were markedly decreased. A monocytosis was present. Based on FAB classification, the myelodysplastic syndrome had converted to AML, classified as FAB-M2 (WHO classification: AML, not otherwise specified (NOS)).

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The patient received induction chemotherapy with seven days of cytarabine and three days of daunorubicin, after which he achieved remission. He had consolidation therapy with five days of cytarabine and two days of daunorubicin.

Following treatment, the patient was in complete remission from secondary AML with normal cytogenetics (intermediate risk). The best chance for long-term disease-free survival would be with an allogeneic stem cell transplant. However, a matched related donor was not available. An unrelated donor was available. This donor was a female in her 50s with a history of six pregnancies, which increases the risk of graft versus host disease, morbidity and mortality. Therefore, the decision was made to transplant the patient utilizing two umbilical cord units. The patient was given a reduced-intensity conditioning regimen of fludarabine 40 mg/m^2 on days −6 to −2, cyclophosphamide 50 mg/kg on day – 6, and total body irradiation of 200 cGy, followed by the double cord (DC) transplant. This regimen, also called nonmyeloablative, uses less intense treatment to prepare for transplant than a standard transplant does, and is appropriate for patients undergoing transplant at an older age (>50 years). Graft versus host disease prophylaxis consisted of tacrolimus and mycophenolate mofetil.

Posttransplant complications included infection with rhinovirus and hemorrhagic cystitis with associated BK virus in blood and urine, treated with cidofivir. Engraftment occurred on day +27 with a WBC of 1.2×10^9/L, and an absolute neutrophil count of >500. There was no evidence of graft versus host disease. A bone marrow biopsy was performed on day +56 following transplant. Myeloid and erythroid precursors demonstrated complete maturation. Megakaryocytes were adequate in number and morphology. An increased number of blasts was not seen, and cytogenetic testing revealed a normal 46,XY karyotype.

Reason for Molecular Testing

Given that this transplant was associated with reduced intensity conditioning of the patient, it was important to define that the engraftment was of donor origin rather than residual recipient hematopoiesis. This can be assessed with chimerism testing on peripheral blood. The chimerism studies in this case were particularly important because an abnormal cytogenetic marker could not be used to identify residual AML. Evaluation of chimerism following transplant allows a determination of the status and engraftment of the infused stem cell product, especially in the situation of a nonmyeloablative preparative regimen. With a DC transplant, chimerism studies also allow evaluation of the status of each individual donor cord transplant. Usually, one donor cord transplant "wins" over the other and becomes the only source of engrafted cells, which also can be determined by chimerism analysis [1].

Question 1: What are the main reasons for selecting BME/chimerism testing?

Test Ordered

Bone marrow engraftment (BME)/chimerism testing was ordered to monitor the levels of donor cells in the patient's CD3+ (T cell lymphocyte) and CD33+ (myelocyte) cell populations, following the double cord blood transplant.

Laboratory Test Performed

Prior to the transplant, peripheral blood collected from the recipient, and donors' cord blood specimens were analyzed to identify informative short tandem repeat (STR) markers, different between donors and recipient. After transplantation, chimerism status was assessed on peripheral blood samples collected on days +18, +32, and +51 following transplant. Chimerism status was monitored on CD3+ T cell lymphocytes and CD33+ myelocytes separated by an immunomagnetic method, using a fully automated cell separator (RoboSep®), and the EasySep® cell labeling reagents (STEMCELL Technologies Inc., Vancouver, Canada). Using this method, more than 90% of the isolated cell fractions were enriched for CD3+ T cell lymphocytes and CD33+ myelocytes, respectively, as assessed by flow cytometry.

Genomic DNA was extracted from peripheral blood WBCs before transplant using the QIAamp DNA MiniKit (QIAGEN Inc., Valencia, CA), as recommended by the manufacturer. After transplantation, DNA was isolated from peripheral blood T lymphocytes and myelocytes. DNA was quantified using standard UV absorption at 260 nm and all samples were

Table 19.1 STR loci and multiplex assays used for the pre- and posttransplant analyses

Pretransplant		Posttransplant	
PPLEX	D16S539	*GSTR*	D16S539
	D7S820		D7S820
	D13S317		D13S317
	D5S818		D5S818
	CSF1PO	*CTTV*	CSF1PO
	THO1		THO1
	TPOX		TPOX
	vWA		vWA
	Amelogenin		
FFFL	F13A01	*FFFL*	F13A01
	FESFPS		FESFPS
	F13B		F13B
	LPL		LPL

diluted to a final concentration of 2 ng/μL in DNA elution buffer (Qiagen).

BME or chimerism status was evaluated using a multiplex fluorescent polymerase chain reaction (PCR) assay and a reference panel of 13 polymorphic microsatellite markers (Promega, Madison, WI), as recommended by the manufacturer. Briefly, 2 ng of DNA were amplified in a total reaction volume of 10 μL, using AmpliTaq Gold with a hot start protocol and a Veriti PCR thermocycler (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Before transplant, the recipient and two donor DNAs were amplified at the following 13 STR loci for the identification of recipient- and donor-specific loci (Table 19.1): D16S539, D7S820, D13S317, D5S818, CSF1PO, THO1, TPOX, vWA, and the amelogenin locus which is used to discriminate between X and Y chromosomes (PowerPlex® 1.2 System) and F13A01A, FESFPS, F13B, and LPL (FFFL *GenePrint*® Fluorescent STR System). The PowerPlex® 1.2 System is also available as two separate quadruplex panels (CTTv and GSTR *GenePrint*® Fluorescent STR Systems) used for posttransplant analysis, as indicated in Table 19.1. After transplant, the CD3+ and CD33+ DNA samples were sequentially analyzed using the CTTv *GenePrint*® Fluorescent STR System harboring the informative marker CSF1PO. The advantage of this approach is that a high level of sensitivity can be maintained because fewer loci are amplified, while simplifying the workflow in the laboratory by using fewer different STR panels, as appropriate, based on the selected informative locus or loci. The PCR products were separated by capillary electrophoresis on an ABI 3130 PRISM Genetic Analyzer (Applied Biosystems). Fragment sizes were determined with GeneMapper 4.0 software (Applied Biosystems). Semiquantitative assessment of mixed chimerism was performed by determining the ratio of peak areas for donor and recipient informative alleles. Using this method, the analytical sensitivity of the test was 1%. Results were reported as percentage recipient and donor, respectively.

Question 2: Why is the STR-PCR test for BME/chimerism assessment viewed as semiquantitative?

Limitations of the Assay

1. The CD3+/CD33+ procedure requires viable cells from EDTA anticoagulated whole blood that is less than 24 hours old.
2. The CD3+/CD33+ enrichment procedure is optimal with at least 20 million cells, with a minimum requirement of two million cells. Insufficient DNA concentration may result in low PCR amplification signal or no amplification.
3. An informative marker must be present for engraftment analysis of posttransplant samples. Some loci are more polymorphic than others. This may be particularly challenging when distinguishing a trio of two cord blood donors and a recipient.
4. The analytical sensitivity achieved can range from <1% to 10% and depends on several factors, including the number of loci amplified and the amount and quality of input DNA. The efficiency of amplification is also influenced by certain loci and genotypes [2].
5. Although rare, chromosome loss in tumor cells during the course of disease may cause corresponding loss of an STR locus. This is a potential source of error in the interpretation of engraftment analysis, especially if only one informative allele is used to monitor engraftment [3].

Results with Interpretation Guideline

In the pretransplant evaluation, STR fragment size analysis demonstrated allele-specific peaks for each donor and the recipient, corresponding to each of the 12 different STR loci tested. The number of the STR

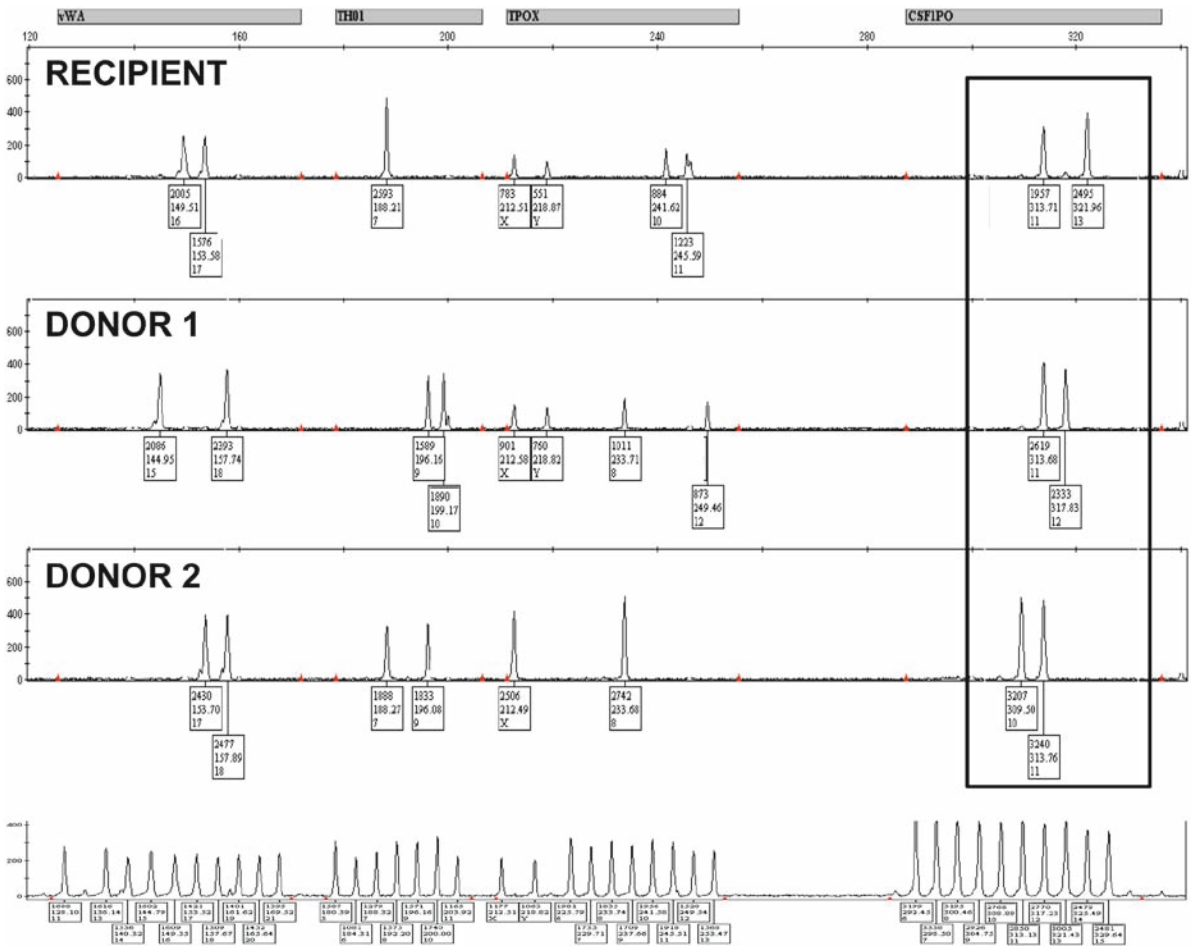

Fig. 19.1 Identification of informative loci using STR-PCR and capillary electrophoresis. STR alleles at 12 loci and amelogenin were amplified using the PowerPlex and FFFL Fluorescent STR Systems (Promega). The PCR products and allelic ladder were separated and detected by capillary electrophoresis on an ABI 3130 instrument. The number of repeats in each allele was compared for the cord blood (CB) donors and the recipient in order to identify the best informative loci, with unique alleles for each of the three individuals. For the purpose of distinguishing the recipient and the CB donors from each other, the CSF1PO marker was the best because it had unique alleles for each of the three individuals and the recipient informative allele was one repeat larger than the donor alleles

repeats for each allele was determined by comparison to an allelic ladder of known sizes (Fig. 19.1). Optimal informative loci have unique donor and recipient alleles that are not in stutter peak locations (nonspecific PCR products generated by Taq polymerase slippage during the PCR amplification that are one STR repeat smaller or larger than a specific allele peak) [2]. Although two loci were informative for discriminating the recipient from each cord blood donor: D16S539 (alleles 9, 10, 11, and 13) and CSF1PO (alleles 10, 11, 12, 13), marker CSF1PO was a better locus in this case because the informative allele of the recipient was located upstream of the donor alleles and the alleles from each CB donor could still be distinguished from each other (Table 19.2). Thus, this locus displayed unique alleles that could be used simultaneously to discriminate and quantify the recipient DNA relative to both cord blood samples: CB A through allele 12 and CB B through allele 10. Locus CSF1PO was then used to analyze engraftment of CD3+ and CD33+ subpopulations following transplant. The shared allele 11 was omitted from the calculation because it stays constant (i.e., all the cells in the samples whether donor or recipient will harbor this allele).

Table 19.2 Determination of informative alleles

Locus	Recipient alleles	CB donor A alleles	CB donor B alleles
D5S818	11, 12	12	11
D13S317	12, 13	12, 13	11
D7S820	9, 10	10, 12	9, 10
D16S539	10, 11	9, 12	12, 13
vWA	16, 17	15, 18	17, 18
TH01	7	9, 10	7, 9
TPOX	10, 11	8, 12	8
[a]CSF1PO	11, 13	11, 12	10, 11
LPL	12	10	10, 12
F13B	8, 10	8, 10	10
FESFPS	10, 11	10, 12	10, 11
F13A01	7	5, 6	7
Amelogenin	X, Y	X, Y	X

[a]Informative STR locus used to distinguish the recipient, CB donor A and CB donor B trio
Single alleles are presumed to be homozygous

Criteria for Selection of Informative Loci

1. Due to nonspecific PCR amplification (stutter peaks), usually one repeat down or up, choose recipient-specific alleles at least two repeats smaller or larger than the donor allele(s).
2. When possible, choose loci producing larger size PCR products to minimize nonspecific background baseline noise from larger amplification products.
3. Loci at which both of the donor and recipient alleles are informative are preferable because both alleles at each locus can be used for the analysis.
4. Primer sets containing more than one informative locus are preferable because they provide independent confirmation of recipient cells.

Posttransplant Analysis

Analysis of CD3+ and CD33+ cells isolated from three whole blood, posttransplant samples showed mixed chimerism (MC) in both cell populations on day 18 posttransplant, followed by complete engraftment on day 32 and preservation of complete donor engraftment on day 51 (Fig. 19.2). The percentage of recipient and donor cells for each of the two cell types is provided in Table 19.3. BME analysis on day 51 also demonstrated that eventually only CB donor A contributed to the donor alleles in this patient (Table 19.3).

Result Interpretation

BME analysis results on posttransplant day 18 demonstrated a high-level of mixed chimerism (88% and 80% of donor T cells and myeloid cells, respectively) followed by complete engraftment on posttransplant days 32 and 51. These results correlated well with the patient's phenotype of engraftment on day +27, as measured by a WBC of 1.2×10^9/L and an absolute neutrophil count >500, and with complete maturation of myeloid and erythroid precursors on posttransplant day 56. Moreover, CB donor A engrafted with loss of CB B, as was demonstrated by the fact that100% of the alleles corresponded to CB A.

Further Testing

Further testing is warranted for monitoring the effects of posttransplant therapies, as well as for clinical management of potential graft rejection, graft versus host disease (GVHD), and for early identification of possible leukemia relapse.

Other Considerations

Depending on the underlying disease and type of transplant, most patients undergoing standard myeloablative hematopoietic stem cell transplant (HSCT) rapidly achieve complete chimerism, defined as complete donor engraftment without recipient cells, which is the goal of this therapy. Subsequently, the detection of small amounts of mixed chimerism may indicate relapse, which is the most common cause of treatment failure. However, the importance of mixed chimerism in the setting of nonmyeloablative HSCT is less clear and does not always correlate well with outcome [4–6]. Major reasons for lack of correlation include analytical variability between assays used, sample types, frequency of BME monitoring, and the underlying disease. Nevertheless, the identification of increasing levels of mixed chimerism (increasing recipient alleles) does, in general, predict a high risk for relapse [7]. Moreover, in cases where a disease-specific marker is not available, BME analysis can be used as a surrogate test for recurrent disease. In contrast to myeloablative transplants, the initial outcome of nonmyeloablative therapy is mixed chimerism. The use of posttransplant

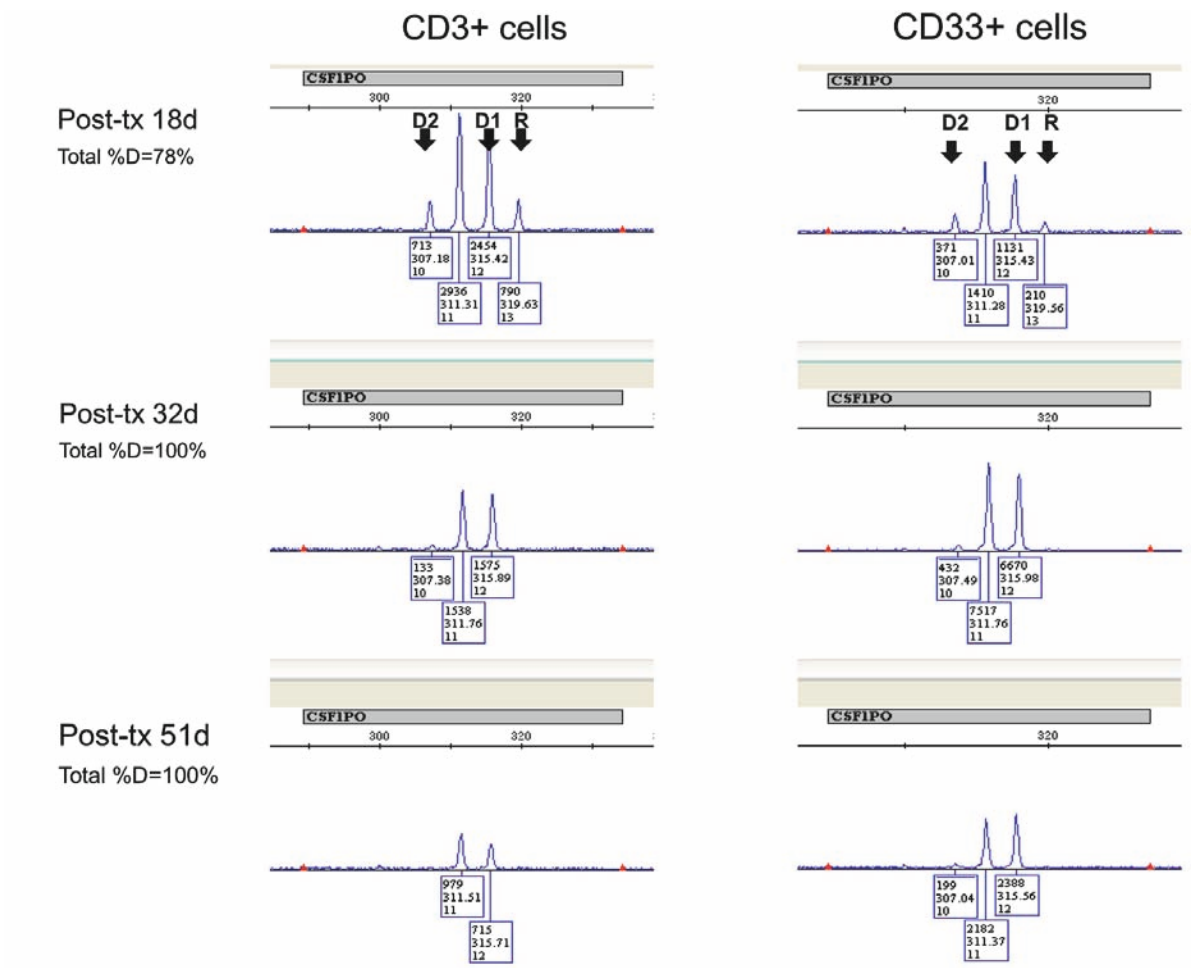

Fig. 19.2 Quantification of estimated percentage of chimerism between the recipient and the CB donor cell populations in post-transplant CD3+ and CD33+ cells, for marker CSF1PO (CTTv Fluorescent STR System, Promega) at three time points post-transplant. The alleles from the recipient and CB donors A and B (noted D1 and D2, respectively) each display a single unique allele used for quantification. A shared allele of 11 repeats was omitted from the quantification. The formulas used to calculate the estimated percentage of the recipient and CB donors A and B (D1/D2) are included at the bottom. "A" represents the area peak of the appropriate allele, designated by the arrows

Table 19.3 Posttransplant BME analysis results by CB donor and cell type

Sample#	Cell type	Days posttransplant	% Recipient (total)	% Donor (total)	% Donor (CB A)	% Donor (CB B)
#1	CD3+	18	12	88	66	22
#2	CD3+	32	0	100	94	6
#3	CD3+	51	0	100	100	0
#1	CD33+	18	20	80	62	18
#2	CD33+	32	0	100	96	4
#3	CD33+	51	0	100	100	0

donor lymphocyte infusions to promote complete donor chimerism requires regular patient monitoring by BME analysis.

Question 3: Is BME analysis/chimerism testing considered a genetic test and does it require the patient's informed consent?

Background and Molecular Pathology

HSCT is used to treat patients with hematologic malignancies, congenital hematologic disorders and aplastic anemia. There is a clear correlation between hematopoietic chimerism (or persistence of both host and donor cells) and eventual relapse [8, 9]. HSCT can use either a myeloablative pretreatment regimen, or a milder nonmyeloablative pretreatment regimen that allows a broader range of patients to receive HSCT therapy, such as older patients or those with prior extensive chemotherapy exposures. Myeloablative transplant can be autologous (using the patient's own stem cells) or allogeneic (when the donor is either related or unrelated). In myeloablative transplants, patients are treated with intensive chemotherapy and/or radiation to kill the tumor cells. This intense treatment also destroys the patient's bone marrow progenitor cells, requiring infusion of stem cells to rescue hematopoiesis. In nonmyeloablative transplants, also referred to as reduced-intensity transplants, patients are given low-dose chemotherapy and immunosuppression to promote engraftment of donor stem cells. The goal is to establish a donor-based cellular immune response leading to a graft versus tumor effect.

Bone marrow was the original source of stem cells for transplantation. Over the past few decades, however, the collection of peripheral blood CD34+ stem cells by leukapheresis has become an important alternative [10]. Currently, the use of peripheral blood stem cells (PBSC) exceeds that of bone marrow for adult transplants [1]. More recently, the use of umbilical cord blood (CB) has become an option for stem cell transplants. However, one of the major disadvantages of this method is the low stem cell count of CB [11]. In an effort to overcome the low stem cell counts, several medical centers have developed protocols involving infusion of umbilical cord blood collections from two donors, which is called a double cord blood transplant (DCBT) [12].

Cord Blood Transplant: Advantages and Disadvantages

Approximately 30% of transplant candidates have a compatible donor. For the remaining 70% of candidates there is a 50–80% chance of finding a compatible donor through national registries. In addition, the search time for a suitable donor is approximately 3.7 months [1]. For these reasons, many eligible patients do not proceed to transplant and there is a need for more abundant and readily accessible sources of stem cells, such as CB, for transplant [13].

One major advantage of CB for transplants is the increased flexibility in the degree of HLA matching. CB units are typically matched by serology at the HLA-A and -B loci, and by high-resolution testing (DNA sequencing) at the HLA-DR locus [11, 12, 14]. With two alleles at each locus, the best match is 6/6. In PBSC or BM transplants, at least a 5/6 match is necessary to avoid severe GVHD. In contrast, CB transplants have been successful with as many as 3/6 mismatches [11, 15]. Also, matching at HLA-C or -DQ and high resolution typing at all HLA loci did not affect two year survival [16] and CB collection has no impact on the mother or the child, reducing significantly the median time to donor availability. Finally, statistics from the New York Blood Center indicate that ~150,000 units would be required to provide a >80% chance of finding a 5–6/6 HLA match for an adult transplant candidate [17].

A key disadvantage of a single CB transplant is the cell count. Both the number of nucleated cells and CD34+ cells are approximately tenfold lower in CB than in BM or PBSC collections [11]. This leads to delayed engraftment with CB transplants and an increase in graft failure, when directly compared to BM transplants [18]. One initial concern was that double CB infusion could lead to graft versus graft effect, preventing engraftment. However, in later reports, it became evident that, typically, cells from only one CB contribute to long-term hematopoiesis. Moreover, hematopoiesis from only a single cord can be detected in patients as early as 21 days after transplant [12].

Cell Lineage-Specific Chimerism Analysis

Follow-up measurement of hematopoietic donor chimerism after allogeneic HSCT is a useful tool for confirming the stability of lymphoid and myeloid donor

engraftment [7, 10]. The lack of association in some studies, however, of mixed chimerism with graft rejection and relapse may reflect the fact that chimerism was not analyzed in the cell subsets responsible for these effects [10]. Testing of cell lineage-specific chimerism is particularly important for patients at high risk of graft rejection or leukemia relapse, that is, in those patients receiving a T-cell depleted peripheral blood graft by CD34+ selection, or a nonmyeloablative HSCT [19, 20]. Detection of an increase in lymphoid and myeloid host cells may provide an indication for early therapeutic interventions, such as donor lymphocyte infusion (DLI) [19]. Moreover, donor T cells play an important role in complications such as GVHD, as well as in beneficial events such as facilitating hematopoietic donor engraftment and graft-versus-leukemia effect (GVL) [20]. Low donor T-cell chimerism on posttransplant day 14 has been associated with an increased probability of graft rejection, and is one of the earliest predictors of BME success or failure [19, 21]. Full T-cell donor engraftment also precedes donor myeloid engraftment and disease regression, consistent with a requirement for >90% donor T-cell chimerism for full expression of the alloresponse [19]. Likewise, serial measurement of host myeloid cells in patients diagnosed with a myeloid malignancy may predict leukemia relapse [22].

The CD3 antigen is expressed on all T cells and is associated with the T-cell receptor. Seventy to eighty percent of human peripheral blood lymphocytes (PBL) and 65–85% of thymocytes express the CD3 antigen. The CD33 antigen is brightly expressed on monocytes and dimly expressed on granulocytes and dendritic cells. The CD33 antigen also is expressed on myeloid progenitor cells, but not on lymphocytes, platelets, erythrocytes, and primitive hematopoietic stem cells. Standard isolation procedures use anti-CD3 and anti-CD33 antibodies attached to magnetic microbeads to isolate nearly pure T-cell and myeloid populations, respectively. Flow cytometry is typically used to assess the purity of the cell isolation. DNA extracted from these cells is used for the BME/chimerism testing after transplant.

BME/Chimerism Assay

The assessment of BME/chimerism status following transplant is based on the ability to distinguish between recipient and donor cells. Several methods have been used [2], however, these methods have been largely replaced by PCR-based methods which are more sensitive, more reproducible and faster. The most commonly used method is PCR amplification of STR loci consisting of a core sequence (ranging from one to eight nucleotides in length), which is tandemly repeated a variable number of times (five to 20 core sequences) [23]. The STRs are highly polymorphic due to the different number of core tandem repeats present in the population. PCR amplification of tetranucleotide STR loci has become the method of choice for DNA-based identity testing in humans [24, 25].

Multiple Choice Questions

1. BME/chimerism analysis can be used for all of the following types of HSCT except:
   A. Allogeneic donor lymphocyte infusion
   B. Autologous hematopoietic cell transplantation
   C. Double cord blood transplant
   D. HLA-matched related donor
   E. HLA-matched unrelated donor
2. STR analysis is used for all of the following applications except:
   A. Maternal cell contamination for prenatal samples
   B. Microbial identity testing
   C. Forensic testing
   D. Parentage testing
   E. Resolution of histology “floater” fragments
3. Stutter peaks detected in STR-PCR analysis are mainly caused by:
   A. Amplification of multiallelic loci
   B. Nonspecific background signal
   C. Nucleotide editing during PCR amplification
   D. Slippage of *Taq* polymerase during PCR amplification of STR loci
   E. STR locus instability
4. All of the following techniques have been used for assessment of BME/chimerism status except:
   A. HLA typing
   B. Northern blotting
   C. SNP analysis
   D. Southern blotting
   E. Y Chromosome analysis

5. Assuming complete engraftment of CB A stem cells in this case, the best locus to use for future BME/chimerism studies will be:
   A. Amelogenin
   B. CSF1PO
   C. D13S317
   D. THO1
   E. vWA

Answers to Questions Embedded in the Text

Question 1: What are the main reasons for selecting BME/chimerism testing?

Major reasons for BME/chimerism testing include: (a) confirmation of initial engraftment following HSCT; (b) monitoring of hematopoietic reconstitution by donor-derived cells; (c) measurement of chimerism in cellular subpopulations to predict graft rejection, GVHD (graft versus host disease) and early relapse; and (d) monitoring effectiveness of posttransplant therapies. BME/chimerism testing also enables the study of cord transplants to elucidate the mechanisms that allow one cord unit to have a survival advantage over the other. This information may be exploited in the future to select the most appropriate cord donors for patients.

Question 2: Why is the STR-PCR test for BME/chimerism assessment viewed as semiquantitative?

The increase in the amount of amplicons during PCR amplification stays exponential for only a limited number of cycles, typically 15–20, after which the amplification rate reaches a plateau (conventional PCR). In this plateau phase, the quantified level of PCR product is no longer proportional to the starting amount of target molecules or cells. This method, also used for the BME/chimerism test, is considered semiquantitative, because quantification is performed at the end of the PCR process (end point) rather than during the linear phase. Factors that affect the PCR amplification rate at the plateau phase include substrate saturation of the enzyme, product strand reannealing and incomplete product strand separation. The accuracy of quantification is further affected by the number of DNA targets amplified, that is, single locus as opposed to multiplex amplification. By contrast, quantitative PCR (qPCR) is kinetically based, that is, PCR product amounts are measured in the exponential phase only where the amplification efficiency is nearly 100%, and the number of amplicons is doubling at each cycle, better reflecting the relative abundance of target nucleic acid in each reaction. The application of real-time PCR for clinical testing enables more sensitive and accurate quantification of mixed chimerism [9].

Question 3. Is BME analysis/chimerism testing considered a genetic test and does it require the patient's informed consent?

Although BME/chimerism testing evaluates germline genetic sequences in a patient, it is not considered a genetic test per se because these sequences are not associated with any particular known disease. Therefore, this testing would not require the patient's informed consent for genetic testing.

Answers to Multiple Choice Questions

1. *The correct answer is B.*

BME/chimerism analysis can be used for all the following types of allogeneic (not from self) HSCT, but not for autologous (self) hematopoietic cell transplantation or syngeneic (identical twins) transplant. Identical twins are considered a complete genetic match for a HSCT and cannot be distinguished by DNA markers.

2. *The correct answer is B.*

Identity testing by STR analysis is used for all the indicated genotyping applications in humans, but is not useful for microbial identification, which is performed by sequencing of the 16S ribosomal RNA gene.

3. *The correct answer is D.*

Stutter peaks are an artifact of STR PCR amplification that may arise from "slippage" within the repeat sequence during the PCR process. Stutter peaks of tetranucleotide STRs are four bp shorter than the main peak and can have a peak area close to 5% of the main peak area [2].

4. *The correct answer is B.*

All the indicated methods, with the exception of northern blotting which is RNA based, are well established DNA applications currently available for identity testing. These methods are based on identification of specific DNA sequences that can distinguish one individual from another. Although northern

blotting allows the detection of species-specific or tissue-specific gene expression, it does not have the required resolution to discriminate one individual from another.

5. *The correct answer is D.*

Assuming complete engraftment of CB A (Donor 1 in Fig. 19.1), the best locus to use for future BME/chimerism studies is THO1. The seven repeat allele of the THO1 locus is unique for the recipient and is located two repeats downstream from the nearest donor allele. D13S317 is not informative. vWA alleles are located one repeat down from both donor alleles. The CSF1PO 13-repeat allele is informative and can be used although it is less optimal, because it is only one repeat larger than the nearest donor allele and tends to yield a weaker amplification signal. The major limitation of the amelogenin locus is its applicability for sex-mismatched transplants only. In this case both the engrafted donor and the recipient were male.

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Mixed Phenotype Acute Leukemia

20

John A. Thorson and Huan-You Wang

Clinical Background

A 45-year-old female presented to her physician with a complaint of pain in the right posterior auricular region. Approximately one year prior to the present visit, she was diagnosed with breast cancer which was treated by lumpectomy, followed by a course of cyclophosphamide and antiestrogen (Tamoxifen) with subsequent radiation therapy. On physical examination, she was found to have significant right cervical lymphadenopathy. Initial laboratory studies included a complete blood cell count showing hemoglobin 9.8 g/dL (reference interval 12–16 g/dL), hematocrit 28.9% (reference interval 36–46%), white blood cell count 59,700/μL (reference interval 4,000–11,000/μL), and platelet count 128,000/μL (reference interval 130,000–400,000/μL). A differential count of the peripheral white blood cells showed 67% blasts, 13% neutrophils, 18% lymphocytes, and 2% monocytes. No eosinophils or eosinophil precursors were found in the peripheral blood or bone marrow, and no mast cells were seen in the bone marrow.

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Flow cytometric analyses of a bone marrow aspirate was performed and revealed 81% blasts positive for CD4, CD11c (partial), CD13, CD19 (partial), cytoplasmic CD22, CD34, CD36, CD45, cytoplasmic CD79a, CD117 (partial), HLA-DR, and TdT, but negative for CD1a, CD2, cytoplasmic CD3, CD5, CD7, CD10, CD14, CD15, CD20, CD33, CD42b, CD64, Glycophorin A, and myeloperoxidase. Cytogenetic analysis revealed the following karyotype: 46, XX, t(4;22)(q12;q11.2)/46XX. Finally, fluorescence in situ hybridization (FISH) assays for *MLL* and *BCR-ABL* translocations were performed, with no evidence of an *MLL* gene rearrangement or a *BCR-ABL* translocation observed. Interestingly, a third *BCR* signal was observed in 85% (170/200) of the nuclei, in agreement with the karyotype finding of a translocation involving 22q11.2.

Taken together, these findings were consistent with a mixed phenotype acute leukemia (MPAL), in which both myeloid (CD4, CD13, CD36, CD117) and B lymphocytic (CD19, cytoplasmic CD22) antigens are present on the same population of blast cells [1]. Therefore, in keeping with strict current WHO terminology, a diagnosis of MPAL, B/myeloid, not otherwise specified (NOS) was rendered.

Reason for Molecular Testing

After completion of the initial cytogenetic tests, there was no evidence for a Philadelphia (Ph) chromosome, that is, a t(9;22)(q34;q11) involving a *BCR-ABL* translocation, or a translocation involving the mixed lineage leukemia (*MLL*) gene at chromosome 11q23. This allowed the disease to be accurately classified as

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MPAL, B/myeloid, NOS, excluding the related entities in which a *BCR-ABL* or *MLL* translocation is present [3]. However, the presence of a translocation involving chromosomes 4 and 22, specifically involving the *BCR* gene (as evidenced by the FISH assay result), was considered by the clinicians to be of potential clinical significance. Of particular interest in this case was the possible involvement of the *PDGFRA* gene, which is located on chromosome 4q12 and encodes the platelet-derived growth factor receptor, alpha polypeptide. Although rare, *BCR-PDGFRA* translocations have previously been described in leukemias [4, 5].

Question 1: What might the clinical significance be of a translocation involving the PDGFRA gene?

As members of the class III receptor tyrosine kinase family, PDGFRA and related proteins including the ABL and KIT tyrosine kinases have been associated with several hematologic and non-hematologic malignancies [6]. In malignancies in which PDGFRA is implicated as a causative factor, the protein is believed to gain enhanced or constitutive activity either through gene amplification resulting in protein overexpression [6], the development of activating mutations in an otherwise intact molecule [7], or by fusion with another cellular protein leading to inappropriate activation via dimerization. An example of the latter situation is seen with the *FIP1L1-PDGFRA* fusion in hypereosinophilic syndrome [8].

Significantly, the small molecule kinase inhibitor imatinib mesylate, first developed as a specific treatment for chronic myelogenous leukemia, has been shown to be an effective inhibitor of PDGFRA activity [9]. *PDGFRA* mutation-positive gastrointestinal stromal tumors have been successfully treated with imatinib [7], generating a high level of interest in the potential use of this drug to treat other malignancies involving an activated receptor tyrosine kinase [10]. With these facts in mind, it was felt that the laboratory demonstration of a *PDGFRA* fusion in this case would provide a rationale for a therapeutic trial of imatinib.

In view of the potential clinical significance of a translocation and/or fusion involving *PDGFRA*, a FISH assay for a *FIP1L1-PDGFRA* fusion (or *CHIC2* deletion) [11] was performed. This test utilizes a set of three hybridization probes specific for a series of genes (*FIP1L1*, *CHIC2*, and *PDGFRA*) which are arranged sequentially, in the centromere to telomere direction, on chromosome 4q12. The *CHIC2*-specific probe is labeled with a red fluorophore and the *FIP1L1* and *PDGFRA* probes are labeled with a green fluorophore. Under normal conditions, the probes are nearly contiguous, and therefore produce a yellow (combination of red and green) fusion signal. If a translocation involving one of the labeled genes is present such that one gene is physically removed from the other two, a physical separation of the probes occurs resulting in signal separation. The results in this case demonstrated a signal separation pattern in 96.5% (193/200) of nuclei, indicating a rearrangement within this region. This finding, together with the t(4;22)(q12;q11.2) revealed by karyotype analysis and refined by the *BCR-ABL* FISH result, was highly suggestive of the presence of a *BCR-PDGFRA* fusion gene being present in this patient; however, definitive proof was still lacking. Therefore, a request to attempt a more specific identification of the translocation partner gene was made.

Test Ordered

In this case, no specific test was ordered. Rather, a request for confirmation at the molecular level of the presence of a *BCR-PDGFRA* fusion gene was made. Although no validated test for this purpose was available in either our laboratory or any outside reference laboratory, it was agreed that the simplest approach to address this question and to confirm the findings on an investigational basis would be an RT-PCR assay, in which the transcript of the suspected fusion gene is targeted by specific oligonucleotide primers, with the goal of amplifying a unique junctional sequence. The use of this type of analysis has previously been reported in the literature to investigate a similar translocation observed in a case of chronic myelogenous leukemia [4].

Laboratory Test Performed

RT-PCR for *BCR-PDGFRA*

In order to confirm the presence of a *BCR-PDGFRA* fusion gene through the use of an RT-PCR assay, a variety of variables need to be considered, relating to the design as well as the performance and potential

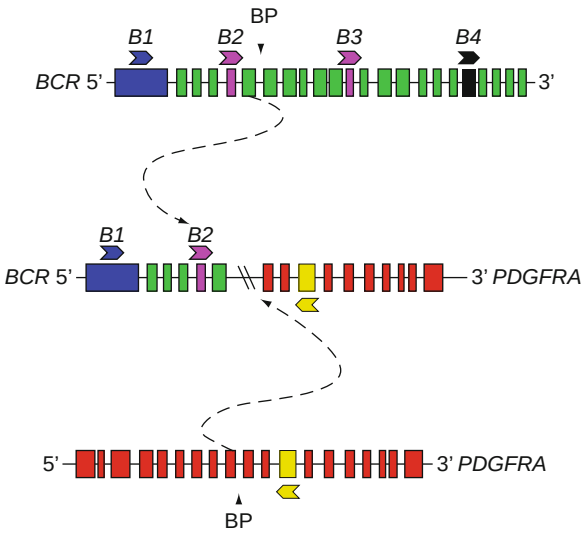

Fig. 20.1 Diagrammatic representation of an RT-PCR strategy for detecting *BCR-PDGFRA* fusion transcripts. Top of the figure: sense primers are located within the *BCR* gene at exon 1 (*B1*), exon 5 (*B2*), exon 13 (*B3*), and exon 19 (*B4*). A single antisense primer is located in exon 12 of the *PDGFRA* gene (*bottom* of the figure). A hypothetical chimeric *BCR-PDGFRA* fusion gene resulting from breakpoints (*BP*) indicated by the vertical arrowheads is depicted in the center of the figure, demonstrating one possible productive PCR between the sense primer *B2* and the antisense *PDGFRA* primer. Other breakpoint locations could produce a fusion gene structure allowing a productive PCR between a different *BCR* sense primer and the antisense *PDGFRA* primer

interpretation of the assay. A significant first concern is the positioning of the PCR primers within the gene sequences. Because the assay is RNA-based, the primers must target exonic regions of each gene. However, depending upon the location of the breakpoints in the gene sequences, variable numbers of exons from each gene can be present on each derivative chromosome, creating uncertainty about the fusion point and the appropriate exons to target in the assay. For instance, if the breakpoint in the *BCR* gene occurs in intron 13 and the break in the *PDGFRA* gene occurs within intron 10, the expected chimeric transcript would consist of *BCR* exons 1–13 fused to *PDGFRA* exons 11–24. In this situation, a primer located in *BCR* exon 15 would not be productive, since exon 15 would not be present in the chimeric transcript. In view of this, the approach described by Baxter et al. [4] was used, in which a series of sense primers are designed to target exons spaced throughout the *BCR* gene (Fig. 20.1). The antisense *PDGFRA* primer site, located in exon 12 just 5′ to the sequence encoding the kinase domain [12], was selected to ensure that a positive result would demonstrate the presence of a hybrid gene potentially capable of producing a functional protein kinase and, therefore, a potential therapeutic target.

Another issue related to primer positioning is the size of the expected PCR product. Again, without prior knowledge of breakpoint locations, it is not possible to predict the product size for purposes of accurate interpretation. In addition, it is possible that the distance between a productive sense and antisense primer pair would be too large to be amplified under the assay conditions. This could result from a transcript comprising a large number of exons or, in rare instances, the inclusion of all or part of an intronic sequence in the processed messenger RNA due to the elimination of a splice site by the gene fusion event.

Finally, compounding the above issues is the lack of any type of positive control material, such as a sample or cell line harboring the transcript, or a clonal construct which represents the fusion gene and is known to be detectable by the assay. Without this type of control, there is no operational means of assessing the integrity of the assay or its ability to produce a result in response to a positive sample.

A residual bone marrow aspirate sample was retrieved and RNA was extracted from the cells using a standard technique. Complementary DNA (cDNA) was prepared by reverse transcription of the RNA using random hexamers to prime the reaction. Aliquots of the cDNA were then amplified by PCR using each of the four *BCR* sense primers paired with the antisense *PDGFRA* primer. As a control for RNA integrity, a separate PCR was performed using primers annealing to the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene. Finally, a set of negative control reactions, using an RNA specimen from a person without leukemia, were performed under the same conditions as used with the patient sample. Because the expression level of any chimeric transcript formed between *BCR* and *PDGFRA* was unknown, PCR was performed using a 40 cycle protocol (in place of a typical 30–35 cycle protocol) to enhance the ability to detect transcripts expressed at a low level. After completion of the PCR, the products were analyzed by electrophoretic separation on a 1% agarose gel followed by ethidium bromide staining for visualization.

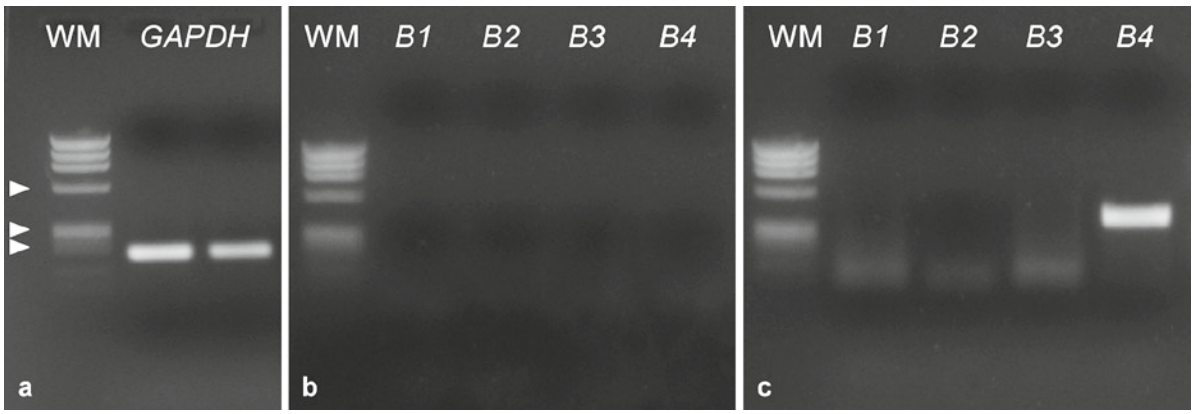

Fig. 20.2 Results from electrophoretic analysis of RT-PCR products (See text for details)

Results with Interpretation Guideline

The results of the electrophoretic analysis of the RT-PCR products can be interpreted with Fig. 20.2. Displayed in panel A are results from the reactions utilizing *GAPDH* specific primers: lane 1, weight markers (WM); lane 2, PCR from control RNA; lane 3, PCR from test patient RNA. Panels B (control RNA) and C (test patient RNA) display results from reactions utilizing combinations of the indicated sense *BCR* primer with the antisense *PDGFRA* primer. In each panel, lane contents are denoted as follows: WM, weight marker; *B1*, *BCR* exon 1 primer; *B2*, *BCR* exon 5 primer; *B3*, *BCR* exon 13 primer; *B4*, *BCR* exon 19 primer. Arrows at the left side of panel A indicate molecular weight markers of 603 bp (top), 310 bp (middle), and 271 bp (bottom).

Question 2: What is the most appropriate interpretation of these assay results?

Result Interpretation

As can be seen in panel A of Fig. 20.2, an amplification product is clearly present from both reactions (control and patient RNA) using the *GAPDH*-specific primers, which produce an expected product of 176 bp. This confirmed that RNA was present and intact (up to a size of 176 bp). As expected, none of the *BCR-PDGFRA* reactions utilizing a control RNA as target produced a product (panel b). In contrast, one of the four reactions performed on the test patient RNA using a sense *BCR* primer (primer *B4*) in combination with the antisense *PDGFRA* primer produced an amplification product of approximately 400 bp in size, indicating the presence, in the patient's sample, of a fusion transcript derived from a chimeric *BCR-PDGFRA* gene (panel c).

Question 3: Do these results confirm the presence of a BCR-PDGFRA fusion gene?

Taken together with the karyotype and FISH assay results, this finding is highly suggestive of the presence of a *BCR-PDGFRA* fusion. However, it does not provide definitive proof of this. As discussed above, because the exact nature in terms of the involved exons of a *BCR-PDGFRA* fusion, if present, is still unknown, accurate interpretation of this assay (based on the size of the expected product) is not possible. The presence of an amplification product, although presumably specific to the sequences of interest, could also be due to nonspecific priming or other artifacts of the PCR process. The absence of amplification products from the negative control sample lessen the likelihood of this, but do not eliminate it completely as each sample is unique.

Question 4: What additional steps might be taken to definitively confirm the identity of the fusion gene?

Further Testing

Although the evidence is strong for the presence of a *BCR-PDGFRA* fusion in this case, the most appropriate next step to definitively confirm this possibility would be to obtain the DNA sequence of the amplified product from the RT-PCR assay. Assuming that significant portions of sequence from both the *BCR* and *PDGFRA* gene could be identified in the fusion product and that sequence

information covering the fusion or junction site was obtained, this would provide acceptable evidence for the presence of a fusion gene. In this case, sequence analysis of the amplified fusion transcript fragment demonstrated a junctional region, in which *BCR* exon 20 is fused in-frame with a portion of *PDGFRA* exon 12, thus confirming the presence of a *BCR-PDGFRA* fusion.

Other Considerations

This case represents an example of an uncommon acute leukemia, which would otherwise carry a poor prognosis [2] if the presumed *BCR-PDGFRA* fusion gene were not present. As the options for treatment of acute leukemia of ambiguous lineage are few and often show limited efficacy, the extended panel of genetic assays was performed with the goal of potentially providing a rationale for a novel treatment strategy, in this case the use of a small molecule kinase inhibitor such as imatinib. It is also important to note that even with definitive evidence of a *BCR-PDGFRA* fusion gene (or fusion transcript) being present, the efficacy of treatment with a tyrosine kinase inhibitor in this situation is uncertain, as only three leukemia cases involving this translocation have been reported previously [4, 5]. Of those three cases, only one received treatment with imatinib [5]. Because our present knowledge of the effectiveness of such treatment is very limited in malignancies other than chronic myeloid leukemia or gastrointestinal stromal tumors, ethical considerations for such trials must weigh the potential for benefit against the likelihood of an outcome more adverse than would be expected with the standard of care.

Background and Molecular Pathology

Classification of acute leukemias relies upon multimodality approaches, which include integration of information from a variety of sources such as clinical presentation, morphologic and immunophenotypic evaluation of blood and bone marrow specimens, and cytogenetic and molecular studies. Based upon the results of these studies, the malignant cells are assigned to a particular lineage (e.g., myeloid versus lymphoid) and differentiation stage. More importantly, selection of treatment options, clinical outcomes, and prognosis are heavily influenced, in some cases determined by the underlying genetic and molecular events in a given patient. A classical example is acute promyelocytic leukemia, in which genetic/molecular proof of the promyelocytic leukemia-retinoid acid receptor α (*PML-RARA*) fusion dictates that optimal therapy should include all-*trans* retinoic acid (ATRA) [13].

Despite advances in the classification of acute leukemia, approximately 4–5% of acute leukemia cases cannot be assigned to a particular lineage, and these are referred to as acute leukemia of ambiguous lineage [14], which includes acute undifferentiated leukemia and, the largest subset of these cases, MPAL. MPAL is defined as a leukemia with blasts that express antigens of more than one lineage (formerly known as biphenotypic acute leukemia) or with separate populations of blasts that are of different lineage (formerly known as bilineal acute leukemia) [14]. MPAL, according to the current WHO classification scheme [1], encompasses MPAL with t(9;22)(q34;q11.2) involving *BCR-ABL1*, MPAL with t(v;11q23) involving an *MLL* rearrangement, MPAL with B/myeloid (NOS), MPAL with T/myeloid (NOS), and MPAL (NOS). Once MPAL with *BCR-ABL1* and *MLL* are excluded, there are no consistent cytogenetic aberrations observed in the remainder of MPAL cases [1]. The prognosis of MPAL is generally considered to be poor [1].

Multiple Choice Questions

1. In the FISH analysis for a *BCR-ABL* translocation, a third *BCR* signal was observed in 170/200 nuclei. What is the most likely explanation for this finding?
   A. A break within the *BCR* gene sequence, separating at least one of the two alleles into two pieces
   B. A cryptic *BCR-ABL* translocation, involving only a small portion of the *ABL* gene
   C. A translocation involving the *BCR* gene
   D. An artifact in which of two or more nuclei overlay each other on the slide
   E. Both A and C
2. In malignancies involving enhanced or constitutive PDGFRA protein activity, what types of mechanisms are thought to account for this inappropriate activation?
   A. Gene amplification leading to protein overexpression
   B. Genomic rearrangements, leading to the fusion of all or part of the PDGFRA protein with another cellular protein

C. A and B
D. The development of activating mutations within the gene sequence, caused by insertions, deletions, or base changes
E. All of the above

3. The RT-PCR assay described in this case was designed to detect unique junctional sequences formed by the fusion of exons from the *BCR* and *PDGFRA* genes. How might the elimination of a splice site by the translocation event impact the ability of this assay to detect a fusion sequence?
   A. Elimination of splice sites has no effect on the structure of fusion transcripts, thus it would have no effect on the described assay
   B. It could prevent appropriate splicing at an intron-exon boundary, leading to the inclusion of all or part of an intron, thus producing an unexpectedly large sequence which might not be amplified under typical PCR conditions
   C. It would alter the sequence of a primer recognition site, preventing priming of the PCR
   D. It would prevent the formation of the fusion transcript, thus leaving no target for the PCR assay
   E. None of the above
4. In the interpretation of the RT-PCR assay results, what is the purpose of the reaction using primers designed to amplify a section of the *GAPDH* transcript?
   A. It eliminates the need for a negative control
   B. It provides evidence for the presence of RNA molecules which are intact, up to the size of the *GAPDH* amplification product
   C. It provides proof that the conditions used for the PCR will not produce artifactual products
   D. Both A and B
   E. All of the above
5. Considering this patient's presentation as well as the immunophenotyping and molecular studies, which of the following entities, as characterized according to the current WHO classification scheme, should be included in the differential diagnosis of this leukemia?
   A. Acute myeloid leukemia with abnormalities of *PDGFRA*
   B. Chronic eosinophilic leukemia
   C. Chronic eosinophilic leukemia, NOS
   D. Chronic myelogenous leukemia, blast crisis
   E. All of the above

Answers to Multiple Choice Questions

1. *The correct answer is E.*

In the assay used to evaluate this case, the *ABL* gene is identified or localized by the presence of a red fluorescence signal while the *BCR* gene is indicated by a green fluorescence signal (Vysis LSI BCR/ABL Dual Color, Dual Fusion Translocation Probe, Abbott Laboratories, Abbott Park, IL). In a normal cell, these genes are located on separate chromosomes, producing a spatial separation of the gene-specific probes and attached fluorophores and allowing each to be visualized individually under fluorescence microscopy. Because a normal cell contains two copies of each chromosome, the expected normal result is two red (*ABL*) signals and two green (*BCR*) signals with no fusion signal. A third *BCR*-specific signal could result from several potential causes. A technical artifact, in which two or more nuclei overlap on the slide and appear in the same visual field under microscopy is one possible cause, but is extremely unlikely in this case since 170 of 200 nuclei displayed this result. Second, an additional copy of the *BCR* gene such as might occur in a state of aneuploidy could produce this result. This is again unlikely in this case because aneuploidy was not observed during the karyotypic analysis. Finally, a translocation producing an internal break in one copy of the *BCR* gene and resulting in two spatially separated but hybridizable sections of the gene could produce this pattern. Of significance, the absence of a yellow fusion (combination of red and green) signal clearly demonstrates that a translocation involving the *ABL* gene is not present. This observation, together with the karyotype results showing a t(4;22), suggest that there may be a fusion of the *BCR* gene with an unidentified gene located on chromosome 4q12.

2. *The correct answer is E.*

Each of the mechanisms listed has been shown to account for increased PDGFRA activity within malignant cells. A comprehensive review of these mechanisms is provided by Blume-Jensen and Hunter [6].

3. *The correct answer is B.*

Splice sites are located at the 5′ and 3′ ends of introns and serve as recognition sites for the cellular splicing machinery responsible for removing introns

and joining exons during RNA processing. Elimination of a splice site could lead to the inclusion of intronic sequences within the messenger RNA. Insertion of a large sequence between the exons recognized by the sense and antisense primers of the PCR assay could produce a target which is too large (usually when greater than approximately 1 kb) to amplify under typical PCR conditions. Therefore, no amplification product would be produced and the assay would appear to be negative, even though a fusion transcript was present.

4. *The correct answer is B.*

The main purpose of the *GAPDH* assay is to demonstrate that RNA template is present and intact up to the size of the product produced. Because RNA is an extremely labile molecule, it can frequently be degraded during or after the extraction process if significant care with proper technique is not exercised. Therefore, it is good laboratory practice to assay the sample for the presence of transcripts from a constitutive or "housekeeping" gene to demonstrate that intact molecules are present. Best practice is to design the control assay so that the product size is equal to or greater than that expected from the assay for the analyte of interest.

5. *The correct answer is E.*

All of the entities listed should be considered in the differential diagnosis of this case. However, chronic myelogenous leukemia, blast crisis, can be easily excluded based upon the absence of *BCR-ABL* as revealed by both conventional karyotype and FISH analysis. Similarly, chronic eosinophilic leukemia, NOS, is excluded because this entity, by definition, should be negative for a rearrangement of *PDGFRA*. In addition, there is no eosinophilia observed either in the peripheral blood or the bone marrow. While chronic eosinophilic leukemia and acute myeloid leukemia with an abnormality of *PDGFRA*, which are classified as parts of myeloid and lymphoid neoplasms with abnormalities of *PDGFRA* as advocated by the current (fourth) WHO classification scheme [15], could potentially be the diagnosis for this case, MPAL is a better choice because the blast population expressed multiple myeloid- and B-lineage antigens, and there was no eosinophilia. Future and more definitive classification of these types of cases will require more in depth investigations.

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Part III
Solid Tumors

Breast Cancer

21

Jennifer Laudadio

Clinical Background

A 58-year-old white female presented to the Breast Care Center following an abnormal screening mammogram. Mammography revealed a suspicious 10 mm focus with calcifications of varying size and density in the upper-outer quadrant of the right breast. The patient complained of bilateral intermittent breast pain, but she had not noted any masses or nipple discharge. She had a benign left breast biopsy five years earlier but had no personal history of cancer. Her mother was diagnosed with breast cancer at the age of 65. Also, a maternal aunt and two maternal great-aunts were diagnosed with breast cancer while in their sixties. On physical examination, no discrete masses were palpated and no palpable lymph nodes were identified. The exam was also negative for skin changes and nipple discharge. The patient was referred for ultrasound guided biopsy.

Histologic examination of the right breast biopsy tissue revealed invasive ductal carcinoma, grade 2 of 3. Solid and comedo types of ductal carcinoma in situ (DCIS) were also present. Microcalcifications were identified in the malignant breast tissue. By immunohistochemistry, the invasive tumor cells were 99% positive for estrogen receptor (ER) expression and 60% positive for progesterone receptor (PR) expression. HER2 immunohistochemistry was scored as 2+ and appropriately reflexed for fluorescence in situ hybridization (FISH) testing. FISH results were equivocal for amplification with a 1.92 ratio of *HER2* signals to centromere 17 control signals (the equivocal range equals 1.8–2.2). The patient was referred to surgical oncology and elected to have bilateral skin sparing mastectomies with right sentinel lymph node mapping.

Pathologic examination of the right breast tissue revealed a 3.5 cm biopsy cavity, but no tumor or lesions were grossly identifiable. Histologically, a 0.6 cm focus of residual intermediate grade invasive ductal carcinoma was found adjacent to the biopsy cavity. DCIS was identified extending beyond the periphery of the invasive lesion. Margins were negative for carcinoma and no lymphovascular space invasion was seen. Examination of the left breast tissue revealed a 0.5 cm focus of intermediate grade DCIS. Five lymph nodes were sampled and were negative for metastatic carcinoma by histologic evaluation. The final TNM pathologic (p) stage was based on tumor size (T), lymph node metastases (N), and distant metastases (M). Since the tumor size was greater than 5 mm but less than 11 mm, it was staged pT1b. The absence of lymph node metastases was staged as pN0. The presence or absence of distant metastasis was not confirmed pathologically so the M stage was not applicable (as per the new Seventh Edition AJCC cancer staging guidelines; previously reported as pMx). HER2 immunohistochemistry was repeated on the tissue from the mastectomy specimen and was again scored 2+. FISH again yielded equivocal results with a 1.9 ratio of *HER2* to control signals.

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The patient did well post-operatively and was seen for follow up in the oncology clinic. Since her tumor was hormone receptor (ER and PR) positive, the oncologist recommended hormonal therapy with an aromatase inhibitor. Meanwhile, due to her family history and bilateral disease, a blood sample was sent to a referral laboratory for *BRCA1* and *BRCA2* comprehensive mutation analysis.

Reason for Molecular Testing

Based on clinicopathologic criteria such as low tumor stage, intermediate tumor grade, the patient's age, positive hormone receptor status, and absence of lymph node involvement, the patient is at a low risk for locoregional or distant breast cancer recurrence. However, gene expression analysis was requested to further clarify the patient's prognosis and the possible need for adjuvant chemotherapy.

Question 1: Which molecular tests of gene expression are commercially available and widely used to help delineate breast cancer recurrence risk?

Test Ordered

Onco*type* DX (Genomic Health Inc., Redwood City, CA) was requested to determine the tumor's gene expression profile.

Question 2: Of the available tests, why was this test selected?

Laboratory Test Performed

The hematoxylin and eosin stained slides from the patient's mastectomy were reviewed in order to select a suitable tissue block for Onco*type* DX testing. A section containing approximately 60% invasive carcinoma cells was selected. This section did not include any necrosis or reactive changes related to the prior biopsy. The formalin fixed paraffin embedded tissue block corresponding to the selected section was packaged and shipped to the CLIA-certified laboratory performing Onco*type* DX.

Upon receipt of the tissue block, testing was performed as published [1]. Briefly, tissue dissection using a sterile blade was performed if non-tumor elements comprised more than 50% of the tissue. This step limited contamination with non-tumor cells and helped prevent false positives or negatives. RNA was extracted from six 10 μm thick sections if microdissection was performed or from three 10 μm thick sections if microdissection was not necessary [1]. Quantitative reverse transcription PCR (RQ-PCR) of 21 genes was then performed using hydrolysis probe chemistry. Sixteen of the genes were informative and five served as reference genes (*ACTB*, *GAPDH*, *RPLPO*, *GUS*, and *TFRC*). The informative genes include proliferation genes (*Ki67*, *STK15*, *Survivin*, *CCNB1*, and *MYBL2*), genes involved in tissue invasion (*MMP11*, *CTSL2*), HER2 related genes (*HER2* and *GRB7*), estrogen related genes (*ER*, *PGR*, *BCL2*, and *SCUBE2*) as well as *GSTM1*, *CD68*, and *BAG1* [1]. The quantitative results of the informative genes were normalized to the reference genes and numerically expressed from 0 to 15 such that a 1 unit increase approximately equals a doubling of RNA level. Individual gene expression levels were then used to determine the Recurrence Score (RS) [1]. The estrogen related genes and *BAG1* are considered favorable and are inversely related to RS. For the remaining informative genes, RS increases as their expression levels increase.

An alternative commercially available test for determining the gene expression profile of breast cancer is MammaPrint (Agendia, Amsterdam, The Netherlands). Unlike Onco*type* DX which is performed on paraffin embedded tissue, MammaPrint requires fresh or snap-frozen tissue. In this patient's case, only paraffin embedded tissue was available for testing. As published, the procedure for the MammaPrint test involves RNA extraction from 30 μm thick sections followed by generation of complementary RNA (cRNA) using T7 RNA polymerase [2]. Five micrograms of total RNA are then labeled using fluorescent dye (Cy3 or Cy5) and mixed with reverse-color fluorescent labeled control RNA [2]. Samples are heated to 60°C in the presence of zinc chloride to generate fragments of 50–100 nucleotides in length. The RNA fragments are added to buffer and hybridized to the custom-designed oligonucleotide array [2]. Subsequently, the array is scanned and fluorescence intensities are measured, quantified, and normalized to the control expression levels. Like Onco*type* DX, MammaPrint testing includes control steps to limit the percentage of non-tumor cells in the tested sample.

The MammaPrint assay uses oligonucleotide microarray technology which allows a single analysis to target a large number of genes and has been cleared by the FDA as a prognostic test. Onco*type* DX is a RQ-PCR assay which uses hydrolysis probe chemistry. This strategy takes advantage of the 5′ exonuclease activity of DNA polymerase. During the annealing step, a probe labeled with a reporter fluorophore at the 5′ end and with a quencher at the 3′ end anneals to the target sequence. When the probe is intact, the quencher is in close enough proximity to the fluorophore to effectively block fluorescence. During extension, however, fluorescence is emitted when the DNA polymerase cleaves the 5′ end of the probe and releases the fluorophore from the quencher. Both Onco*type* DX and MammaPrint employ methodologies currently used for routine clinical testing, but both tests are only offered at a single centralized reference laboratory each, and utilize proprietary data analysis.

Results with Interpretation Guideline

The Onco*type* DX results were received and reported a breast cancer Recurrence Score equal to 13. The *ER* score was 10.6, *PR* score 6.1, and *HER2* score 10.6.

For interpretation, the recurrence score is used to determine breast cancer recurrence risk as follows: the predicted risk is low if the RS is less than 18, intermediate if the RS is less than 31 but greater than or equal to 18, and high if the RS is 31 or greater. The *ER* and *PR* score positive–negative cutoffs are 6.5 and 5.5, respectively, with expression levels greater than these values being positive. For *HER2*, a numeric expression value greater than or equal to 11.5 is positive, from 10.7 to 11.4 is equivocal, and less than 10.7 is negative.

Question 3: How do the Oncotype DX® results correlate with clinicopathologic risk?
Question 4: How do the Oncotype DX® results correlate with the ER, PR, and HER2 immunohistochemistry results?

Result Interpretation

The results indicate that the patient is at low risk for recurrence of her breast cancer. Specifically, the report states a RS of 13 is associated with 9% (95% confidence interval 6–11%) average rate of distant recurrence at 10 years, after 5 years of tamoxifen therapy. A low RS score, as seen in this patient, is associated with a high *ER* group score, low proliferative group scores, and low invasion group scores. In this case, the low risk RS correlates with the previously described low risk clinicopathologic features. The patient's RS also predicts that administering chemotherapy in addition to hormonal therapy will not be of additive benefit.

According to the Onco*type* DX results, the tumor is *ER* and *PR* positive which correlates with the results of immunohistochemistry testing. For this specific patient, *ER* RNA expression is 4.1 expression units above the positive–negative cutoff, and *PR* RNA expression is 0.6 expression units above the positive–negative cutoff. The reported percent of cells ER and PR positive by immunohistochemistry is 99% and 60%, respectively. *HER2* is negative according to Onco*type* DX results whereas findings with immunohistochemistry and FISH are equivocal. However, the Onco*type* DX result is only 0.1 expression units below the equivocal range.

Further Testing

Due to the patient's bilateral disease and family history, the patient was referred to a genetic counselor who recommended testing for hereditary breast ovarian cancer syndrome. A blood sample was sent to a referral laboratory for comprehensive *BRCA1* and *BRCA2* mutation analysis. Without a known familial mutation to target, full gene sequencing was performed for both genes. In addition, *BRCA1* analysis includes testing for five common rearrangements because large deletions or insertions may be missed by sequencing. For example, if a deletion encompassed the entire sequence targeted by one primer pair, only the wild-type sequence would be amplified and sequenced. In this patient's case, no mutation was discovered in either *BRCA1* or *BRCA2*.

Based on the patient's age, clinical, and family history, the result was not unexpected. Characteristics associated with hereditary breast ovarian cancer syndrome include diagnosis of breast cancer prior to the age of 50, diagnosis of breast cancer in family members before the age of 50, a personal or family history of ovarian cancer, bilateral disease, and Ashkenazi Jewish descent. In this case, the patient was diagnosed

Table 21.1 Comparison of two gene expression profile tests used to determine breast cancer recurrence risk

	Onco*type* DX	MammaPrint
Methodology	RQ-PCR	Microarray
Number of genes tested	21	70
Sample type accepted	Formalin fixed paraffin embedded tissue	Fresh or snap-frozen tissue
Testing location	Centralized reference lab	Centralized reference lab
Clinical characteristics recommended for testing	Any age, lymph node negative, ER positive OR postmenopausal, lymph node positive, ER and/or PR positive	Patients with stage 1 or 2 carcinoma less than 5.0 cm in size, lymph node negative, ER positive or negative
Results generated	RS reported from 0 to 100 with risk increasing continuously as RS increases. Patients also grouped as low, intermediate, or high risk	Binary results classify patients as low or high risk
Other	*ER, PR, HER2* scores reported	FDA cleared

at 58 years old. She did have four relatives with breast cancer, but none of these relatives were diagnosed before age 60. Our patient was not of Ashkenazi Jewish descent and there was no history of ovarian cancer or male breast cancer in her family. However, given that she had bilateral disease (invasive carcinoma in the right breast and DCIS in the left breast), testing for a *BRCA1* or *BRCA2* mutation was reasonable.

Other Considerations

In order to correctly interpret the Onco*type* DX results, the presence and importance of pre-analytic variables needs to be considered. The time and type of fixation, age of the paraffin block, storage and shipping conditions are all variables that are difficult to control and could, theoretically, affect results. Additionally, consideration should be given to how well the patient matches the study populations used during assay validation. For example, this patient was being treated with an aromatase inhibitor, but a majority of the initial patients in the Onco*type* DX studies were treated with tamoxifen. Recently, however, the results have been reported to be applicable to patients treated with aromatase inhibitors, as well [3].

Background and Molecular Pathology

Clinicopathologic risk factors for breast cancer prognosis include tumor stage (based on size) and grade, histologic tumor type, lymph node status, hormone receptor status, and patient age. While other tests are available, two commercially offered gene expression assays are widely used to further help delineate a patient's risk of recurrence (Table 21.1). These genetic signatures may also aid in predicting response to adjuvant chemotherapy. The Onco*type* DX assay is an RQ-PCR test performed on formalin fixed paraffin embedded tissue. The test targets 21 genes, 16 informative and five reference genes, selected by investigating 250 candidate genes implicated in carcinogenesis. Although obtaining quality RNA from formalin fixed paraffin embedded tissue can be challenging, the procedure yielded adequate RNA and subsequent expression profiles in 95–99% of samples [1, 3–5]. Onco*type* DX results are expressed as a recurrence score ranging from 0 to 100 and categorize patients as low risk, intermediate risk, or high risk for tumor recurrence so that low risk is associated with a low recurrence score. Molecular determination of prognosis correlates with certain clinicopathologic features. Poorly differentiated (high grade or grade 3) tumors are associated with high risk scores, and small tumors tend to generate low risk scores [4, 6]. However, more than a third of small tumors (less than 2.0 cm) are classified as intermediate or high risk [4, 6]. Results are reproducible with a recurrence score standard deviation of 0.72 within block and 2.2 between blocks from the same patient [1].

Initial studies of Onco*type* DX included patients with ER positive, lymph-node negative breast cancer treated with tamoxifen. The percentage of patients classified as low risk, intermediate risk, and high risk was 51%, 22%, and 27%, respectively [1]. Similar distributions were seen in subsequent studies [4, 6]. In this population, RS was prognostic with improved survival in low molecular risk patients as compared to

Table 21.2 Survival estimates associated with Onco*type* DX recurrence score

Reference	Clinical features	10 year disease free survival (%)			10 year breast cancer specific survival (%)			10 year overall survival (%)		
		Low RS	Inter. RS	High RS	Low RS	Inter. RS	High RS	Low RS	Inter. RS	High RS
Paik et al. [1]	ER+ LN− Tamoxifen	93.2	85.7	69.5	–	–	–	–	–	–
Albain et al. [5]	Postmenopausal ER+ LN− Tamoxifen	60	49	43	–	–	–	77	68	51
Habel et al. [6]	ER+ LN− Tamoxifen	–	–	–	97.2	89.3	84.5	–	–	–
Dowsett et al. [3][a]	Postmenopausal ER+ and/or PR+ LN− Tamoxifen or AI	96	88	75	–	–	–	88	84	73
Dowsett et al. [3][a]	Postmenopausal ER+ and/or PR+ LN+ Tamoxifen or AI	83	72	51	–	–	–	74	69	54

Inter Intermediate, *LN* lymph node metastases, *AI* aromatase inhibitor
[a]9 year survival estimates

both intermediate and high risk patients (Table 21.2). Recurrence score also functioned as a continuous variable such that risk for recurrence increases as RS increases [1]. Recently, the prognostic value of Onco*type* DX RS was confirmed in expanded patient populations including postmenopausal women with lymph node metastases as well as patients treated with aromatase inhibitors (Table 21.2). RS was a successful prognosticator of recurrence, regardless of the type of hormonal therapy administered [3].

In addition to utility as a prognostic marker, Onco*type* DX predicts response to chemotherapy. Identification of patients who are unlikely to benefit from chemotherapy can spare these women the toxic effects, potential complications, and expense. Addition of chemotherapy to hormonal therapy increased recurrence free survival in molecular high risk patients but did not have a similar benefit in low risk patients [4]. The magnitude of chemotherapy benefit increased continuously as the RS increased [4]. Predictive ability was demonstrated in patients with node negative ER-positive breast cancer but also in postmenopausal women with lymph node metastases [4, 5]. This chemotherapy benefit was seen regardless of the number of positive lymph nodes.

Onco*type* DX also provides separate *ER*, *PR*, and *HER2* scores. For *HER2* expression, RQ-PCR performed well in comparison to FISH with an overall *HER2* positive and negative concordance of 97% [7]. RQ-PCR correlated well with immunohistochemistry for determining ER and PR positivity. Concordance rates ranged from 88% to 100% [8, 9]. Numerous factors may account for the discordant results and should be considered when interpreting RQ-PCR results. RQ-PCR measures RNA levels, but immunohistochemistry detects protein expression and sensitivity can vary between antibodies. Immunohistochemistry incorporates morphology so that only carcinoma cells are scored but interpretation involves subjectivity. An advantage of RQ-PCR is the elimination of subjectivity, but the results may be impacted by non-tumor cells present in the sample.

An alternative to RQ-PCR for determining breast cancer gene expression is a microarray test marketed as MammaPrint. The test determines expression of 70 genes selected from initial studies using oligonucleotide microarrays covering approximately 25,000 genes [2]. The 70 included genes involve cell cycle signaling, angiogenesis, invasion, and metastasis. Interestingly, since the genes involved in the design of

Table 21.3 Survival estimates and MammaPrint risk classification

Reference	Clinical features	5 year disease free survival (%)		5 year breast cancer specific survival (%)		5 year overall survival (%)	
		Low risk	High risk	Low risk	High risk	Low risk	High risk
van der Vijver [13][a]	≤ 52 years old LN− or LN+ ER− or ER+	85	51	–	–	95	55
Knauer et al. [14]	LN− or LN+ ER− or ER+ ET or ET+ chemo	95	82	97	87	–	–
Mook et al. [10]	LN− or LN+ ER− or ER+ ≤2.0 cm in size	95	80	99	88	–	–
Mook et al. [10]	LN− or LN+ ER− or ER+ ≤1.0 cm in size	98	86	100	90	–	–

LN lymph node metastases, *ET* endocrine therapy, *chemo* chemotherapy
[a]10 year survival estimates

the test were not specifically chosen because of their involvement in breast carcinogenesis, previously identified genes associated with breast cancer outcomes, such as *ER*, *HER2*, and *CCND1* are not included. MammaPrint was cleared for marketing in the USA in 2007 by the Food and Drug Administration (FDA) for use on fresh or frozen tissue samples. Using tissue snap frozen within 1 h of surgery, 81% of samples yielded quality RNA for testing [10, 11]. The assay generates binary results classifying breast cancer patients as either low risk or high risk for recurrence. Low molecular risk correlates with clinicopathologic features as this group tends to include smaller, lower grade, ER positive tumors [12].

Patients diagnosed with ER positive or negative breast cancer at 61 years of age or younger were included in validation studies of MammaPrint [2, 11, 13, 14]. In different study populations, 37–54% of breast cancers were classified as being of low recurrence risk [10, 11, 14]. Overall survival, breast cancer specific survival, and disease free survival were significantly better in patients classified as low molecular risk versus high risk (Table 21.3) [10, 11, 13, 14]. The prognostic value of the MammaPrint test was further evaluated in a predominantly older postmenopausal population. In this patient population, the negative predictive value of the test was 100% [12]. Of 27 patients classified as low risk, none developed disease recurrence [12]. However, there was no statistically significant difference in disease free survival between the low risk and high risk groups and the positive predictive value was only 12% [12].

Recently, MammaPrint was shown to have predictive value for chemotherapy. High risk patients receiving both chemotherapy and endocrine therapy demonstrated a significantly longer distant disease free survival and breast cancer specific survival [14]. The five year breast cancer specific survival was 81% for high risk patients receiving only endocrine therapy but 94% for high risk patients receiving chemotherapy in addition to endocrine therapy. Distant disease free survival was 76% for high risk patients receiving only endocrine therapy versus 88% for high risk patients receiving both endocrine and chemotherapy [14]. Addition of chemotherapy did not show a similar benefit in the low risk group.

In summary, Onco*type* DX is well studied and marketed for use in patients of any age with lymph-node negative, ER positive breast cancer. Postmenopausal women with lymph node positive, hormone receptor positive breast cancer are also candidates for testing. Onco*type* DX is an effective prognostic and predictive test. MammaPrint is FDA cleared for women of any age with lymph node negative disease regardless of hormone receptor status. This test also has prognostic value in patients with lymph node metastases. The predictive utility of MammaPrint is emerging. By including both ER positive and ER negative cases, MammaPrint is applicable to a larger number of patients but Onco*type* DX has the advantage of using

formalin fixed paraffin embedded tissue samples. In the USA, these specimens are much more likely to be routinely available for testing than fresh or frozen tissue. The predictive value of both tests is limited by the chemotherapy regimens included in the studies. Furthermore, Onco*type* DX studies on patients treated with aromatase inhibitors are limited and many studies of MammaPrint do not specify the type of endocrine therapy. The two tests are similar in that they are performed in a central laboratory, limiting the ability to perform external proficiency testing.

Gene expression analysis in breast cancer can provide information complementary to other clinicopathologic risk factors and help guide treatment decisions. A potential issue with the use of these tests is that the result may be discordant with the clinicopathologic determined risk. This increases the challenge for oncologists, who must counsel patients and recommend therapeutic options. Also, the consideration of test results should occur in the context of how well the patient relates to the validation population in terms of disease status, age, and ethnicity. Study subjects used in the validation studies of Onco*type* DX were predominantly Caucasians in the USA, whereas MammaPrint validation subjects were European. In 2009, an Evaluation of Genomic Applications in Practice and Prevention (EGAPP) working group found insufficient evidence to make a recommendation for or against the use of gene expression profiles to improve outcomes in breast cancer patients [15]. They concluded that an association between Onco*type* DX RS and recurrence was supported by the evidence as well as an association between RS and response to chemotherapy [15]. The group found adequate evidence to support an association between MammaPrint results and risk of future metastases [15]. Prospective validation will be of value.

Multiple Choice Questions

1. How many informative genes are included in the Onco*type* DX assay?
   A. 5
   B. 16
   C. 21
   D. 70
   E. 250
2. True or False: Formalin fixed paraffin embedded tissue is an acceptable specimen for MammaPrint.
3. Which patient is a candidate for Onco*type* DX testing?
   A. Postmenopausal, ER negative, no lymph node metastases
   B. Postmenopausal, ER positive, two positive lymph nodes
   C. Premenopausal, ER negative, no lymph node metastases
   D. Premenopausal, ER negative, two positive lymph nodes
   E. Premenopausal, ER positive, two positive lymph nodes
4. If the Onco*type* DX recurrence score is high, then which group of genes has low expression levels?
   A. *ER*
   B. *GRB7*
   C. *HER2*
   D. Invasion
   E. Proliferation
5. In comparison to Onco*type* DX, MammaPrint:
   A. Heavily weights *HER2* in the data analysis
   B. Includes an intermediate risk category
   C. Is FDA cleared
   D. Is only validated for ER positive tumors
   E. Uses real time quantitative RT-PCR

Answers to Multiple Choice Questions

1. *The correct answer is B.*

Onco*type* DX is a 21 gene RQ-PCR assay that includes 16 informative genes and five reference genes. The genes were selected from 250 genes involved in carcinogenesis. MammaPrint is a microarray covering 70 genes.

2. *The correct answer is False.*

Fresh and frozen tissues are acceptable specimens for MammaPrint. Onco*type* DX is performed on formalin fixed paraffin embedded tissue.

3. *The correct answer is B.*

Patients of any age with ER positive, lymph node negative breast cancer are candidates for Onco*type* DX testing. Additionally, postmenopausal women with ER positive, lymph node positive disease are acceptable. Patients eligible for MammaPrint testing are those with

lymph node negative, Stage 1 or 2 carcinomas that are less than 5.0 cm in size. MammaPrint may be performed on both ER positive and negative tumors. Of interest, outside of the USA, MammaPrint testing is approved for patients with up to three positive lymph nodes.

4. *The correct answer is A.*

High recurrence scores are associated with low *ER* expression levels. High *HER2*, *GRB7*, proliferation, and invasion scores result in elevation of RS.

5. *The correct answer is C.*

MammaPrint is cleared by the FDA as a prognostic test. *HER2* is not included in the set of genes examined by the MammaPrint assay. MammaPrint does not include an intermediate risk category. Onco*type* DX is validated for ER positive tumors only whereas MammaPrint is suitable for ER positive and negative cases. MammaPrint is a microarray and Onco*type* DX uses RQ-PCR.

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Lung Adenocarcinoma

22

Lynette M. Sholl and Neal I. Lindeman

Clinical Background

A 45-year-old female nonsmoker presented to her primary care physician complaining of dry cough, pleuritic pain, and headache. Chest X-ray revealed an opacity in the left lower lobe of the lung. Chest CT scan showed a 3.7-cm mass in the left lower lobe and mediastinal adenopathy. Cranial MRI demonstrated a 6.5-cm mass in the occipital lobe, with additional smaller cerebellar lesions. A brain biopsy was performed, demonstrating metastatic adenocarcinoma with TTF-1 immunoreactivity, consistent with a primary tumor in the lung. The patient was treated with platinum–taxane based chemotherapy, with radiologic progression of disease. The patient was subsequently referred to a tertiary care center for therapeutic consultation.

Question 1: What is the role of molecular testing in this clinical context?

Reason for Molecular Testing

Molecular testing was performed to guide therapy selection, specifically with regards to the use of an EGFR tyrosine kinase inhibitor (EGFR-TKI), such as erlotinib.

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Test Ordered

Formalin-fixed, paraffin-embedded tumor samples were sent to detect activating mutations in exons 18 through 21 of the *EGFR* gene, the region that encodes the cytoplasmic tyrosine kinase domain of the epidermal growth factor receptor. These mutations include single nucleotide missense mutations and small in-frame deletions or insertions/duplications.

Laboratory Test Performed

Several methods are employed for *EGFR* testing, all of which begin with nucleic acid isolation and amplification by polymerase chain reaction (PCR). The methods vary, however, by the strategy employed to detect the mutations in the amplified product.

Question 2: What are the possible approaches to mutation analysis in this context?
Question 3: What are the advantages and limitations of the available techniques?

Sequencing-based methods were used initially to discover the mutations and are, therefore, the "gold standard." Sequencing can detect any of the common mutations, including drug resistance mutations and rare novel variants. However, this method is fairly labor-intensive and slow, with a turnaround time of several days to 2 weeks, depending on the laboratory volume and schedule. More importantly, however, sequencing is insensitive in heterogeneous samples because normal DNA sequences from admixed benign elements such

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as inflammatory and stromal cells can interfere with the ability to detect the mutant sequence. Mutant sequence is difficult to discern clearly by most Sanger sequencing methods when present in less than approximately 25% of the total DNA. Because the mutant sequence is typically present on only one of the tumor alleles, interpretation of sequencing results can be challenging in samples that contain fewer than 50% tumor cells. Unfortunately, benign elements commonly outnumber malignant cells in biopsy specimens, especially those taken from metastatic sites. Although this is mitigated somewhat by co-occurrence of polysomy, if present, on the mutant allele, adequate analysis typically requires manual dissection of samples from unstained slides by a pathologist or specially trained technologist in order to enrich the tumor content of the analyzed material to at least 50%. The need for pre-analytic dissection introduces delay, cost, and complexity to the testing, and renders many of the available specimens (especially cytology samples and very small biopsies) insufficient for analysis. The limitations of sequencing may, in fact, require patients to undergo a second diagnostic procedure to procure adequate tissue for testing.

An alternative approach is targeted amplification, hybridization, or enzymatic digestion to differentially detect individual mutant and wild-type sequences. These methods are considerably more sensitive to low levels of mutation, even in highly heterogeneous or frankly paucicellular samples, and pre-analytic dissection is often unnecessary. However, these assays generally must be custom-designed for each specific mutation tested, are typically limited to the most common mutations (exon 19 deletions and the c.2573T > G (p.Leu858Arg) mutation in exon 21), and do not detect the less common Gly719 mutations in exon 18, any novel mutations, or the exon 20 resistance mutations, which collectively account for ~10% of mutations.

Mutation screening approaches, such as heteroduplex analysis, melting curve analysis, or denaturing high-performance liquid chromatography, offer the potential to rapidly identify samples that harbor mutations, and limit the pool of samples that go on to confirmatory analysis by sequencing. These methods must be sufficiently robust to distinguish between point mutations and benign SNPs in order to be useful. Several SNPs are quite common in the regions of interest, particularly in exon 21, and these must be taken into consideration when considering this strategy. Also, if samples are preselected on clinical grounds prior to analysis (i.e., the laboratory is primarily testing samples from nonsmokers), the pretest probability of finding a mutation could be high enough that a pre-screening technique is unnecessary and too time-consuming.

Finally, several groups have demonstrated an association between *EGFR* copy number gain and response to EGFR-TKIs that largely overlaps with the correlation between mutation and response [1]. In most studies, copy number gains were assessed by fluorescence in situ hybridization (FISH) or chromogenic in situ hybridization (CISH), both of which are technically simpler and more widely available than is sequence analysis. However, analysis of copy number in phase III clinical trials failed to predict outcome as consistently as mutation analysis [2]. Some of the discrepancies in the predictive value of FISH/CISH in published studies may be related to methodologic and/or interpretive differences, but underlying biology may also be involved. Most FISH/CISH positive cases show "high" polysomy of chromosome 7, rather than specific amplification of the *EGFR* locus. This is in contrast to *HER2/ERBB2* in breast cancer, in which there is a focused amplification of a region containing that specific gene. When focused amplification of *EGFR* occurs, it does so preferentially on the mutated allele, is associated with more advanced and higher grade disease as compared to non-amplified tumor, and it has been associated with a more dramatic response to TKI therapy [3, 4]. These findings suggest that true amplification is a significant step in the process of tumorigenesis and may actually predict response. However, the significance of polysomy is less clear. Polysomy of chromosome 7 may confer a growth advantage to tumor cells that may be partially inhibited by EGFR-TKIs, but other oncogenes on this chromosome (namely *MET* on 7q) are likely to remain active.

Results with Interpretation Guideline

The forward (top) and reverse (bottom) strands were sequenced using Sanger dideoxy terminators labeled with different fluors for each nucleotide (Fig. 22.1). The reference sequence appears above the forward strand and below the reverse strand. Single nucleotide missense mutations would appear as superposition of two peaks in the same position, each of lesser height than adjacent peaks, with the difference in height loosely proportional to the percentage of tumor cells bearing the mutation. Deletions and/or insertions would

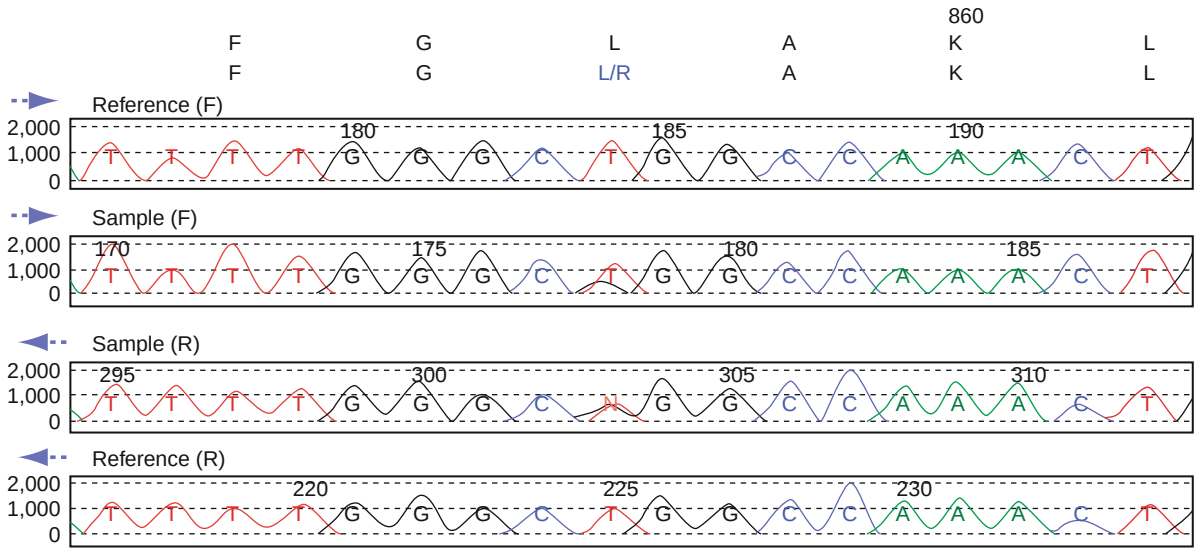

Fig. 22.1 *EGFR* sequencing result (exon 21). Sanger dideoxynucleotide sequence traces for part of exon 21, showing a T > G transversion at nucleotide 2573 causing a leucine to arginine mutation at codon 858 (Leu858Arg). Patients with this mutation do benefit from treatment with an EGFR kinase inhibitor

show a more complex pattern, with superposition of complete mutant and wild-type sequences that diverge at the boundary of the deleted/inserted sequence. The boundaries from the complementary strands thus define the region of the deletion or insertion.

Result Interpretation

This sequence shows a missense point mutation in exon 21, resulting in a leucine to arginine amino acid substitution at codon 858 (c.2573T > G (p.Leu858 Arg)). Missense point mutations involving *EGFR* codon 858 are seen in ~20% of *EGFR* mutant lung cancers. Tumors with this mutation respond well to treatment with erlotinib. Based upon this result, the patient was treated with erlotinib.

Question 4: What results might contraindicate treatment with erlotinib?

Further Testing

KRAS mutations in lung tumors occur predominantly in codons 12 (91.7%), 13 (5.7%), and 61 (2.2%); these are the same sites as seen in other cancers (e.g., colon and pancreas) [5]. Like *EGFR*, *KRAS* is an oncogene. Because of its location downstream of EGFR, proliferative signals emanating from a mutated KRAS protein will not be inhibited by EGFR blockade. As a result, and as clinical evidence demonstrates, *KRAS* mutant lung cancers do not respond to EGFR inhibition [6].

The implementation of laboratory-developed *EGFR* mutation analysis is hampered by intellectual property agreements. In contrast, *KRAS* mutation analysis is not, and it is therefore an attractive surrogate for *EGFR* analysis, insofar as it is able to identify TKI nonresponders. This approach should be used with caution however, in light of data suggesting that *EGFR* wild-type patients will do worse with TKI therapy, regardless of the *KRAS* mutation status [7]. There are currently no effective agents targeting KRAS activation although new trials are underway to study the role of specific inhibitors of downstream molecules (i.e., MEK, BRAF) in *KRAS* mutant tumors.

In addition, patients with sensitizing *EGFR* mutations eventually relapse on therapy, usually after an interval of approximately one year. On relapse, ~50% will show an additional *EGFR* mutation, the c.2369C > T (p.Thr790Met) substitution in exon 20. Second-generation EGFR inhibitors are in trial to determine if they have improved efficacy against the Thr790Met mutant EGFR, and testing for Thr790Met

is done on a research basis to help the selection of patients with relapse for those trials. In addition, FISH-based testing of the *MET* locus on chromosome 7 is also performed on a research basis for patients who have relapsed, as ~20% of patients who relapse have polysomy involving this oncogene [8]. Trials of MET inhibitors are underway for these patients.

Other Considerations

A molecular classification paradigm is emerging in lung cancer that is, in some ways, similar to that which has been embraced by hematopathologists for the classification of lymphomas. It is no longer sufficient to separate lung cancer into small cell and non-small cell lung carcinomas (NSCLC). NSCLC, particularly adenocarcinoma, has been shown to consist of several distinct molecular subtypes, for which targeted therapeutics are in trial or are being developed. Although *EGFR* is the best understood of the molecular targets in NSCLC, there is a rapidly expanding menu of other oncogenes that hold promise as therapeutic targets in this disease. Certain patients have shown dramatic responses to an inhibitor of the ALK kinase, which is activated by a chromosomal inversion (*EML4-ALK*) in a distinct group of lung cancers with neither *EGFR* nor *KRAS* mutations [9]. We predict that just as EGFR mutation has become integrated into the diagnostic workup for lung adenocarcinoma, so too will *ALK* analysis, by either immunohistochemistry or FISH. Other oncogenes including *BRAF*, *PIK3CA*, and *HER2* are being evaluated for their potential as diagnostic classifiers as well as therapeutic targets. As technologies evolve, so too will our ability to screen tumors for a wide range of molecular alterations. These advances will drive a "personalization" of the therapeutic approaches in lung cancer and other malignancies. While histology will undoubtedly remain the backbone of cancer diagnostics, molecular classification will become increasingly critical to tumor categorization and thus to patient care.

Background and Molecular Pathology

Lung cancer is the most lethal cancer in the United States, causing more deaths than the next four cancers (colorectal, breast, pancreas, prostate) combined, according to 2010 American Cancer Society statistics [10]. Treatment is determined primarily by the morphologic type and clinical stage. The outcomes are typically poor, particularly in advanced-stage non-small cell lung cancer, where median survival is four to nine months and 18 month survival is approximately 5% [11]. Beginning in 2003, however, significantly improved outcomes were reported in a subset (~20%) of adenocarcinomas treated with drugs (erlotinib and gefitinib) targeting the EGFR tyrosine kinase. Subsequently, several studies demonstrated that the presence of somatic mutations in the tyrosine kinase domain of the *EGFR* gene rendered the tumors susceptible to these targeted therapies, and molecular testing of *EGFR* has since become the standard of care for patients with lung adenocarcinoma [12–14].

EGFR (ERBB1, HER1) is a transmembrane growth factor receptor in the ErbB family of receptors that also includes ERBB2 (HER2/neu), which has been well established as a diagnostic and therapeutic target in breast cancer [15]. All ErbB family receptors have an extracellular ligand-binding domain, a dimerization–activation domain, and all but ERBB3 have a cytoplasmic tyrosine kinase domain. Upon ligand binding, ErbB family receptors dimerize (either as homodimers or as heterodimers with other family members), activating the tyrosine kinase and triggering several downstream pathways that promote growth, most notably the PI3K/AKT/mTOR and RAS/RAF/MAPK pathways [16].

Because of its growth-promoting properties and its frequent overexpression in a variety of tumors, including ~60% of lung cancers [17], *EGFR* had long been implicated as an oncogene. Moreover, its location in the cell membrane and accessible ligand binding and kinase domains render it an attractive target for anticancer therapies. Two strategies emerged for inhibiting EGFR signaling: monoclonal antibodies that block the ligand-binding domain, and small molecules that occupy the ATP-binding groove of the tyrosine kinase. The antibody-based therapies have yielded disappointing results in lung cancer, in contrast to the treatment successes seen with trastuzumab targeting Her2/ERBB2 in breast cancer. In contrast, the tyrosine kinase inhibitors gefitinib and erlotinib were very promising in phase I and phase II studies [18]. In phase III trials involving unselected patients with refractory, advanced-stage NSCLC, however, these drugs failed to demonstrate a significant benefit. While

these EGFR-TKIs showed, at best, marginal benefit in the overall population, a subset of patients, such as the patient described in this case, had dramatic and sustained responses. The responsive patients tended to be female nonsmokers with adenocarcinoma, especially the bronchioloalveolar-type. Asian ethnicity was also associated with response [19]. Still, these clinical factors alone did not adequately predict outcome, as some men and smokers also responded [19]. EGFR protein overexpression by immunohistochemistry also failed to predict response to EGFR-TKIs.

The best predictors of response to gefitinib and erlotinib were shown to be somatic gain-of-function *EGFR* mutations that occurred in several "hot spots" in exons 18 through 21. Around 90% of EGFR-activating mutations are either short in-frame deletions involving a conserved Leu Arg Glu Ala (LREA) motif in exon 19 or a missense point mutation (Leu858Arg) in exon 21. Other, less frequent mutations include a variety of missense mutations in exon 18 at Gly719 and in exon 21 (c.2582T > A (Leu861Gln)). The association between *EGFR* mutation status and response to TKI therapy was subsequently confirmed in multiple large clinical trials [20, 21].

These kinase domain mutations trigger activation of the EGFR tyrosine kinase independent of ligand binding or receptor protein overexpression, leading to proproliferative and anti-apoptotic signaling. The tumor cells are thought to become "addicted" to the oncogenic stimulus and thus exquisitely sensitive to inhibition of EGFR signaling [22]. In addition to increasing dependency on EGFR signaling in mutant cells, these activating mutations also stabilize the chemical interaction between drug and the kinase, as compared to wild-type *EGFR*. Therefore, EGFR-TKIs inhibit mutant EGFR more effectively than wild-type EGFR, a fortuitous relationship that enhances efficacy while reducing toxicity.

After the initial reports of drug-sensitizing mutations, an important subclass of mutations was detected in exon 20 of the *EGFR* gene. These include relatively uncommon insertion/duplication mutations and a more common missense point mutation, Thr790Met. Similarly to the mutations in exons 18, 19, and 21, which predict response to EGFR-TKIs, exon 20 mutations trigger EGFR activation. However, they also typically confer *resistance* to erlotinib and gefitinib. The Thr790Met mutation occurs secondarily in approximately 50% of patients who relapse on therapy after an initial response, whereas the exon 20 insertion/duplication mutations are more common in patients who fail therapy from the start [23, 24].

Multiple Choice Questions

1. Which of the following molecular diagnostic methods is most suitable for *EGFR* testing of a pleural fluid cytology sample?
   A. Allele-specific PCR
   B. FISH
   C. Immunohistochemistry
   D. Real-time quantitative RT-PCR
   E. Sanger dideoxynucleotide sequencing
2. If *EGFR* mutation analysis is unavailable, what other information would be most useful for determining whether or not a patient with lung adenocarcinoma should be treated with erlotinib?
   A. Asian ancestry, female gender, no smoking history
   B. FISH showing disomy with an *EGFR* probe
   C. FISH showing polysomy with an *EGFR* probe
   D. Presence of a *KRAS* codon 12 mutation (G12A)
   E. Strong immunohistochemical staining for EGFR
3. A patient with an exon 19 deletion in *EGFR*, diagnosed initially by allele-specific PCR of exons 19 and 21, relapsed on erlotinib after a partial remission of 16 months. Which of the following molecular tests is now recommended?
   A. FISH for *EML4-ALK* translocation
   B. FISH to assess *EGFR* copy number
   C. *KRAS* mutation analysis
   D. Repeat allele-specific PCR to assess loss of the *EGFR* exon 19 deletion
   E. Sanger dideoxynucleotide sequencing to detect an *EGFR* exon 20 mutation
4. A 62-year-old Italian-American male with a 42 pack-year (two packs/day for 21 years) history of smoking presents with a new diagnosis of metastatic lung adenocarcinoma. How should this patient be managed?
   A. Counsel that the prognosis is good, if the patient stopped smoking
   B. *EGFR* testing to determine the likelihood of response to erlotinib
   C. Empiric trial of erlotinib, with *EGFR* testing after 4–6 weeks
   D. Platinum-based chemotherapy
   E. Refer to a clinical trial of an experimental agent

5. EGFR inhibitors performed poorly in phase III clinical trials, because:
   A. Benefits are seen in a subset of patients who were not preselected in the trials
   B. EGFR inhibitors do not provide a significant benefit in lung cancer
   C. Entry criteria for trials required strong immunohistochemical staining for EGFR, while most patients who respond to treatment have negative immunohistochemistry
   D. Trials were conducted in the United States, while the patients who respond to treatment are Asian
   E. Trials were focused on patients who smoked

Answers to Multiple Choice Questions

1. *The correct answer is A.*

Pleural fluids often contain an admixture of reactive mesothelial cells intermingled amongst the cancer cells, and these samples often cannot be dissected for enrichment. For samples such as this, mutation-specific approaches, such as allele-specific PCR are preferable to Sanger sequencing.

2. *The correct answer is D.*

KRAS and *EGFR* mutations are mutually exclusive. If a *KRAS* mutation is found, the patient does not have an *EGFR* mutation and will not benefit from erlotinib. FISH and clinical features may suggest a better chance of a treatment response, but are not as predictive as a *KRAS* or *EGFR* mutation.

3. *The correct answer is E.*

Approximately 50% of patients who relapse after an initial response to erlotinib have a secondary mutation in exon 20, which is often not detected by allele-specific methods because this mutation is not an included target in these assays.

4. *The correct answer is B.*

Although *EGFR* mutations are less common in patients with this clinical presentation, they occur not uncommonly, and such patients should still be tested and treated with erlotinib if a mutation is found.

5. *The correct answer is A.*

The initial trials were performed on all patients with NSCLC, before it was known that *EGFR* mutation was the indicator of likely response. Although significant benefits were seen in the *EGFR* mutant patients in these studies, they accounted for only ~10% of the study population, insufficient to demonstrate an overall survival benefit for the drug when administered to all patients with NSCLC.

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Melanoma

23

Martin P. Powers

Clinical Background

A 61-year-old patient with a history of stage III melanoma presented with multiple subcutaneous nodules and multiple positron emission tomography (PET) positive tumors. A fine needle aspiration (FNA) of a subcutaneous nodule was performed and showed sheets of malignant cells with prominent nucleoli and intranuclear inclusions consistent with metastatic melanoma.

Question 1: What molecular testing may be useful on this specimen?

Reason for Molecular Testing

Inhibitors for BRAF have been developed specific to the oncogenic *BRAF* mutation V600E (c.1799T>A or p.Val600Glu). To determine whether this patient is eligible for BRAF inhibitor therapy, his tumor will be tested for this mutation.

Test Ordered

The test ordered was *BRAF* V600E mutation detection by real-time PCR and melting curve (probe dissociation) analysis.

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Question 2: What other testing can give this same information, and what are the advantages and disadvantages of other methods?

Laboratory Test Performed

Melting temperature analysis is one way to identify point mutations. DNA sequencing and allele-specific methods such as allele-specific PCR, real-time PCR with allele-specific hydrolysis probes, allele-specific hybridization, or allele-specific primer extension, are among the alternative approaches. DNA sequencing has the advantage of analysis of all nucleotides in the sequenced region. However, it requires at least 10–20% of mutant DNA in a background of wild-type DNA. Allele-specific methods typically have better sensitivity, but require development of a specific probe or primer for each mutation that is to be analyzed. For our patient, a melting curve test was performed.

PCR primers were designed to amplify an ~250 bp region surrounding the *BRAF* V600E region in exon 15. Amplification was detected using adjacent dual hybridization fluorescence resonance energy transfer (FRET) probes [1]. The sensor probe was 24 nucleotides long and a 100% match with the wild-type *BRAF* sequence between nucleotides c.1791 to c.1814 and labeled at its 3′ end with FAM. The anchor probe was 29 nucleotides long, labeled at its 5′ end with LC-Red640 and was a perfect match to the wild-type (WT) *BRAF* sequence from c.1816 to c.1844 (Fig. 23.1a). The sensor probe melted off the wild-type sequence with a temperature of about 64.5°C and from the c.1799T>A mutation at approximately 59.5°C.

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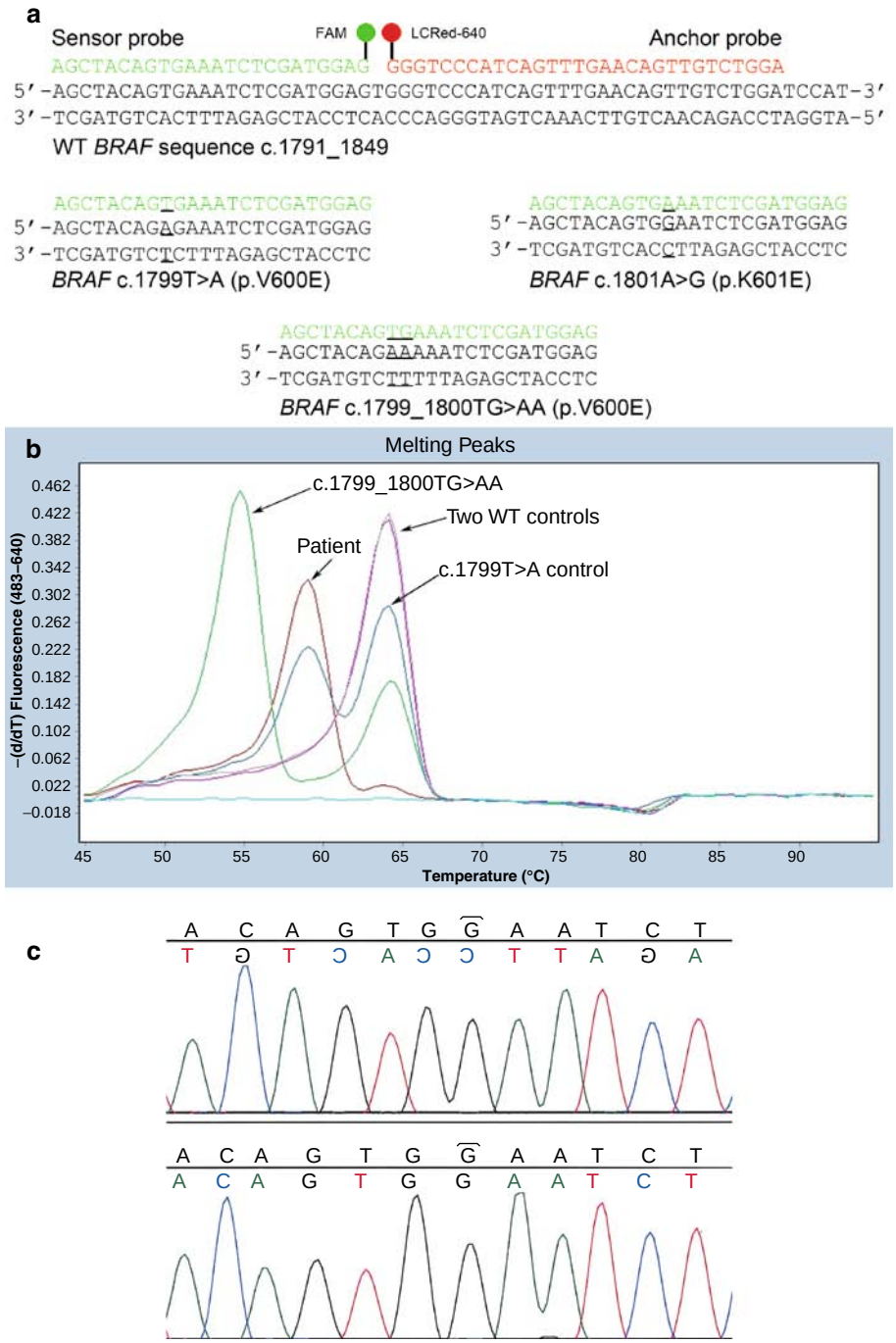

Fig. 23.1 (**a**) Representations of sensor and anchor probe on wild-type *BRAF* sequence, and sensor probe with mutant sequences. In this figure, both the sense and antisense strands of the *BRAF* sequence are shown in addition to the probe that matches the sense strand. In hybridization, the probe would only hybridize to, and melt off of, the antisense strand. Mismatches are underlined. (**b**) Negative derivative of the melt curve using *BRAF* wild-type dual hybridization FRET probes. (**c**) Sequence tracing of our patient's sample in the forward (*bottom*) and reverse (*top*) direction

Extracted DNA was mixed with primers and probes and amplified in the LightCycler 480 (Roche) with real-time detection of amplification product during the annealing phase of each cycle (not shown). Fluorescence was detected via FRET [2]. In FRET, the fluorescent signal is only detected when both probes are adjacent to each other (Fig. 23.1a). The signal is generated by using a wavelength that excites one probe's fluorophore and by detecting fluorescence at the wavelength that is emitted by the fluorophore of the other probe. Fluorescence will only be detected if both probes are adjacent and in the proper orientation for the excitation and emission of the first probe's fluorophore, which then excites the second probe's fluorophore, which in turn emits the light that is detected.

Post-amplification melt curve analysis is performed by cooling the sample to 45°C and then slowly raising the temperature to 95°C at 0.1°C per second with constant acquisition of fluorescence. As the temperature increases, the sensor probe is eventually no longer able to hybridize to the target, and the fluorescent signal is lost. The point at which half of the fluorescence is lost is the melting temperature, which is best represented by taking the negative derivative of the melt curve (Fig. 23.1b). Instead of identifying the point on a graph where half of the fluorescence is lost for some of the targets and not the others, the negative derivative method identifies the temperatures at which the melting is happening most "quickly," or where the most fluorescence is lost between two temperature points. This method also identifies the melting point for each individual target in the mix (in this example, both wild-type and mutant sequences after amplification). This method allows for sensitive detection of the mutation through the detection of a melting peak different from wild-type DNA and for an easy one-step work flow. Extracted tumor DNA is mixed with PCR master mix, probes, and primers, and then amplification, detection, and melting curve analysis are all performed in one automatic step on the real-time PCR instrument in a closed system. Final data review and analysis is performed on the computer.

Results with Interpretation Guideline

Two negative controls (wild-type *BRAF* sequence), a c.1799T>A positive control, a no template control and our patient were included in the testing, along with a different, rare, mutation (discussed below) (Fig. 23.1b). The measured melting temperatures are listed in Table 23.1. In validation studies, the wild-type allele melted at 64.5°C plus or minus 0.5°C and the V600E mutant melted at 59.5°C plus or minus 0.5°C. Probe dissociation ranges are established for each instrument, in individual laboratories.

Table 23.1 Melting temperatures with "wild-type" probe

Sample	Melting peak 1	Melting peak 2
Positive control	59.15°C	64.08°C
Our patient	59.02°C	–
Negative control	64.20°C	–
No template control	–	–
c.1799_1800TG>AA	54.72°C	64.15°C

Question 3: How do you explain the presence of a wild-type melting peak in the positive control and the lack of a wild-type melting peak in our patient?

Result Interpretation

The result of our patient was consistent with the presence of a *BRAF* V600E mutation. Our positive control was from a patient sample and this patient sample most likely had some contaminating normal cells. Although blunt microdissection for tumor is performed from the formalin fixed paraffin embedded (FFPE) tissue, the tumor may still have some normal endothelial cells and other stromal elements. Also, *BRAF* p.V600E is a dominantly acting oncogenic mutation. It is an activating mutation, and the presence of only one mutant copy is required to promote oncogenesis. The wild-type allele may still be retained in the tumor tissue.

Question 4: Is this test specific for BRAF V600E, and are you confident that the patient has a V600E mutation and not something else?

Further Testing

Based on the database, the Catalogue of Somatic Mutations in Cancer (COSMIC) [3, 4], the c.1799T>A (V600E) *BRAF* mutation is the most common mutation seen in melanoma but other mutations in the region have been reported. In the region between c.1791 and c.1814 (the region under our sensor probe), 94% of all

reported mutations are c.1799T>A. Of the single point mutations, >98% are c.1799T>A. Some of the remaining mutations are more complex and would not be expected to show a melt curve similar to c.1799T>A. The next three most common mutations reported are the double nucleotide changes c.1798_1799GT>AA, c.1798_1799GT>AG, and c.1799_1800TG>AA, which would have two mismatches with our probe and would be expected to melt at a temperature significantly lower than 59.5°C ± 0.5°C. One example of c.1799_1800TG>AA (p.V600E) is included in Table 23.1 and Fig. 23.1b with a melting temperature of the mutant peak almost 10°C lower than the wild-type peak. Reported eight times in the database (0.4% of reported mutations), and the fifth most common mutation in this region in melanoma, is mutation c.1801A>G (p.K601E). Because this is a single base pair change near nucleotide 1799, the melting temperature may be similar to the one for mutation c.1799T>A. Therefore, the probe may not be 100% specific for c.1799T>A. The PCR product of our patient was sequenced using the Sanger-dideoxy method and a c.1801A>G (p.K601E) mutation was indeed found with no detection of any wild-type BRAF sequence in the background (Fig. 23.1c). Thus, our patient appeared homozygous for this sequence change within the limits of detection for the assays performed.

Question 5: How can we explain the lack of a wild-type signal in our patient?

Actually, a closer look at our patient's melting temperature curve (Fig. 23.1b) reveals a subtle bump near a temperature at which a wild-type allele would be expected to melt. However, this signal was not strong enough to be called a mutation by eye or the software (Fig. 23.1c). There are several possible explanations. First, there were very few contaminating normal cells because this fine needle aspirate (FNA) contained tumor cells almost exclusively, with only a few contaminating blood cells. A rough estimate would have at least 100 tumor cells for each white blood cell. Therefore, there was very little contamination from normal DNA. However, the tumor cells can still retain a wild-type copy of *BRAF* and the absence of the wild-type *BRAF* signal may have been caused by amplification of the mutant *BRAF* sequence, or by loss of the wild-type *BRAF* sequence. Both are possibilities in melanoma. Methods that measure the gains and losses of genomic material, such as comparative genomic hybridization (CGH) or array-based comparative genomic hybridization (aCGH), have shown that many melanomas have gains or amplification of the genomic DNA that includes the *BRAF* locus (7q34), presumably of the mutant allele [5–7]. Therefore, the number of copies of the mutant *BRAF* locus may be significantly greater than the number of wild-type copies. In addition, whole genome single nucleotide polymorphism (SNP) analysis on melanoma cell lines (which may not reflect true in vivo melanoma) has shown loss of heterozygosity (LOH) on the long arm of chromosome 7 (7q), including LOH of the entire 7q arm. This suggests that loss of the wild-type copy of *BRAF* is possible in patients with melanoma, as well [8].

Question 6: Is this patient a candidate for the anti-BRAF drug PLX4032?

Other Considerations

As highlighted above, an abnormal melt curve with a wild-type probe may not be 100% specific for the mutation in question; a change in melting temperature from wild-type could also represent another change underneath the probe region. Therefore, the assay may be a reasonable screening tool for a variety of mutations underneath the probe sequence. Given the predominance of V600E as the most common mutation, however, is it even necessary to confirm the specificity of the melt curve for this mutation? In melanoma, as reported in the COSMIC database [3], 98.3% of all single-point mutations between nucleotides c.1791 and c.1814 (the region covered by the sensor probe) are c.1799A>T. Therefore, a possible estimate of the specificity of this probe, when it shows a melting temperature change consistent with a single-point mutation, for c.1799A>T is 98.3% (in melanoma). The true specificity may in fact be higher than this since the database may be biased toward rarer mutations. If an unbiased assessment of all point mutations in *BRAF* in melanoma were performed, c.1799A>T may account for an even higher percentage. Therefore, this test is nearly 100% specific for the c.1799T>A mutation in melanoma. Also highlighted is the near perfect specificity of sequencing as opposed to more indirect methods. The specificity may change by tumor type. Based on the COSMIC database [3], in thyroid cancer, another tumor often tested for *BRAF* mutations,

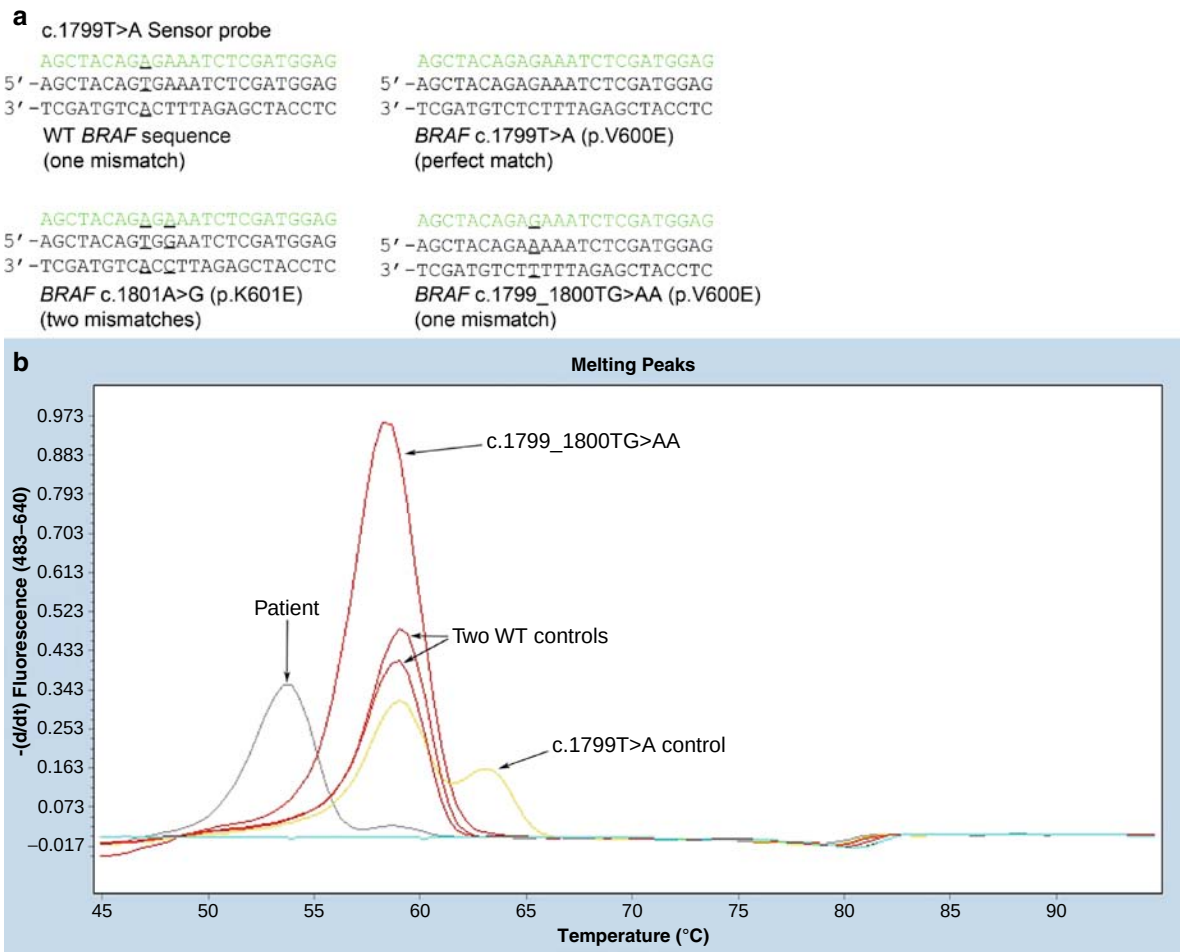

Fig. 23.2 (**a**) Representations of a c.1799T>A specific sensor probe on wild-type and mutant *BRAF* sequences. Mismatches are underlined. (**b**) Negative derivative melting peaks of our patient and other sequences using the c.1799T>A specific probe

>99.7% of the point mutations reported in the area covered by our probe are c.1799T>A.

Sequencing is one way to confirm the mutations. Another method for confirmation is to have a mutant-specific probe in the real-time assay, as opposed to a wild-type-specific probe (Figs. 23.2a and 23.2b). If the probe is specific to the c.1799T>A mutation, it will have a higher melting temperature with the mutation than with the wild-type sequence. This would increase the specificity of the assay for c.1799T>A and would detect point mutations at nucleotides other than 1799 as two nucleotide changes. An example of this is our patient, who carried the wild-type nucleotide at c.1799 and an A>G mutation at c.1801 (Fig. 23.2 and Table 23.2). Using a mutation-specific probe, however, may limit the ability to "see" other point mutations at c.1799 (such as T>G and T>C, which are extremely rare mutations) as they might melt similarly to wild-type. Some double mutations, such as c.1798_1799GT>AA and c.1799_1800TG>AA, which are not uncommon in melanoma, may also melt similarly to wild-type, as

Table 23.2 Melting temperatures with "c.1799T>A" specific probe

Sample	Melting peak 1	Melting peak 2
Positive control	59.05°C (wild-type)	64.15°C (V600E)
Our patient	53.27°C (c.1801A>G)	–
Negative control	59.26°C	–
No template control	–	–
c.1799_1800TG>AA	58.44°C	

they would have one nucleotide change that matches the probe and another nucleotide change that is a mismatch with the probe for a net one nucleotide difference between the probe and the target DNA sequence (Fig. 23.2a). In this case, the V600E mutant that results from c.1799_1800TG>AA now shows a melting temperature analysis profile peak that is a little wider than wild-type with a melting temperature just under 59°C, most likely reflecting the melting of the probe from the wild-type and mutant sequences together under one peak (Fig. 23.2b and Table 23.2). The mutant peak most likely melts off a little earlier than the wild-type peak, due to the disruption of a G-C bond versus an A-T bond, but this discrimination is too subtle for this melting curve assay (Fig. 23.2a). In conclusion, the specificity of the melt curve is determined by the probe used and only alleles that match completely with this probe can be detected with near 100% reliability. Other mutations may be missed or erroneously called something else if the mutation frequencies of all the possible alleles under the probe are not known.

Background and Molecular Pathology

Melanoma is a malignant tumor of melanocytes and is deadly if not caught in its earliest stages. Melanomas may arise on chronically or intermittently sun-exposed skin, including acral areas (palms, soles, and nailbeds), mucosal surfaces, and the eye. Several characteristic genetic and genomic changes have been seen in the melanocytic tumors and some of these are relatively specific to the different subtypes of melanoma [9]. Such genetic alterations can be used in the differential diagnosis of melanoma [10]. Melanomas often exhibit activation of the RTK-RAS-RAF-MAPK pathway. The most common activating change in melanoma is a mutation in the *BRAF* gene. This change is not specific for melanoma and cannot be used for melanoma diagnosis, as it is often seen in benign melanocytic nevi as well. However, testing for *BRAF* mutations has gained importance in predicting the response of melanoma patients to BRAF inhibitors. One such inhibitor is PLX4032. This inhibitor has shown a response rate of up to 80% in patients with *BRAF* V600E mutated melanoma [11]. This inhibitor was developed specifically to the V600E mutation and has less activity against wild-type *BRAF* [12]. Current clinical trials require that patients have the V600E mutation. Also, the importance of a *BRAF* mutation in patients treated with BRAF inhibitor drugs has been highlighted by in vitro studies with PLX4032. PLX4032 has been shown to promote the growth of melanoma cells with wild-type BRAF, possibly through the activation of another RAF family member, CRAF [13–16]. Therefore, treatment with such a drug may be detrimental to patients without a *BRAF* V600E mutation. The effect of an inhibitor drug on a different *BRAF* mutation such as K601E, as seen in our patient, is unknown.

Multiple Choice Questions

1. Real-time PCR with FRET probes and melting curve analysis is useful for detecting mutations in all of the following circumstances EXCEPT … (select the one best answer)
   A. A three nucleotide-specific deletion
   B. A 2 bp insertion
   C. Multiple mutations in a very narrow region (i.e., one or two codons)
   D. Multiple mutations scattered throughout the gene in many different exons
   E. One single recurrent point mutation
2. Which method would NOT be effective in identifying the c.1799T>A, p.V600E point mutation in *BRAF* (select the one best answer)?
   A. Allele-specific PCR
   B. Allele-specific primer extension
   C. DNA sequencing
   D. FISH (fluorescent in situ hybridization)
   E. Melting curve analysis
3. V600E is the only *BRAF* mutation seen in which of the following tumors (select the one best answer)?
   A. Colorectal carcinoma
   B. Melanoma
   C. Thyroid carcinoma
   D. All of the above
   E. None of the above
4. A *BRAF* V600E mutation can be used in all of the following clinical circumstances EXCEPT … (select the one best answer)
   A. Diagnosing melanoma
   B. Predicting a more aggressive thyroid cancer
   C. Predicting response to a BRAF inhibitor
   D. Predicting resistance to anti-EGFR therapy in colorectal cancer
   E. Ruling out Lynch syndrome in a colorectal carcinoma specimen

5. Which of the following methods is the most specific to detect all the point mutations that are possible between nucleotides c.1795–1803 in the *BRAF* sequence? (select the one best answer)
   A. Allele-specific hybridization to a probe (i.e., reverse dot blot)
   B. DNA sequencing
   C. Dual hybridization (FRET) melt curve analysis
   D. High-resolution melting
   E. PCR with allele-specific primers

Answers to Multiple Choice Questions

1. *The correct answer is D.*

Dual hybridization FRET probes will show a shift in melting temperature if there are small or single changes underneath the probe. Therefore, they are useful for screening or identification of point mutations or small insertion/deletions in either one specific base or a narrowly defined area. Large changes may prevent probe hybridization if too many mismatches are present. If FRET probes are to be used to screen for multiple mutations in different exons, multiple different probes will need to be created for each possible area that has a mutation. This may not be feasible or practical in a large gene with mutations scattered throughout.

2. *The correct answer is D.*

Melting temperature analysis is not the only way to identify point mutations. DNA sequencing can be done as shown above, as can other allele-specific methods such as allele-specific PCR, real-time PCR with other allele-specific probes such as hydrolysis probes, allele-specific hybridization or allele-specific primer extension. FISH uses very large probes hybridized to interphase or metaphase nuclei and such probes do not discriminate single base changes. DNA sequencing has the advantage of seeing all the possible mutations in the region sequenced and is very specific as the mutations are directly visualized. However, it may be limited by sensitivity and may not detect a mutant in a background of too many wild-type alleles. The other allele-specific methods have excellent analytic sensitivity (the ability to detect mutant sequence in a wild-type background), but a specific probe or primer must be developed for each mutation that is being analyzed.

3. *The correct answer is E.*

BRAF V600E comprises more than 98% of the single base pair point mutations in each of these tumors; however, other *BRAF* mutations have been reported, the significance of which is less well characterized. Although not specifically discussed in the text, *BRAF* mutations are seen in about 11% of colorectal cancer. Most of these mutations are *BRAF* c.1799T>A, but some of the rarer mutations are seen in colorectal carcinoma, as well [3].

4. *The correct answer is A.*

BRAF mutations are seen in melanoma as well as in benign melanocytic nevi. Therefore, they can't be used to reliably diagnose a melanoma. However, they do predict a response to BRAF inhibitor therapy, and they can predict resistance to EGFR therapies in colorectal carcinoma and predict more aggressive disease in papillary thyroid carcinoma [17, 18]. As mentioned in Lynch syndrome Chapter 27, mutations in *BRAF*, if seen in colorectal carcinoma, can be used to rule out Lynch syndrome.

5. *The correct answer is B.*

Per the COSMIC database, to date, 13 point mutations and 14 different complex mutations have been reported in this region [3]. Sequencing would be expected to detect all of these variants if the number of tumor cells is adequate to allow detection by sequencing and if the PCR and sequencing primers were designed to be outside the region of interest. Allele-specific methods can detect these mutations as well, but an individual allele-specific primer or probe would need to be designed for each mutation and if unexpected (not previously reported), mutations arise in a tumor, they could be picked up by sequencing, but would most likely be missed by the allele-specific methods. Melting-based protocols can only tell you that a mutation is present and not necessarily which specific mutation is present, although certain melt curve profiles may suggest specific mutations.

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Thyroid Cancer

24

Matija Snuderl and Jennifer L. Hunt

Clinical Background

A 75-year-old male complained of three weeks of dyspnea and a small lump in the back of his left neck. The patient's history was notable for coronary artery disease, treated with coronary artery bypass surgery two years ago, and a papillary thyroid carcinoma (PTC) diagnosed 25 years ago and treated with a total thyroidectomy. The patient had also had a slowly growing lung metastasis from the papillary carcinoma that was treated with partial lung lobectomy 10 years ago. Other history included prostatic carcinoma diagnosed 15 years ago, treated with prostatectomy and local radiation. Clinically, the patient had been followed with serum prostate-specific antigen (PSA), which had been slowly rising over the previous year. The patient had a remote history of smoking occasionally but had quit smoking in his late twenties. A combined CT and PET scan demonstrated high uptake in the posterior neck lesion and revealed additional lesions with high metabolic uptake in the hilum of the left lung, left pelvis, and abdominal para-aortic lymph nodes. A diagnostic bronchoscopy was performed, and on examination, an endobronchial lesion in the left middle lobe bronchus was discovered, with compression of the bronchus intermedius.

Question 1: What is the differential diagnosis? Which clinical tests could narrow the differential diagnosis?

Reason for Molecular Testing

The lung and neck lesions were biopsied and pathology evaluation revealed a moderately to poorly differentiated squamous cell carcinoma. Immunohistochemical staining demonstrated that tumor cells were strongly positive for keratin 7 and negative for keratin 20 and thyroglobulin. Scattered cells were weakly positive for p63 and TTF-1. Prostate-specific antigen was negative. Morphologic and immunohistochemical features excluded metastatic prostate carcinoma. Histologic comparison to the patient's prior papillary thyroid carcinoma revealed that the current lesion did not resemble the thyroid tumor. Morphologic and immunohistochemical studies favored a primary squamous cell carcinoma of the lung. The differential diagnosis also included metastatic papillary thyroid carcinoma with either squamous metaplasia or anaplastic dedifferentiation (Fig. 24.1). Distinction between a new primary lung carcinoma and a widely metastatic thyroid carcinoma was critically important for clinical management and to determine prognosis.

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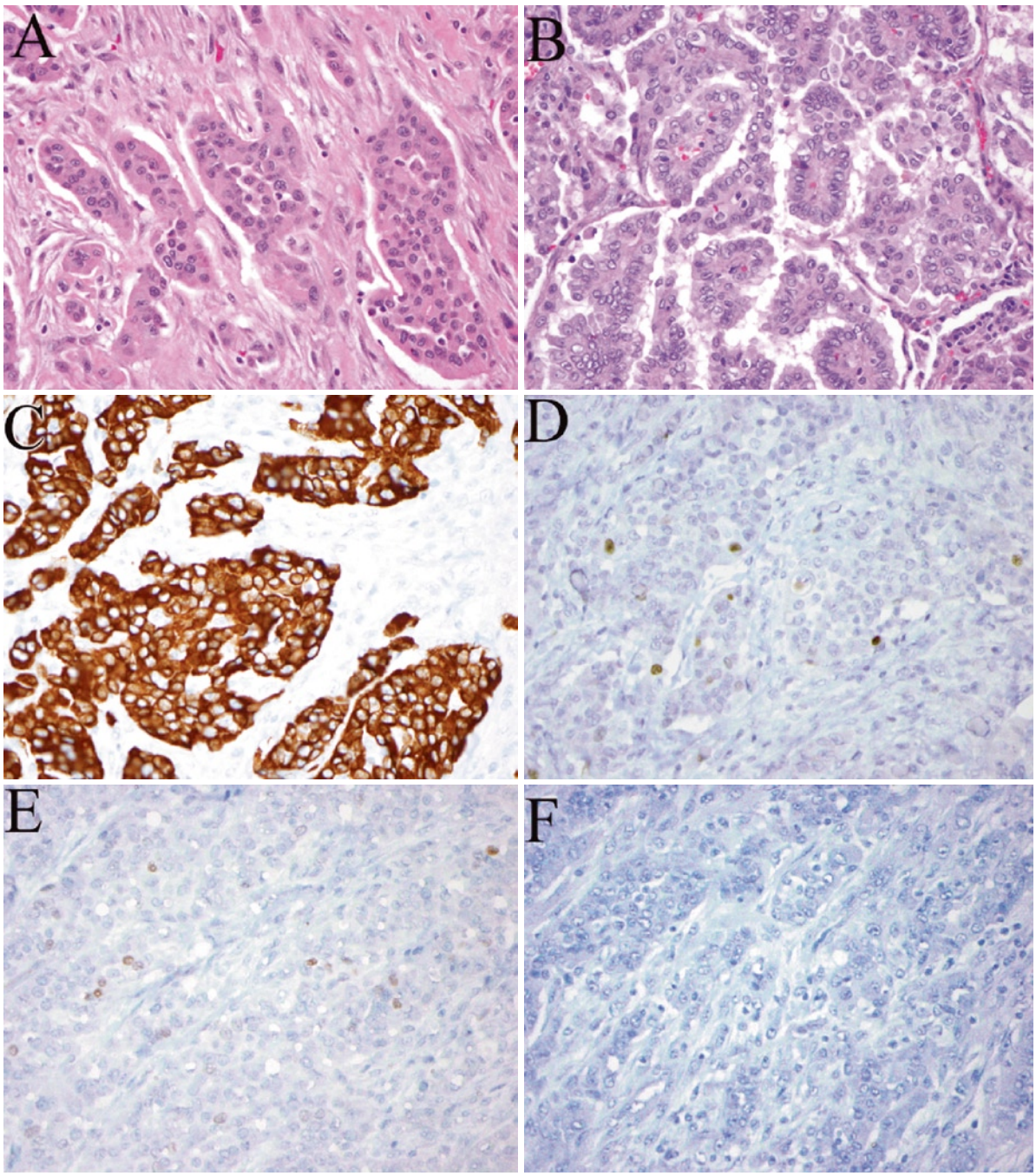

Fig. 24.1 Hematoxylin and eosin stain of the current neck node lesion revealed features of a poorly differentiated carcinoma with abundant pink cytoplasm, stippled chromatin, and reactive desmoplastic stromal reaction. Features of PTC were not recognized (**a**, 400x). Patient's primary thyroid tumor revealed classic histological features of papillary thyroid carcinoma with papillary architecture, round nuclei with cleared chromatin, and nuclear grooves (**b**, 400x). By immunohistochemical studies (**c**-**f**, all 400x), tumor cells were positive for cytokeratin 7 (**c**), scattered cells were positive for squamous differentiation marker p63 (**d**), rare cells were weakly positive for thyroid transcription factor 1 (TTF-1) (**e**), and tumor cells were negative for thyroglobulin (**f**). Results of capillary electrophoresis show normal genomic DNA (**g**: forward primer, (**h)**: reverse primer) and the tumor DNA (**i**: forward primer, (**j)**: reverse primer). A point mutation is highlighted by arrows

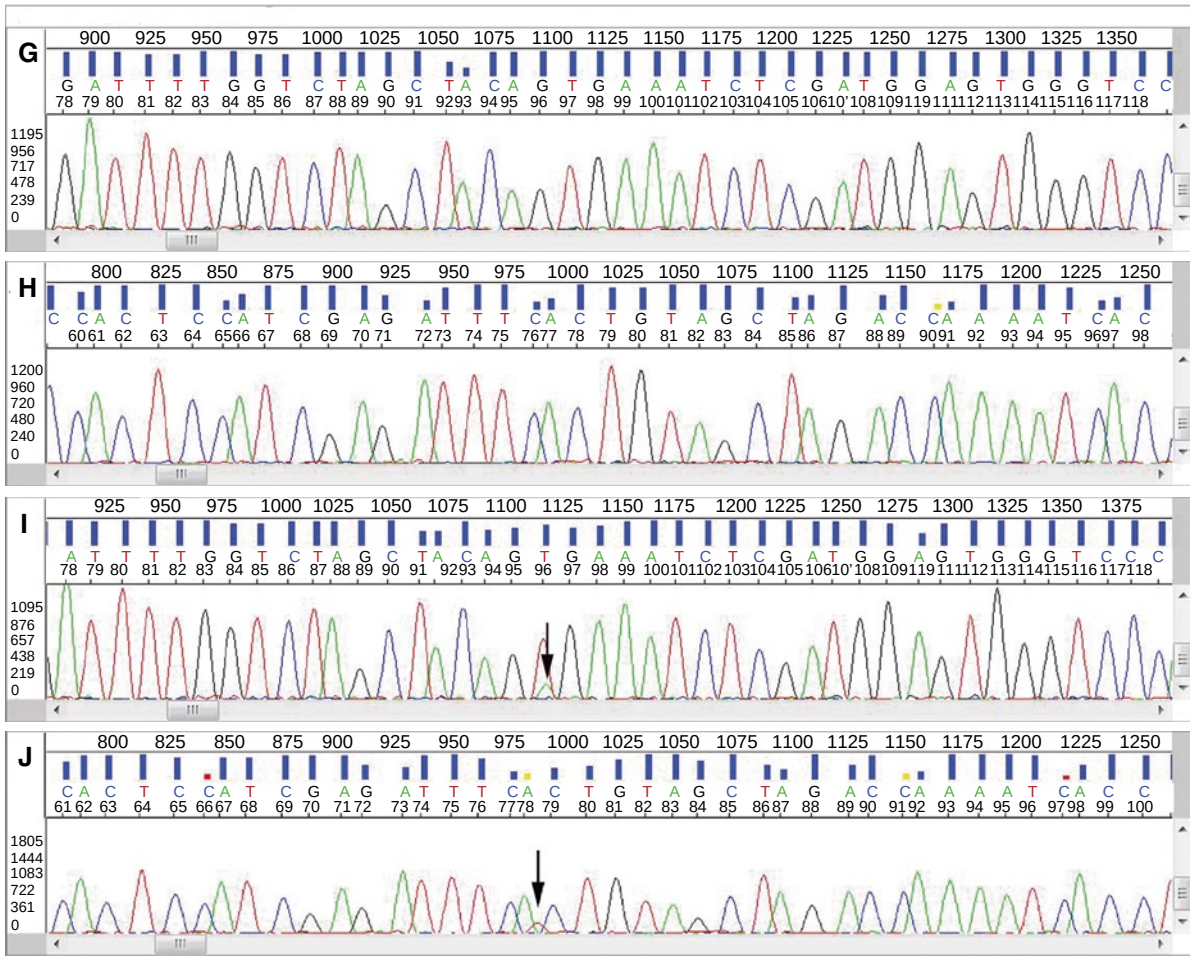

Fig. 24.1 (continued)

Test Ordered

Genotyping analysis for *BRAF*, *EGFR*, *KRAS*, *NRAS*.

Question 2: Is this an appropriately ordered test?

Laboratory Test Performed

Somatic mutations in tumor DNA can be detected by several different techniques, including allele-specific polymerase chain reaction (PCR), traditional gene sequencing, pyrosequencing, and mutation detection using single base extension sequencing assays. Within the allele-specific assays, it is often possible to design multiplex assays to detect multiple mutations in commonly mutated genes at the same time. In addition, PCR reactions using forward and reverse primers flanking exons of interest can be utilized to detect in-frame activating deletions of tumor-associated genes.

Results with Interpretation Guideline

Testing results are summarized in Fig. 24.1.

- Interpretation guidelines for morphological and immunohistochemistry studies: hematoxylin and eosin stain of patient's lung metastasis (A) and his original thyroid tumor (B), immunohistochemical stains for cytokeratin 7 (C), p63 (D), thyroid transcription factor 1 (TTF-1) (E), and thyroglobulin (F).
- Interpretation guidelines for studies by PCR with capillary electrophoresis: normal genomic DNA is used as a control (G: forward primer, H: reverse

primer) and these sequences are compared with the tumor DNA (I: forward primer, J: reverse primer). A point mutation was defined as an alteration of the nucleotide from the genomic DNA.

Result Interpretation

The current neck node tumor revealed morphological features of a poorly differentiated carcinoma without features of papillary thyroid carcinoma. By immunohistochemical studies, tumor cells were positive for cytokeratin 7, scattered cells were positive for squamous differentiation marker p63, rare cells were weakly positive for thyroid transcription factor 1 (TTF-1) and tumor cells were negative for thyroglobulin. Morphologic and immunohistochemical results were not supporting a diagnosis of metastatic papillary thyroid carcinoma, although de-differentiation of the original tumor could not be excluded.

Results of capillary electrophoresis indicated a point mutation (arrows) at the same location in both directions. The mutation is represented by the smaller peak in the sequence. Analysis of the nucleotide peaks created by traditional gene sequencing from tumor DNA revealed a *BRAF* mutation in exon 15 at codon 600 (GTG > GAG, nucleotide change c.1799T > A; amino acid change p.Val600Glu or p.V600E) in the patient's lung metastasis and neck lymph node. No mutations were observed in *KRAS*, *NRAS*, or *EGFR*.

BRAF mutations are rare in lung tumors, but very common in papillary thyroid carcinomas and in anaplastic thyroid carcinomas. *RAS* mutations are less common in papillary carcinoma, but are often seen in smoking-related tumors in the lung. *EGFR* mutations are seen in lung carcinomas, but generally only in those with adenocarcinoma morphology and associated with specific patient demographics (young, nonsmoking, female patients). Molecular studies, demonstrating a *BRAF* mutation in this tumor sample in the context of the morphology and the clinical scenario, suggest that the tumor represents metastatic thyroid carcinoma with either squamous metaplasia or anaplastic transformation.

Question 3: Does the test result provide a definitive diagnosis?

Further Testing

It was still important to identify the sequencing profile of the primary papillary carcinoma of the thyroid. Demonstrating concordance between the current tumor material and the prior papillary carcinoma would strengthen the argument against a de novo lung squamous cell carcinoma. The original thyroid tumor from 25 years ago and the lung metastasis from 10 years ago, both with conventional papillary thyroid carcinoma morphology, were retrieved from the archives and sequencing for *BRAF* was performed. Analysis of nucleotide peaks using tumor DNA revealed the identical *BRAF* mutation in exon 15 (c.1799T > A, p.V600E).

Background and Molecular Pathology

PTC is the most common malignancy of the thyroid gland, representing nearly 80% of all malignant thyroid tumors. PTC affects women more commonly than men. Patients usually present with a painless thyroid mass. Metastatic disease is common at the time of presentation, but is usually limited to the presence of central compartment lymph node metastases. Distant metastases are much less common. The treatment usually includes total thyroidectomy, often followed by a cure rate of over 95% [1].

The diagnosis of PTC is based on the morphologic and cytologic features of the tumor cells. The classic cellular features of PTC include nuclear clearing, elongation and enlargement, nuclear overlapping, groove formation, and cytoplasmic intranuclear inclusions. Variant morphologies have also been identified, and these are usually defined by the growth pattern and other cytological features. The most common variants include the follicular variant and the tall cell variant. Less common variants include the diffuse sclerosis, columnar cell, and cribriform morular variants.

The molecular changes in PTC have been extensively studied in the past decade. The most common molecular changes implicated in the development of the PTC have included *RET/PTC* rearrangements and somatic point mutations in the *RAS* and *BRAF* genes.

Although *RAS* mutations are quite common in follicular-derived neoplasms (follicular adenoma and

follicular carcinoma), they are much less frequently seen in PTC. Because they are so common in other benign and malignant tumors of the thyroid, RAS mutations cannot be used to make the diagnosis of PTC and have not been shown to have any prognostic significance.

RET/PTC rearrangements are present in approximately 30% of PTC [2], although regional and epidemiological differences in the prevalence exist. Multiple different mutations have been described, representing translocations between the *RET* gene, located on chromosome 10q21, and different partner genes, which are grouped under the generic name "PTC" genes (for papillary thyroid carcinoma) [3, 4]. Currently, there are more than ten different partner genes identified within the *RET/PTC* category of mutations, with most common partner genes being the *H4* gene (*PTC1*) and the *ELE1* gene (*PTC3*) [5], both located on chromosome 10. While *RET/PTC1* rearrangement predominates in sporadic tumors [2], radiation-induced tumors often carry *RET/PTC3* rearrangements [6]. Tumors that are radiation induced are known to have genetic instability, which is likely the cause of complex chromosomal abnormalities [7, 8]. Currently, there are two approaches to designing assays for the *RET/PTC1* translocation, including reverse-transcription and polymerase chain reaction (RT-PCR) based assays and fluorescent in situ hybridization (FISH) assays. Both are difficult to perform on routine paraffin-embedded tissues, due to sample size and quality issues. The FISH assay is also somewhat challenging to interpret, because the most common variant translocations are actually intrachromosomal rearrangements, with both partner genes located on the same chromosome. For these reasons, most laboratories do not offer this as a clinical test.

BRAF gene mutations are extremely common in PTC, but they are absent from follicular lesions of the thyroid (follicular adenoma or follicular carcinoma). The most common PTC mutation is seen in more than 50% of the conventional PTC cases. The mutation is a somatic point mutation in exon 15, at codon 600, nucleotide 1799, where the normal T nucleotide is replaced with an A [9–11]. Interestingly, different morphologic variants of PTC have quite different profiles of *BRAF* mutation. Notably, the tall cell variant has a high rate of *BRAF* positivity, whereas the follicular variant has a very low frequency of the *BRAF* mutation [9, 12]. *BRAF* mutations are also not typically associated with radiation and are essentially mutually exclusive with *RET/PTC1* translocations [13]. Other tumors that have been shown to have *BRAF* gene mutations include melanoma, pilocytic astrocytoma, and some colon cancers (particularly nonhereditary tumors with microsatellite instability).

Testing for *BRAF* mutations is relatively straightforward and can be done using a variety of sequencing-based and PCR-based approaches. Perhaps the most commonly used method is a DNA sequencing assay, which includes PCR of exon 15, followed by sequencing of the PCR product to detect the point mutation. This assay is robust and usually simple to interpret, but lacks sensitivity in mixed tumor cell populations. Another approach is to perform allele-specific PCR, which has the added advantage of better sensitivity in mixed populations. Finally, other methods, including single base extension sequencing, pyrosequencing, probe-dissociation testing, and some kit-based assays have been described.

Although *RET/PTC* rearrangements and *RAS* mutations do not have known prognostic significance, the *BRAF* mutation is associated with more aggressive tumor features, including extrathyroidal extension and lymph node metastases [14, 15]. Several drugs that target the *BRAF* gene are currently in clinical trials for treating melanomas (which also harbor *BRAF* mutations) [16]. The potential for targeted therapy has stimulated interest for potentially treating aggressive PTC, particularly those that do not respond to traditional approaches using radioactive iodine therapy.

Multiple Choice Questions

1. Abnormalities of *BRAF* are commonly seen in the following tumors
   A. Melanoma
   B. Papillary thyroid carcinoma
   C. Pilocytic astrocytoma
   D. None of the above
   E. All of the above
2. When comparing *BRAF* and *RAS* mutation analysis in thyroid tumors
   A. Both mutations are equally prevalent in all thyroid lesions
   B. *BRAF* is more common in papillary lesions, *RAS* is more common in follicular lesions
   C. *RAS* mutation analysis is useful in distinguishing benign and malignant follicular thyroid tumors

D. Testing for *RAS* and *BRAF* can reliably distinguish between follicular and papillary thyroid tumors
E. The value of *BRAF* and *RAS* testing is diagnostic but has no impact on prognosis

3. *RET/PTC* rearrangement in thyroid tumors
   A. Is commonly found in both papillary and follicular thyroid tumors
   B. Is never associated with radiation exposure
   C. Is a single mutation with two known partner genes
   D. Is the most common molecular abnormality in papillary thyroid cancers
   E. Suggests poor prognosis in patients with papillary thyroid cancer
4. Papillary carcinoma of the thyroid
   A. Is an uncommon malignancy with poorly understood molecular biology
   B. Is associated with a poor prognosis
   C. Is not associated with radiation
   D. Is treated with surgery alone
   E. None of the above
5. What is the advantage of doing multiplex assays for tumors?
   A. Diagnosis is enhanced, because most tumors are defined by a typical mutation profile that has little variability from patient to patient
   B. Laboratory operations can be improved because it allows for a single tube reaction to assess multiple genes at the same time
   C. Quality control is better in multiplex assays, because if one gene is mutated and others are not, it suggests a technical problem
   D. With multiplex molecular testing, there is no need for microscopic analysis
   E. All of the above

Answers to Questions Embedded in the Text

Question 1: What is the differential diagnosis? Which clinical tests could narrow the differential diagnosis?

The patient has a complex medical and oncological history. Prostate adenocarcinoma rarely metastasizes to the lungs and a slowly increasing PSA would not suggest widely metastatic disease. However, PSA screening is neither specific nor sensitive for metastatic prostatic adenocarcinoma. The patient had a prior papillary thyroid carcinoma metastatic to the lung and therefore already has defined aggressive thyroid carcinoma. Based on the imaging studies, another primary cancer such as lung carcinoma must be included in the differential, despite the patient's remote smoking history. Fine needle aspiration of the superficial neck nodule could be performed, with the added advantage that an office-based procedure with immediate cytopathologist interpretation could yield preliminary results within minutes. The best initial approach for the lung lesion would include bronchoscopy with a biopsy for morphologic interpretation.

Question 2: Is this an appropriately ordered test?

The morphologic pattern seen on histology did not correlate well with either of the patient's known primary tumors (prostate or thyroid). Both imaging and biopsy results were more suggestive of a new primary lung carcinoma, with probable squamous differentiation. However, given the history of aggressive papillary thyroid carcinoma and the fact that papillary carcinomas can display squamous metaplasia or squamoid anaplastic de-differentiation, the possibility of metastasis should also be strongly considered. This differential diagnosis was explored using immunohistochemical staining. Unfortunately, the staining profile was fairly nonspecific and did not strongly differentiate these two possibilities. A molecular approach was subsequently undertaken. *EGFR* mutations are seen in lung carcinomas, though usually are associated with adenocarcinomas in a unique patient population (younger patients, nonsmokers, and females). Typical smoking-associated lung cancers can also harbor *RAS* gene mutations. *BRAF* and *RAS* gene abnormalities are commonly seen in thyroid tumors. *BRAF* is rarely present in lung carcinomas, and those that have this mutation show features of micropapillary adenocarcinoma. Primary squamous cell carcinoma of the lung would be very unlikely to have *BRAF* point mutations.

Question 3: Does the test result provide a definitive diagnosis?

Presence of the *BRAF* mutation makes the diagnosis of a primary lung squamous cell carcinoma less likely. However, to further strengthen the suggestion of a metastasis from the papillary thyroid carcinoma, it is helpful to compare the current sequencing results from the lymph node in the neck and the lung biopsy, with

the molecular profile of the primary thyroid tumor and the previous lung metastasis. The presence of the same mutation is very good evidence that this represents the same tumor, despite the morphologic differences with the more poorly differentiated squamous features. Molecular studies alone cannot establish a definitive diagnosis in this case. Correlation with the clinical scenario, radiologic studies, and, most importantly, the morphologic and immunohistochemical staining profile is essential.

Answers to Multiple Choice Questions

1. *The correct answer is E.*

BRAF abnormalities are commonly seen in melanoma, papillary thyroid carcinoma, pilocytic astrocytoma, a subset of colon carcinomas, and rare head and neck cancers. *BRAF* is most commonly activated via the described point mutation. However, other mechanisms such as chromosomal 7q34 tandem duplication leading to a *KIAA1549* and *BRAF* fusion gene, as well as a three base pair insertion (TAC) at codon 598 in *BRAF*, which leads to an additional threonine residue inserted near the mutational hotspot valine at position 600 (p.A598_T599insT), have also been described. These alternative mutations are particularly typical for pilocytic astrocytomas [17, 18].

2. *The correct answer is B.*

RAS mutations can be observed in both follicular and papillary tumors and therefore cannot be used reliably to differentiate between them. However, *RAS* mutations are much more common in follicular-derived tumors and are only seen in a small subset of papillary carcinomas. The *RAS* gene can also be mutated in benign follicular thyroid lesions and is therefore not a practical diagnostic tool for predicting malignancy. *BRAF* mutations are the more common change in papillary carcinoma.

3. *The correct answer is D.*

RET/PTC rearrangement is the most common molecular aberration in papillary thyroid carcinomas, but not in follicular carcinomas. PTC stands for "papillary thyroid carcinoma" a group of at least ten genes that can rearrange with the *RET* gene on chromosome 10. *RET/PTC* rearrangement does not have clear prognostic significance. While *RET/PTC* can be present in spontaneous tumors, the mutation has been associated with previous radiation exposure, in the setting of both therapeutic radiation (particularly when children are exposed to radiation) and in the setting of nuclear accidents.

4. *The correct answer is E.*

None of the answers is correct. Papillary thyroid carcinoma is the most common malignant tumor of the thyroid and its underlying molecular changes are relatively well understood. Previous radiation exposure has been well documented to be causative in the development of PTC. These tumors most often present as a localized nodule. Although primary therapy includes surgery with total thyroidectomy, most patients are also treated with radioablation using radioactive iodide. The prognosis is generally recognized to be excellent for patients with papillary carcinoma, particularly when features of aggressive disease are absent.

5. *The correct answer is B.*

The main advantage of a multiplex assay is the convenience for laboratory operations to evaluate multiple genes and their mutations simultaneously, with less hands-on time and lower cost. Although many tumors have characteristic or common mutations, tumorigenesis is often polygenic and may not yet be entirely understood. The diagnosis of any tumor is often not possible based on only the mutation profile. Molecular tests are best used to support or confirm a pathology diagnosis, and should not typically be seen as a replacement for morphology. All assays should be designed with adequate quality controls; the presence or absence of mutations should not serve as a quality control element, except in the context of known positive and negative samples that are run in the assay as designated control specimens.

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Renal Cell Carcinoma

25

Federico A. Monzon

Clinical Background

A male in his 50s with a history of prostate cancer presented for follow-up one year after prostatectomy. The follow-up computed tomography (CT) scan of the abdomen revealed a left renal solid enhancing mass. Under the clinical diagnosis of a renal neoplasm suspicious for malignancy, a left robotic-assisted partial nephrectomy was performed. Grossly, the tumor was confined to the kidney, measured 4.0 cm in greatest dimension and showed a homogenously tan-yellow cut surface with friable tissue and necrosis. Microscopically, the tumor demonstrated cells arranged in closely packed tubules and focal papillary architecture (Fig. 25.1a). Tumor cells displayed uniform, round to oval nuclei (Fuhrman nuclear grade 2) and had scant eosinophilic cytoplasm. Foamy macrophages and scant extracellular mucin were noted (Fig. 25.1b). No lymphovascular invasion was identified. The tumor was reported as Renal Cell Carcinoma, not otherwise specified.

Question 1: What is the differential diagnosis?

Reason for Molecular Testing

The differential diagnosis, based on the morphological features of this tumor, included papillary renal cell carcinoma (pRCC), type 1, solid variant, versus a mucinous tubular and spindle cell carcinoma of the kidney (MTSCC). Both tumors can display tubular and papillary architecture along with the presence of mucin [1]. MTSCC tumors commonly contain spindle cell areas but these are not a requirement for diagnosis. The immunohistochemical (IHC) profiles for these tumors are quite similar and therefore cannot be used to arrive at the diagnosis in this case [2]. However, the chromosomal profiles for these two tumors are quite different: pRCC frequently shows trisomies of chromosomes 7 and 17 whereas MTSCC is characterized by losses of multiple chromosomes (Fig. 25.2) [3]. Given that pRCC is the second most aggressive renal tumor (after clear cell RCC) with a known capacity to develop metastasis, and that MTSCC is a tumor of low malignant potential, establishing a definite diagnosis has important implications for patient management.

Test Ordered

Molecular methods to evaluate chromosomal abnormalities in morphologically challenging renal tumors have emerged as a reliable alternative to IHC and conventional cytogenetics [4, 5]. Recently, microarray-based tools have been developed that provide high-resolution, genome-wide assessment of tumor genomes and reveal chromosome copy numbers. These array-based copy number platforms (a.k.a. virtual karyotyping or array-based karyotyping) can be employed to detect chromosomal imbalances in renal cell tumors [5]. Given the uncertainty of the diagnosis in our patient, a virtual karyotype was ordered

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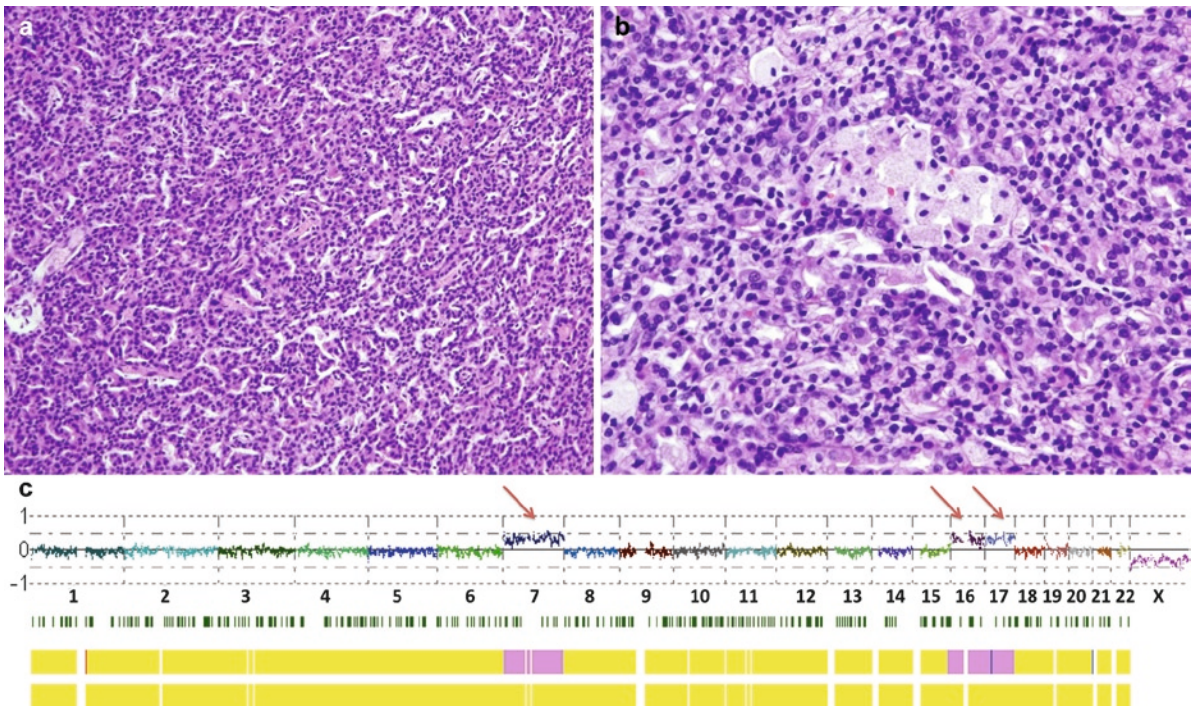

Fig. 25.1 Morphologic characteristics and chromosomal profile of the renal tumor. (**a**) Tumor cells arranged in closely packed tubules (10X). (**b**) 20X view of tumor cells showing scant eosinophilic cytoplasm, foamy macrophages, and extracellular mucin. (**c**) Whole genome view of virtual karyotype from this case. The uppermost plot represents the estimated copy number as a log 2 ratio averaged over 30 SNPs; green bars represent heterozygote calls (AB calls), the third bar represents a color-coded Hidden Markov Model (HMM) for copy number (*yellow* copy number 2, *pink* copy number 3, *aqua* copy number 1), and the bottom bar is a color-coded HMM for LOH (*yellow* no LOH, *blue* LOH). Note the trisomy of chromosomes 7, 16, and 17 (*arrows*)

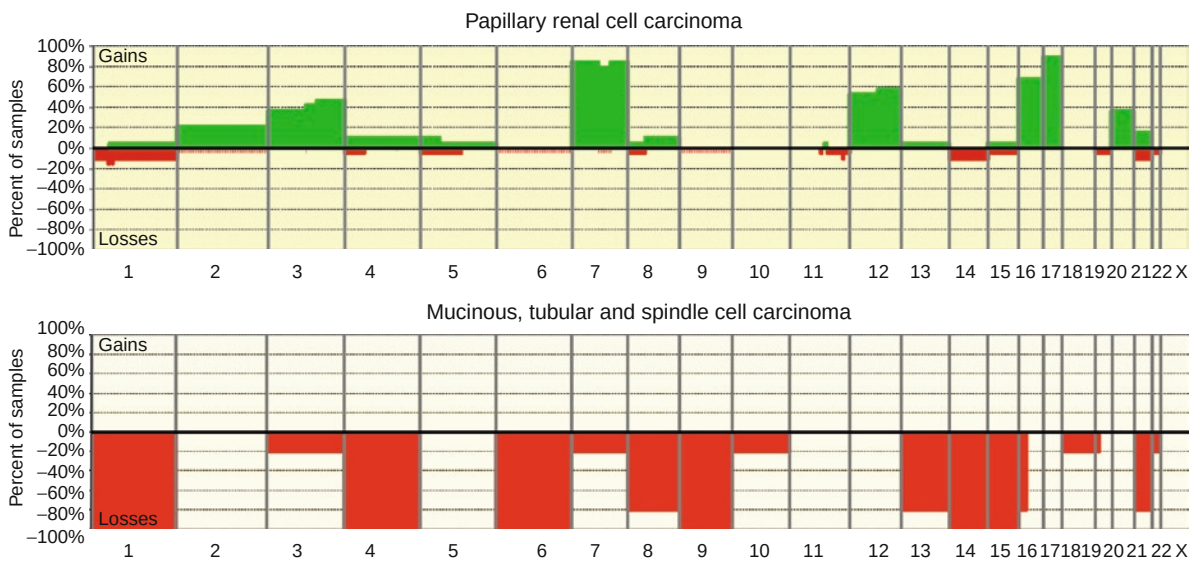

Fig. 25.2 Chromosomal gains and losses in pRCC and MTSCC. Cumulative frequency of chromosomal lesions in papillary renal cell carcinoma (*Top*) and mucinous, tubular, and spindle cell carcinoma (*Bottom*). Gains are indicated as positive values (*green*) and losses as negative values (*red*)

and performed with a 10 K Xba SNP Microarray (Affymetrix, Santa Clara, CA).

Question 2: What is the advantage of a whole genome test?
Question 3: What are the limitations and advantages of array-based approaches for chromosome copy number analysis?

Laboratory Test Performed

Several techniques have been utilized for genome-wide scanning of chromosomal imbalances in renal tumors, including comparative genomic hybridization (CGH), array CGH, and SNP arrays. Array CGH has been used to accurately classify RCCs that were difficult to distinguish by histologic type, based on specific genetic alterations [6]. A limitation of array CGH, however, is that it cannot detect regions of copy neutral loss of heterozygosity (LOH) or acquired uniparental disomy (aUPD) which has been reported to constitute 50–80% of the LOH in human cancers [7]. Array CGH also shows poor performance with formalin-fixed, paraffin-embedded (FFPE) samples [8]. SNP arrays are similar to array CGH in that genomic DNA is amplified, labeled, and applied to an array that contains oligonucleotide probes optimized to identify alleles at specific SNP loci. As with all array technologies, the genome can be represented on the array at different resolutions (from 10,000 to ~1 million SNP probes). An advantage of SNP arrays is that, in addition to copy number information, they can also provide genotypes at each SNP locus, which can then be used to determine regions of LOH/aUPD. Most of the most recent designs for SNP arrays actually combine SNP and copy-number-only probes, in order to increase genomic representation of SNP poor regions for copy number assessment. One of the advantages of SNP arrays includes the ability to use either fresh or FFPE tissues [9]. SNP arrays have been used successfully to detect chromosomal copy number variations in several types of cancer, including RCC [5]. Virtual karyotyping by one of these methods has the ability to classify morphologically challenging renal tumor cases up to 90% of the time [3, 10].

An important parameter to keep in mind when performing array-based karyotyping is the selection of areas to be tested. Areas with high tumor content must be chosen for DNA extraction. Contamination with nonneoplastic elements, such as inflammatory or stromal cells, will dilute the signal of clonal chromosomal aberrations. When the samples used are tumor resections and large needle core biopsies, one can easily obtain adequate amounts of DNA to perform virtual karyotypes on SNP arrays.

Results with Interpretation Guideline

Data acquired from array-based karyotyping are usually in the form of signal intensities for each of the probes included in the array. These signals need to be transformed to copy number and allele data (in the case of SNP arrays). LOH and copy number estimates (the log 2 ratio of test to reference samples) were obtained using a publicly available analysis package, Copy Number Analyzer for Affymetrix GeneChip arrays (CNAG 3.0) [11]. This software allows for the use of non-paired references, which eliminates the need of normal tissue from the same patient to obtain normalized copy number data. The quality control parameters evaluated in each run, to determine adequate assay performance, are: a signal detection rate (the percentage of features in the array that show adequate fluorescence intensity) above 95%, a SNP call rate (rate of successful allele identification) above 85%, and a standard deviation (SD) of log 2 ratios across the array under 0.6 [12].

The virtual karyotype exhibited gains of chromosomes 7, 16, and 17 (Fig. 25.1c). The pattern of chromosomal gain/loss was compared to the patterns of genomic changes typically observed in pRCC and MTSCCs (Fig. 25.2) [3]. The most frequent chromosomal imbalances observed with array-based karyotyping can be obtained from the scientific literature and some online databases (Table 25.1). However, a comprehensive resource linking this information to clinical significance is not yet available, although some of the databases do include clinical correlations. Note that the apparent one copy loss of the X chromosome (Fig. 25.1c) reflects the fact that this sample belongs to a male patient (XY). This can be used as a quality control measure. Although most SNP arrays do not include probes for the Y chromosome, it is also not advisable to use the Y chromosome as a control, because it is frequently lost in several malignancies.

Table 25.1 Most frequently used databases for chromosomal alterations in cancer

Database	Creator	URL
ACTuDB: A database for the integrated analysis of array-CGH and clinical data for tumors	Institut Curie Paris, France	http://bioinfo-out.curie.fr/actudb/index.php
Atlas of Genetics and Cytogenetics in Oncology and Haematology	University Hospital Poitiers, France	http://atlasgeneticsoncology.org/index.html
Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer	National Cancer Institute Bethesda, USA	http://cgap.nci.nih.gov/Chromosomes/Mitelman
Online CGH Tumor Database	Institute of Pathology University Hospital Charité Berlin, Germany	http://amba.charite.de/~ksch/cghdatabase/index.htm
Progenetix – Genomic Profiling in Cancer	University of Zurich Zurich, Switzerland	http://www.progenetix.net/index.shtml

Thus, given the fact that interpretation of virtual karyotyping in cancer has to take into account issues of tumor representation in the specimen, expected chromosomal imbalances, and the clinical significance of the specific chromosomal abnormalities, it should only be interpreted with deep understanding of the technical aspects of array-based copy number analysis, significant experience with this technique, and knowledge of the specific disease entity.

Result Interpretation

The pattern obtained for this tumor matched the pattern seen in pRCC, with common gains of chromosomes 7, 16, and 17. Thus, based on this assay, the patient was diagnosed with pRCC. The diagnosis issued, based on morphology on this case, would be categorized as an "unclassified" RCC, based on the World Health Organization (WHO) classification of renal neoplasms [13]. Unfortunately, the usage of the term "unclassified" renal cell carcinoma carries significant prognostic implications that do not apply to the majority of morphologically non-classifiable renal tumors [14]. Thus, efforts to provide classification for these tumors are important for directing adequate patient care. In the case presented herein, the morphologic and immunohistochemical features had narrowed the differential diagnostic possibilities for this tumor to a pRCC versus MTSCC diagnosis. The distinction between these two entities is clinically significant, because one of them is a malignant tumor with recognized metastatic potential and the other one a tumor of low malignant potential. The chromosomal profiles from pRCC and MTSCC are quite different, with pRCC showing trisomies that readily allow distinction from an MTSCC's profile. Gain of the p arm of chromosome 1 has been associated with higher mortality in pRCC [15]. Therefore, the absence of this genetic lesion in this patient can be used as a good prognostic factor. Although appearance of additional chromosomal abnormalities has been reported with progression of clear cell and chromophobe RCCs to sarcomatoid tumors [16, 17], this has not been shown in pRCC. In addition, progression of renal tumors is usually evaluated by imaging techniques and tumor tissue is generally not available for testing in patients with metastatic tumors.

Further Testing

No further testing was recommended at this time. However, pRCC can present within the hereditary papillary renal cell carcinoma (HPRC) and hereditary leiomyomatosis renal cell carcinoma (HLRCC) syndromes [18]. HPRC is characterized by the presence of multifocal and bilateral pRCCs and is caused by mutations in the *MET* proto-oncogene (hepatocyte growth factor receptor). Mutations in the *MET* gene result in ligand-independent tyrosine kinase activation leading to downstream activation of the hepatocyte growth factor (HGF) pathway. HLRCC is a syndrome characterized by cutaneous and/or uterine leiomyomas and pRCC. It is caused by a germline mutation in the fumarate hydratase gene (*FH*) located on chromosome

1. Therefore, if a second pRCC were to be detected in this patient, or if other family members develop pRCC and/or multiple cutaneous or uterine leiomyomas, especially at a young age, an investigation for a possible familiar variant of pRCC should be performed, including genetic counseling and testing for *MET* and/or *FH* mutations.

Background and Molecular Pathology

Each morphologic subtype of renal cell tumors has characteristic recurrent chromosomal abnormalities [19]. Clear cell RCCs show deletions in the short arm of chromosome 3. pRCCs usually display trisomies of chromosomes 7 and/or 17 with or without loss of the Y chromosome. Chromophobe RCCs have a hypodiploid chromosomal complement, with monosomies of chromosomes 1, 2, 6, 10, 13, 17, and 21. Oncocytomas (OC) can show a normal diploid karyotype or copy number alterations in chromosomes 1 and Y, along with translocations involving 11q13. Given the recurrent nature of these chromosomal imbalances, they are being used as a diagnostic aid in the classification of renal tumors [4, 5].

Ancillary studies such as IHC, while reliable when evaluating renal tumors with classic morphology, often fail to unequivocally categorize morphologically challenging renal tumors. As discussed in this case, IHC staining patterns fail to resolve pRCC from MTSCC [1, 2]. Molecular methods used to evaluate chromosomal abnormalities in renal tumors have recently emerged as a reliable alternative to IHC and conventional cytogenetics. FISH has been successfully used to differentiate between specific renal tumor subtypes [4]. However, results from interphase FISH on formalin-fixed, paraffin-embedded (FFPE) tissues are limited by the presence of necrosis and/or the sectioning of nuclei [20]. In addition, FISH on 5-μm sections of FFPE tissue frequently underestimates chromosomal anomalies when compared to the analysis of entire nuclei. This artifact may give an underestimation of trisomies and an overestimation of monosomies if the dimensions of the analyzable nuclei are not correctly evaluated [20]. An additional limitation of FISH is the low genomic coverage; in the majority of cases, multiple FISH probes are required to differentiate between different renal tumor subtypes, thus significantly increasing the cost associated with this testing.

Recently, microarray-based tools have been developed that provide high-resolution, tumor genome-wide chromosome copy number assessment. These array-based copy number platforms, such as array-based comparative genomic hybridization (aCGH) or SNP arrays, can be employed to detect chromosomal imbalances in renal cell tumors. SNP arrays provide chromosome copy number data and can also provide genotypes, which can be used to determine regions of LOH that would go undetected by aCGH and FISH. Although the clinical significance of copy neutral LOH has not been well established, copy neutral LOH in 17p has been associated with homozygous mutations in *TP53* and conferred significantly shorter survival times in patients with glioblastoma [21]. It is also important to understand the limitations of array-based technologies: aCGH and SNP arrays are unable to detect tetraploidy with certainty (although certain features of virtual karyotypes created with SNP arrays can suggest tetraploidy), and they cannot detect inversions or balanced translocations. In the context of renal tumors, this is consequential because virtual karyotyping cannot be used to identify the presence of translocations involving Xp11.2 or t(6; 11)(p21; q12). These translocations define a specific group of renal neoplasms characterized by translocations involving the microphthalmia transcription factor (*MiTF*)/transcription factor E (TFE) family, which usually present in children and young adults [22].

Correct classification of renal tumors is critical for an accurate diagnosis and to provide information for prognosis and patient management, including eligibility to clinical trials of new targeted therapies. Renal cell tumor subtypes can be reliably distinguished by their patterns of chromosomal gains and/or losses. Virtual karyotyping with SNP arrays has proven to be a useful ancillary study for the diagnosis of morphologically challenging renal tumors. An advantage to the use of whole genome chromosomal analysis is the ability to observe additional chromosomal imbalances beyond those used for subtype classification. In ccRCC, loss of 14q has been associated with high-grade tumors, advanced disease, and poor prognosis [23]. Loss of 9p has also been associated with high grade/stage ccRCC and was recently identified as an independent prognostic factor in multivariate analysis for survival [24, 25]. In pRCC, gain of 1p has been associated with higher mortality [15].

Multiple Choice Questions

1. Which of the following statements about renal cell tumors is correct?
   A. Clear Cell RCC is characterized by gain of chromosomes 3, 9, and 14
   B. Each subtype of renal tumor is characterized by specific recurrent chromosomal abnormalities
   C. Mucinous, tubular, and spindle cell carcinoma shows no chromosomal imbalances
   D. Papillary RCC is characterized by loss of chromosomes 7 and 17
   E. Unclassified renal tumors have a relatively good prognosis
2. Limitations of fluorescence *in situ* hybridization (FISH) are:
   A. Limited genomic coverage
   B. Underestimation of genomic lesions in FFPE tissues due to sectioning of nuclei
   C. Whole genome coverage
   D. All of the above
   E. A and B
3. Which of the following is NOT a characteristic of virtual/array-based karyotyping:
   A. Ability to work with fresh or FFPE tissues
   B. Detection of balanced chromosomal translocations
   C. Detection of chromosomal gains and losses
   D. LOH/UPD detection with SNP arrays
   E. Whole genome coverage
4. Classification of morphologically challenging renal tumors is:
   A. An academic exercise
   B. An attempt by molecular pathologists to displace surgical pathology
   C. Important for diagnostic purposes and for patient management
   D. Important for diagnostic purposes but not for patient management
   E. Not important at all
5. Virtual (array-based) karyotyping can be a useful test to confirm the diagnosis of all these renal tumors, except:
   A. Eosinophilic renal cell tumors (clear cell and chromophobe RCCs)
   B. Oncocytic renal cell tumors (oncocytoma and chromophobe RCC)
   C. Papillary tumors (papillary RCC, clear cell papillary RCC, and MTSCC)
   D. Spindle cell tumors (papillary RCC and MTSCC)
   E. Xp11.2 translocation carcinomas

Answers to Multiple Choice Questions

1. *The correct answer is B.*

Each morphologic subtype of renal cell tumors has characteristic recurrent chromosomal abnormalities. Clear cell RCCs show deletions in the short arm of chromosome 3, frequent gains of chromosomes 5 and 7, and frequent loss of 9 and 14. Papillary RCCs usually display trisomies of chromosomes 7 and/or 17 with or without loss of chromosome Y, as well as frequent gain of chromosomes 12 and 16. Chromophobe RCCs carry monosomies of chromosomes 1, 2, 6, 10, 13, 17, and 21. Oncocytomas can have a normal diploid karyotype or carry copy number alterations in chromosomes 1 and Y, along with translocations involving 11q13.

2. *The correct answer is E.*

Limited genomic coverage and underestimation of genomic lesions in FFPE tissues due to sectioning of nuclei are limitations of FISH. One of the advantages of FISH is the ability to detect translocations.

3. *The correct answer is B.*

Virtual (array-based) karyotyping cannot detect balanced chromosomal translocations.

4. *The correct answer is C.*

Correct classification of renal tumors is critical for an accurate diagnosis and to provide information for prognosis and patient management, including eligibility for clinical trials of new targeted therapies.

5. *The correct answer is E.*

Virtual (array-based) karyotyping can be a useful test to confirm the diagnosis of most renal tumors, except for those characterized by balanced chromosomal translocations. This type of genetic lesion is NOT detectable by current array designs, although new array designs capable of detecting specific translocations have already been proposed to address this shortcoming.

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Gastrointestinal Stromal Tumor

26

Jennifer Laudadio

Clinical Background

A 60-year-old female initially presented to a local urgent care center complaining of abdominal pain. Ultimately, an abdominal CT scan was obtained revealing a large upper abdominal mass of uncertain origin. The patient was referred to the regional academic medical center for possible surgical intervention. On evaluation, she complained of a two to three month history of early satiety, bloating, nausea, and intermittent abdominal pain. Her past medical history was significant for hypertension and hyperlipidemia. Her mother had been diagnosed with breast cancer at 62 years old, and there was no other family history of cancer. On physical examination, a left upper quadrant mass was palpable without associated tenderness. Review of the outside imaging confirmed a heterogeneous upper abdominal mass measuring 20 × 17 cm. The origin of the tumor was difficult to ascertain but was considered possibly pancreatic or retroperitoneal. Laboratory data showed normal carbohydrate antigen 19-9 (CA19-9), carcinoembryonic antigen (CEA), and alpha-fetoprotein (AFP) levels. An image-guided percutaneous needle biopsy was scheduled.

Pathologic evaluation revealed a hypercellular spindle cell neoplasm with mild nuclear atypia and scattered mitotic activity (Fig. 26.1a). By immunohistochemistry, the tumor cells were positive for vimentin, CD34, and CD99. CD117 (KIT) was strongly and diffusely positive (Fig. 26.1b). The tumor was negative for desmin, smooth muscle actin, S100, pan-melanoma, pan-keratin, EMA, and CD45.

Question 1: Based on these findings, what is your diagnosis?

A diagnosis of gastrointestinal stromal tumor (GIST) was rendered, and the patient was taken for surgical resection of the mass with the possibility of needing a distal pancreatectomy, nephrectomy, partial colectomy, and/or partial gastrectomy. At the time of surgery, no evidence of metastatic disease was identified in the abdominal cavity. As resection proceeded, it became evident that the tumor was arising from the posterior gastric wall. The mass was also adherent to the spleen and the tail of the pancreas. Ultimately, the mass was resected with a partial gastrectomy, distal pancreatectomy, and splenectomy. No gross residual disease remained.

Upon gross examination, the mass measured 28 × 21 × 14 cm and was attached to the stomach, pancreas, and spleen. The neoplasm was solid, cystic, and well-circumscribed. Serial sectioning revealed hemorrhage and necrosis. The mass involved the gastric submucosa, but no mucosal involvement was identified. Representative histological sections were submitted and confirmed the diagnosis of GIST arising in the gastric wall. Invasion of the pancreatic or splenic parenchyma was not identified histologically. The tumor had spindle cell morphology and, in areas, a myxoid background (Fig. 26.1c). Moderate nuclear pleomorphism was identified, and mitotic activity averaged 10 per 50 high-power fields. Margins were negative.

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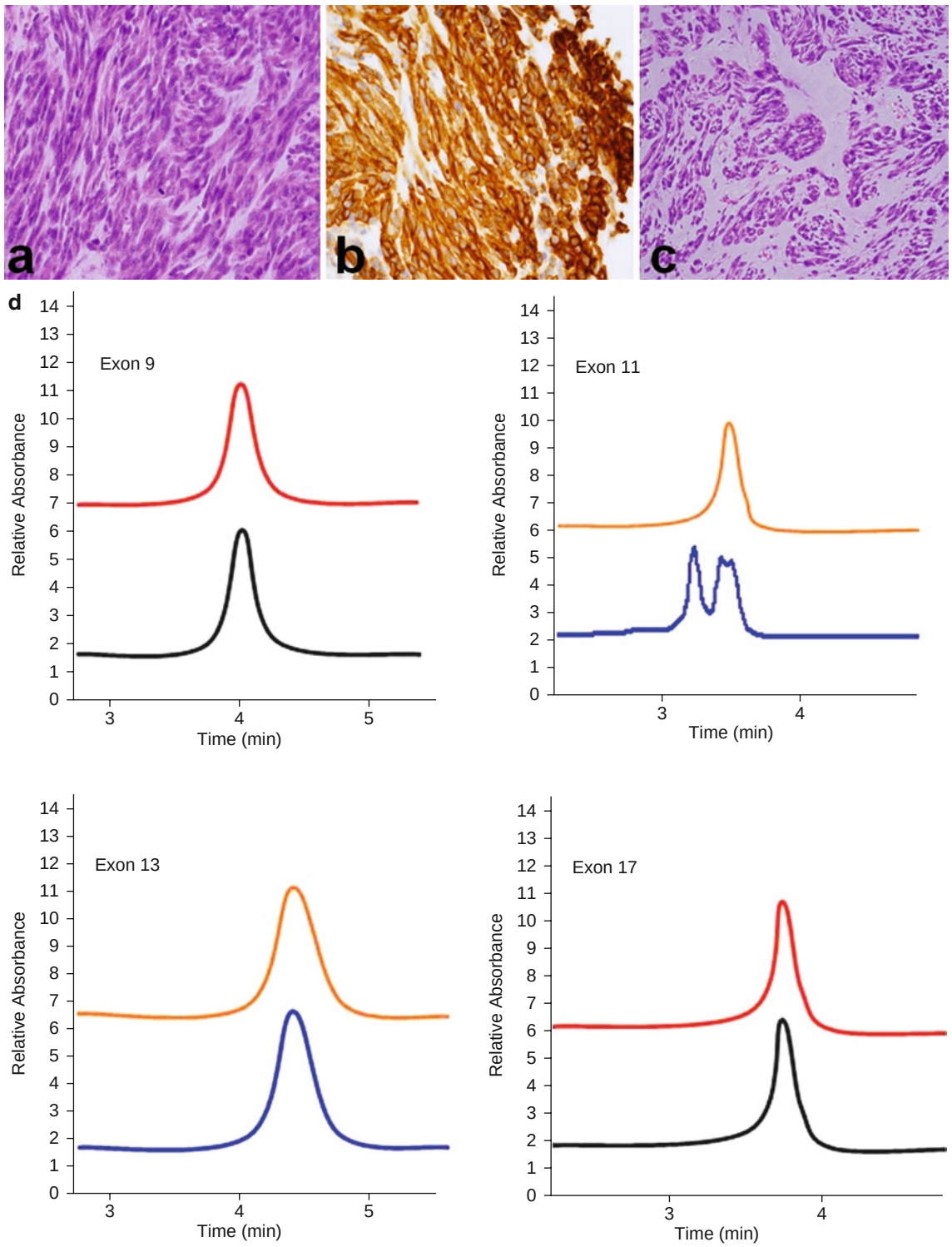

Fig. 26.1 *KIT* exon 9, 11, 13, and 17 dHPLC chromatograms from a spindle cell GIST. (**a**) Spindle cell neoplasm with mitotic activity. (**b**) Strong and diffuse KIT expression detected by immunohistochemistry. (**c**) Low power view showing the myxoid background. (**d**) Chromatograms for *KIT* exons 9, 11, 13, and 17. For each exon, the patient sample is the bottom curve and the wild-type control is the top curve

The patient did well postoperatively and was discharged after seven days. Due to the tumor size and mitotic activity, the neoplasm was considered to have a high malignant potential. The patient was referred to oncology for clinical follow-up.

Reason for Molecular Testing

This patient is at a high risk of tumor recurrence and future metastasis. Based on the results of a study of more than 1,000 gastric GISTs, 86% of patients with tumors larger than 10 cm with more than five mitoses per 50 high-power fields had progressive disease [1]. However, adjuvant treatment with imatinib mesylate has been shown to significantly improve recurrence-free survival following primary resection of localized GIST [2]. Based on this information, the oncologist recommended treatment with imatinib. He contacted the pathologist and requested mutation analysis to help guide treatment and further delineate prognosis.

Question 2: Which gene (or genes) is the oncologist interested in testing and how may the results impact therapy?
Question 3: Do the immunohistochemical results negate the need for mutation analysis?

Test Ordered

KIT mutation analysis was ordered, specifically looking for mutations in exons 9, 11, 13, and 17. Reflex testing for platelet-derived growth factor receptor alpha (*PDGFRα*) exon 12 and 18 mutations was requested if *KIT* mutation analysis was negative.

Question 4: Was it appropriate to request the tests in this manner, or should PDGFRα mutation testing be performed regardless of the KIT result?

Laboratory Test Performed

KIT mutation testing was performed using denaturing high-performance liquid chromatography (dHPLC), and Sanger sequencing was performed to confirm any abnormal findings. Unstained slides from a representative section of the tumor were submitted from a formalin-fixed, paraffin-embedded block. Comparison to a hematoxylin and eosin-stained slide was used to identify tumor tissue on the unstained slides. A sterile scalpel blade was used to scrape tissue into a microcentrifuge tube. This "macro-dissection" step protected against a false-negative result due to contamination with excess non-tumor cells. Tissue was deparaffinized and DNA manually extracted from the sample. DNA yield was determined by measuring the absorbance at 260 nm, and purity determined from the absorbance at 260/280 nm.

DNA was amplified using previously published primers for *KIT* exons 9, 11, 13, and 17 [3]. Each exon was amplified in a separate tube using 0.1 μg of DNA. Negative and wild-type controls were included for each reaction. Amplification was confirmed by agarose gel electrophoresis. Aliquots of PCR products were then scanned for mutations at two different partially denaturing temperatures using a Transgenomic WAVE dHPLC system (Transgenomic Inc, Omaha NE). Any abnormal chromatogram pattern was interpreted as positive, and sequencing was then performed on the same PCR product to confirm the presence of a mutation. When interpreting the confirmatory *KIT* sequence, any detected mutation was compared to known single nucleotide polymorphisms (SNPs) using the National Center for Biotechnology (NCBI) database of genetic variation.

The WAVE dHPLC system is an ion-pair, reverse-phase, high-performance liquid chromatography method capable of comparing two alleles by separating heteroduplex and homoduplex DNA fragments. In the presence of a heterozygous mutation, heating and cooling of the PCR products will result in annealing of the mutant strand to the complementary wild-type strand, forming a heteroduplex. The heteroduplex fragment is formed in addition to mutant and wild-type homoduplexes. Under partially denaturing temperatures, the WAVE dHPLC chromatography has different retention times for the heteroduplex and homoduplex fragments. Mutations can be delineated with run times of just 5 to 6 minutes. Scanning for mutations using dHPLC eliminates the need to sequence all implicated exons. Confirmatory sequencing is necessary to identify the specific mutation present in the sample.

An alternative testing strategy would be to sequence each exon without first performing mutation scanning. However, dHPLC is faster, less labor intensive, less expensive, and able to detect lower levels of mutant alleles [3, 4]. Denaturing HPLC has been shown to have a sensitivity of 100% as compared to traditional sequencing for *KIT* mutations [4]. However,

interpretation subjectivity remains even with dHPLC because some pattern changes may be very subtle. All subtle but suspicious findings should therefore be sequenced for confirmation. Using more than one partially denaturing temperature is also important because a small percentage of mutations are detectable at only one of several temperatures [4]. A disadvantage of using dHPLC is that the presence of homozygous mutations, which are rare but do occur, cannot be detected, leading to false-negative results.

High-resolution melting temperature analysis of PCR amplicons is an alternative method to scan for mutations in *KIT* and *PDGFRα*. Similar to dHPLC, this methodology differentiates heteroduplexes from homoduplexes. On high-resolution melting temperature analysis, the melt curve of the heteroduplex fragments will vary from the homoduplex fragments because of the base pair mismatch(es). Like dHPLC, this technique has been shown to have 100% sensitivity when compared to sequencing [5]. Because run times are only about 2 minutes, high-resolution melting analysis has the advantage of rapid turnaround time. Also, the risk of contamination is limited as the PCR and subsequent melting analysis are performed in the same vial. Both dHPLC and high-resolution melt analysis require specialized instrumentation.

Results with Interpretation Guideline

The chromatograms for *KIT* exons 9, 11, 13, and 17 at a single temperature are shown in Fig. 26.1d. For each exon, the chromatogram obtained from the second temperature is not shown but revealed consistent findings. In each chromatogram, the pattern generated from the wild-type control is on top and the patient sample is on the bottom.

Question 5: Is there indication of a KIT mutation? If so, in which exon(s)?

Result Interpretation

The results indicate a mutation in *KIT* exon 11. Based on the chromatogram data, there is no evidence of a mutation in exon 9, 13, or 17. In GISTs, mutations in *KIT* exon 11 are the most common. The location of the mutation in exon 11 correlates with the gastric location and spindle cell morphology found in this case.

Further Testing

Follow-up sequencing of exon 11 was performed to confirm the presence of a mutation and to identify the specific mutation. In this case, sequencing confirmed a deletion of nucleotides 1687–1701 (c.1687_1701del) based on GenBank reference sequence X06182. This deletion corresponds to codons 556–560 (p.Gln556_Val560del).

Other Considerations

Mutations of *KIT* and *PDGFRα* are known to be mutually exclusive of one another and ordering *PDGFRα* testing as a reflex for cases found to be *KIT* wild-type is appropriate. In this case, with a mutation confirmed in *KIT* exon 11, testing of *PDGFRα* exons 12 and 18 is not necessary.

Background and Molecular Pathology

Gastrointestinal stromal tumors are the most common mesenchymal tumor of the gastrointestinal tract and arise from the interstitial cells of Cajal. GISTs most frequently are located in the stomach or small intestines but also occur in the esophagus, rectum, omentum, and mesentery, among other locations. Both *KIT* and *PDGFRα* mutations have been identified in GISTs, and mutations are invariably in-frame. Both genes encode type III transmembrane receptor tyrosine kinases. Ligands for KIT and PDGFRα are stem cell factor and PDGF, respectively. Ligand binding results in phosphorylation of tyrosines in the kinase domains, leading to activation of downstream signaling pathways. Mutations result in constitutive activation of the kinases in the absence of ligand binding.

The majority of GISTs (60–85%) are associated with *KIT* mutations [4, 6–8]. GIST-associated mutations have been identified in exons 9, 11, 13, and 17, which encode the extracellular, juxtamembranous, tyrosine kinase 1, and tyrosine kinase 2 domains of the KIT protein, respectively. Exon 11 mutations are the most frequent with c.1690_1695delTGGAAG (p.Trp557_Lys558del) being most common [9, 10]. More than 90 exon 11 mutations have been described and consist of insertions, deletions, duplications, and substitutions, but deletions

Table 26.1 GIST genotype correlation with clinicopathologic features

Mutation site	Common tumor location	Common tumor morphology	Response to imatinib	Response to sunitinib
KIT exon 9	Small intestinal	–	Improved tumor response and PFS with *high dose* imatinib	Improved tumor response, PFS and OS compared to exon 11 mutated and wild-type GISTs
KIT exon 11	Trp557_Lys558del Gastric Tyr568del or Tyr570del Small intestinal	Spindle	Improved PFS, OS, and tumor response compared to exon 9 mutated and wild-type GISTs. Higher risk of secondary mutations	–
KIT exon 13	Non-gastric	Spindle	–	–
KIT exon 17	Non-gastric	Spindle	–	–
PDGFRα	Gastric	Epithelioid	–	–

PFS progression-free survival, *OS* overall survival

are most common. Interestingly, deletions cluster at the 5′ end of *KIT* exon 11 and duplications cluster at the 3′ end [9]. Single nucleotide substitutions have been reported in four exon 11 codons: 557, 559, 560, and 576 [9]. *KIT* exon 9 mutations are less common, and have been reported to occur in 8% of CD117-positive GISTs [8]. The most common mutation in exon 9 is a duplication of six nucleotides (c.1525_1530dupGCCTAT) corresponding to codons 502 and 503 (p.Ala502_Tyr503dup) [4, 8, 9]. Mutations in *KIT* exons 13 and 17 are rare, being detected in just 1–2% of GISTs [8, 11]. The most common primary exon 13 mutation is an A>G point mutation at nucleotide 1,945 (c.1945A>G) resulting in p.Lys642Glu [11]. Substitutions involving codon 822 are the most common mutations detected in exon 17 with rare substitutions identified involving codons 816, 820, and 823 [11]. In GISTs found to be wild-type for *KIT*, *PDGFRα* mutations may be identified. Approximately 3–7% of GISTs have been found to harbor *PDGFRα* mutations [6–8]. Mutations are seen in either exon 12 or 18, which, respectively, correspond to the juxtamembrane domain and the activation loop of the protein. The majority of *PDGFRα* mutations involve exon 18 [4, 9]. In a proportion of GISTs, approximately 10–15%, no mutation is identified in *KIT* or *PDGFRα*. These are referred to as "wild-type" GISTs.

Certain genotypes have been shown to correlate with clinicopathologic features of GISTs including tumor location and morphology (Table 26.1). Specifically, exon 9 *KIT* mutations are more frequently associated with small intestinal tumors, and *PDGFRα* mutations are more often associated with gastric GISTs [4]. Exon 11 mutations have been shown to be predominantly of gastric origin although this is not uniform among all exon 11 mutations [6]. Mutation p.Trp557_Lys558del is more often associated with gastric GISTs whereas deletion of one of the tyrosine residues at position 568 or 570 (p.Tyr568del or p.Tyr570del) is more frequently associated with small intestinal GISTs [10]. Both mutation types were associated with spindle cell morphology, similar risk category, and similar objective response to imatinib therapy [10]. Both exon 13 and 17 mutations have been shown to more often be associated with non-gastric GISTs and spindle cell tumor morphology [11]. *PDGFRα* mutations are associated with epithelioid morphology [6]. The most specific immunohistochemical marker of GISTs is KIT (CD117), but genotype has not been found to correlate with KIT expression [6, 9]. This supports the concept that mutations do not affect expression but rather tyrosine kinase function. Approximately 5% of GISTs are KIT negative by immunohistochemistry, and mutation testing may be useful in these cases to establish a definitive diagnosis. In one study of 13 KIT negative or weak cases, nine harbored *PDGFRα* mutations, one was wild-type, and three had *KIT* mutations [5]. Furthermore, of nine wild-type GISTs, eight were positive for KIT expression [5].

Familial GIST has been described with various *KIT* mutations identified. These kindreds have multiple GISTs, skin hyperpigmentation, and hyperplasia of the interstitial cells of Cajal. While not familial, GISTs are also a component of the Carney triad, which includes paragangliomas, pulmonary chondromas, and GISTs.

Carney triad is frequently diagnosed in younger patients and is not associated with *KIT* or *PDGFRα* mutations. A small percentage of patients with Neurofibromatosis 1 also develop GISTs, but these are similarly not associated with *KIT* or *PDGFRα* mutations.

For patients with a diagnosis of GIST, tumor size, location, and mitotic activity are the best known predictors of survival, but genotype can also be predictive of prognosis and response to therapy with kinase inhibitors. Two large trials correlated genotype with prognosis and found superior progression-free survival and overall survival in patients with *KIT* exon 11 mutations as compared to exon 9 mutations and wild-type cases [8, 12]. Whether or not the specific type of exon 11 mutation affects prognosis has not yet been resolved.

Surgery is the first-line treatment for GISTs. They are insensitive to chemotherapy and radiation. Imatinib mesylate is a selective inhibitor of KIT and PDGFRα used for GIST therapy in cases of metastatic disease or for tumors not amenable to surgery. Tyrosine kinase inhibitors, imatinib and sunitinib are also used for treatment of patients status post-resection with high risk of recurrence. In patients with advanced disease treated with imatinib, *KIT* exon 11 mutations have been found to be a positive predictive factor of objective tumor response (complete or partial), time to tumor progression, and overall survival [8]. Tumors with exon 11 mutations are also significantly more likely to show complete or partial response to imatinib than those with exon 9 mutations or wild-type GISTs [8]. One study also found improved progression-free survival in patients with exon 9 *KIT* mutations treated with high-dose imatinib as compared to standard dose [12], and one study found patients with exon 9 mutations, but not exon 11 or wild-type cases, to have significantly higher response rates to higher doses of imatinib [8]. A higher dose of imatinib (800 mg) has consequently been recommended for patients found to have exon 9 mutations.

Some patients do develop resistance to imatinib due to secondary mutations in *KIT* exons 13, 14, and 17. These resistance-associated mutations are most often single nucleotide substitutions. GISTs with primary exon 11 mutations are significantly more likely to develop secondary mutations in response to imatinib than are cases with primary exon 9 mutations [7]. Sunitinib is a second-generation tyrosine kinase inhibitor approved for use in patients who fail treatment with imatinib. In response to sunitinib in patients who failed imatinib, tumors with primary exon 9 mutations are significantly more likely to achieve an objective response [7]. When treated with sunitinib, patients with primary exon 11 mutations have significantly lower progression-free survival and overall survival than patients with exon 9 or wild-type GISTs. These results are converse to those seen for imatinib, in which patients with exon 11 mutations show a more favorable response. Interestingly, one mutation in *PDGFRα*, c.2664A>T (p.Asp842Val), has been shown to confer resistance to both imatinib and sunitinib [7].

In summary, GIST genotype has been shown to be predictive of prognosis and response to therapy. Therefore, clinical testing of primary tumors should be seriously considered, especially for those at high risk of recurrence or metastatic disease. The majority of GISTs are related to oncogenic *KIT* mutations, and *PDGFRα* mutations are much less frequent. The same signaling intermediaries are activated by mutated KIT and PDGFRα proteins, suggesting that one could replace the other in tumor promotion. This may provide the biological basis for why mutations in these two genes are mutually exclusive of one another. Furthermore, simultaneous mutations involving more than one exon of either gene have only been identified in GISTs previously treated with kinase inhibitors. Therefore, performing *KIT* testing first with reflexive testing of *PDGFRα* in cases negative for a *KIT* mutation is both cost effective and efficient. Another acceptable testing strategy is to initially test only *KIT* exons 9 and 11, because exon 13 and 17 mutations are rare.

Multiple Choice Questions

1. Compared to traditional sequencing for *KIT* and *PDGFRα* mutations, dHPLC is
   A. Faster
   B. Less accurate
   C. Less sensitive
   D. More expensive
   E. More labor intensive
2. Which gene and exon is most commonly mutated in GISTs?
   A. *KIT* exon 9
   B. *KIT* exon 11
   C. *KIT* exon 17
   D. *PDGFRα* exon 12
   E. *PDGFRα* exon 18

3. What is the most common *KIT* exon 11 mutation?
   A. Deletion of tryptophan and lysine at codons 557–558 (p.Trp557_Lys558del)
   B. Deletion of tyrosine residue at codon 568 (p.Tyr568del)
   C. Duplication of alanine and tyrosine at codons 502 and 503 (p.Ala502_Tyr503dup)
   D. Duplication of tryptophan and lysine at codons 557–558 (p.Trp557_Lys558dup)
   E. Single nucleotide substitution at nucleotide 1945 (c.1945A>G corresponding to p.Lys642Glu)
4. True or False: KIT immunohistochemical expression predicts mutation status?
5. Which genotype correlates with the best response to standard dose imatinib?
   A. Genotype does not affect response to imatinib
   B. *KIT* exon 9 mutations
   C. *KIT* exon 11 mutations
   D. *PDGFRα* mutations
   E. Wild-type

Answers to Multiple Choice Questions

1. *The correct answer is A.*

DHPLC has been shown to be faster, less expensive, as accurate, and less labor intensive than traditional sequencing. Also, dHPLC can detect mutant alleles at levels as low as 10% as compared to approximately 20% for sequencing.

2. *The correct answer is B.*

KIT exon 11 is most frequently mutated in GISTs. Exon 9 mutations are less common and exon 17 mutations are rare. *PDGFRα* mutations are less common than *KIT* exon 11 mutations, but most *PDGFRα* mutations are in exon 18.

3. *The correct answer is A.*

Most *KIT* exon 11 mutations are a deletion of codons 557 and 558. Deletion of a specific Tyrosine residue at either codon 568 or 570 is a less common exon 11 mutation. Duplication of codons 502 and 503 is the most common *KIT* exon 9 mutation. Duplication of codons 557 and 558 is not known to occur. Most KIT exon 11 duplications occur at the 3′ end of the exon involving codons 571–591. Single nucleotide substitution in codon 642 is the most common *KIT* exon 13 mutation.

4. *The correct answer is False.*

KIT immunohistochemistry expression does not correlate with genotype. KIT negative cases have been found to harbor either *PDGFRα* or *KIT* mutations. Also, wild-type GISTs may be positive for KIT expression.

5. *The correct answer is C.*

When treated with standard dose imatinib, *KIT* exon 11 mutations had a better tumor response than GISTs with *KIT* exon 9 mutations or wild-type GISTs. However, patients with primary exon 11 mutations were more likely to develop secondary *KIT* mutations and associated imatinib resistance. *KIT* exon 9 mutations were found to benefit from higher dose imatinib.

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Lynch Syndrome

27

Martin P. Powers and James P. Grenert

Clinical Background

A 42-year-old woman underwent an endometrial biopsy for dysfunctional uterine bleeding. The biopsy demonstrated a International Federation of Gynecology and Obstetrics (FIGO) grade 2 endometrioid endometrial adenocarcinoma. A total abdominal hysterectomy with bilateral salpingo-oophorectomy (TAH-BSO) was performed and confirmed a superficially invasive endometroid adenocarcinoma, FIGO grade 2, with a prominent peritumoral lymphocytic infiltrate. There was no metastatic disease in 25 lymph nodes examined.

Question 1: Does this patient meet the criteria for Lynch syndrome screening?
Question 2: Would the criteria change if she had colorectal carcinoma?

Reason for Molecular Testing

Because of a concern for Lynch syndrome in a patient with endometrial carcinoma under the age of 50 (one of the Lynch syndrome screening criteria), microsatellite instability (MSI) testing was performed on this patient's tumor. The criteria for Lynch syndrome screening for both endometrial carcinoma and colorectal carcinoma are similar (Tables 27.1 and 27.2). However, the histological features that are associated with MSI-high colorectal cancer are more clearly established than those in endometrial cancer (discussed further in the Background and Molecular Pathology section).

Test Ordered

Microsatellite testing by multiplex PCR and capillary electrophoresis using nearly monomorphic mononucleotide repeat markers (BAT-25, BAT-26, MONO-27, NR-21, and NR-24) and two highly polymorphic pentanucleotide repeat markers (Penta C and Penta D) (MSI Analysis System v1.2, Promega, Madison, WI, USA) was ordered.

Question 3: Are there any other tests, either molecular or nonmolecular, that can be used to screen for the phenotypes associated with Lynch syndrome?
Question 4: Which other microsatellites can be tested by PCR, and how do they perform as compared to the quasi-monomorphic markers listed above?

Laboratory Test Performed

A slide of formalin-fixed, paraffin-embedded (FFPE) tissue from the TAH-BSO was used for the DNA extractions. Separate areas of invasive endometrial adenocarcinoma (tumor DNA) and normal myometrium (normal DNA) were identified on the slide and macro-dissected individually for the two extractions. Each DNA sample was then subjected to a multiplex

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Table 27.1 The Revised Bethesda Guidelines for testing colorectal tumors for microsatellite instability (MSI)

Tumors from individuals should be tested for MSI in the following situations:
1. Colorectal cancer diagnosed in a patient who is less than 50 years of age
2. Presence of synchronous or metachronous colorectal, or other HNPCC-associated tumors,[a] regardless of age
3. Colorectal cancer with the MSI-H histology[b] diagnosed in a patient who is less than 60 years of age
4. Colorectal cancer diagnosed and one or more first-degree relatives has an HNPCC cancer diagnosed before the age of 50
5. Colorectal cancer diagnosed and two or more first- or second-degree relatives with HNPCC-related tumors, regardless of age

From Umar et al. [12]

[a]Hereditary non-polyposis colorectal cancer (HNPCC)-related tumors include colorectal, endometrial, stomach, ovarian, pancreas, ureter and renal pelvis, biliary tract, and brain (usually glioblastoma as seen in Turcot syndrome) tumors, sebaceous gland adenomas and keratoacanthomas in Muir–Torre syndrome, and carcinoma of the small bowel

[b]Presence of tumor infiltrating lymphocytes, Crohn's-like lymphocytic reaction, mucinous/signet-ring differentiation, or medullary growth pattern

Table 27.2 Society of Gynecologic Oncologists Education Committee Statement on risk assessment for inherited gynecologic cancer predispositions

1. Patients with a greater than approximately 20–25% chance of having an inherited predisposition to endometrial, colorectal, and related cancers, and for whom genetic risk assessment is recommended
(a) Patients with endometrial or colorectal cancer who meet the revised Amsterdam criteria as listed below:
(i) At least three relatives with a Lynch/HNPCC-associated cancer (colorectal cancer, cancer of the endometrium, small bowel, ureter, or renal pelvis) in one lineage
(ii) One affected individual should be a first-degree relative of the other two
(iii) At least two successive generations should be affected
(iv) At least one HNPCC-associated cancer should be diagnosed before age 50
(b) Patients with synchronous or metachronous endometrial and colorectal cancer with the first cancer diagnosed prior to age 50
(c) Patients with synchronous or metachronous ovarian and colorectal cancer with the first cancer diagnosed prior to age 50
(d) Patients with colorectal or endometrial cancer with evidence of a mismatch repair defect (i.e., MSI or immunohistochemical loss of expression of MLH1, MSH2, MSH6, or PMS2)
(e) Patients with a first- or second-degree relative with a known mismatch repair gene mutation
2. Patients with a greater than approximately 5–10% chance of having an inherited predisposition to endometrial, colorectal, and related cancers and for whom genetic risk assessment may be helpful
(a) Patients with endometrial or colorectal cancer diagnosed prior to age 50
(b) Patients with endometrial or ovarian cancer with a synchronous or metachronous colon or other Lynch/HNPCC-associated tumor[a] at any age
(c) Patients with endometrial or colorectal cancer and a first-degree relative with a Lynch/HNPCC-associated tumor[a] diagnosed prior to age 50
(d) Patients with colorectal or endometrial cancer diagnosed at any age with two or more first- or second-degree relatives[b] with Lynch/HNPCC-associated tumors[a], regardless of age
(e) Patients with a first- or second-degree relative[b] who meets the above criteria

From Lancaster et al. [13]

[a]Lynch/HNPCC-related tumors include colorectal, endometrial, stomach, ovarian, pancreas, ureter and renal pelvis, biliary tract, and brain (usually glioblastoma as seen in Turcot syndrome) tumors, sebaceous gland adenomas and keratoacanthomas in Muir–Torre syndrome, and carcinoma of the small bowel

[b]First- and second-degree relatives are parents, siblings, aunts, uncles, nieces, nephews, grandparents, and grandchildren

PCR protocol with amplification of seven different PCR products associated with microsatellite markers (BAT-25, BAT-26, MONO-27, NR-21, NR-24, Penta C, and Penta D) in one PCR tube. The primers for Penta C and NR-24 are labeled with the same fluorescent dye, TMR (tetramethylrhodamine). The Penta D and BAT-26 primers are labeled with FL (BODIPY-FL: 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene – a variant of fluorescein). The BAT-25, MONO-27, and NR-21 primers are all labeled with JOE (2′,7′-dimethoxy-4′,5′-dichloro-6-carboxyfluorescein). This allows for a simple one-tube PCR for each of the tumor and normal

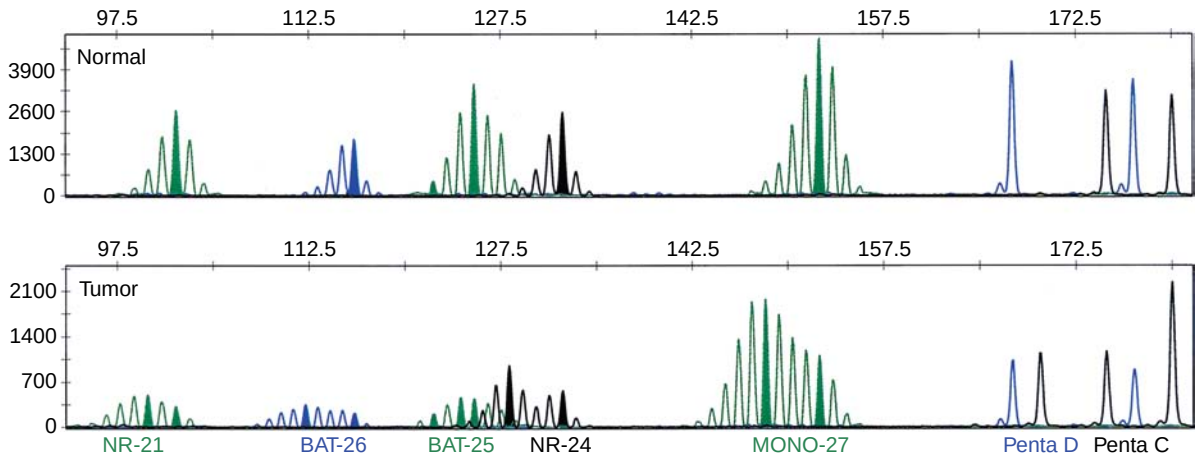

Fig. 27.1 MSI results. The PCR products for all seven microsatellite markers from both the normal DNA (*top*) and tumor DNA (*bottom*) are included. The markers from left to right are: NR-21 (*green*), BAT-26 (*blue*), BAT-25 (*green*), NR-24 (*black*), MONO-27 (*green*), Penta D (*blue*), and Penta C (*black*). In the tumor, notice the presence of three Penta C peaks. Also notice a taller peak to the left side of the main BAT-25 peak (as determined from the normal sample), and the relative height of the peaks on the left side of BAT-25 compared to the main BAT-25 peak, highlighted in *green*

DNA samples. After PCR, the products are run on an ABI 3130xl instrument with 600 bp size standards and a 50-cm capillary array with POP7 polymer. This polymer increases the apparent size of the PCR products as called by the GeneMapper software, but doesn't affect the ability to detect microsatellite instability. As opposed to a test for the loss of mismatch repair (MMR) genes or proteins in the tissue by molecular methods or by immunohistochemistry, MSI testing is utilized to determine whether the tumor has the phenotype of microsatellite instability, which results from the genetic loss of the mismatch repair (MMR) machinery. This test is only for the phenotype of MSI, which is seen in the tumors of Lynch syndrome almost 100% of the time, but it is also seen in a fraction of the sporadic (i.e., nonhereditary) colorectal or endometrial carcinomas. Because these are much more common than Lynch syndrome, the MSI phenotype is actually observed more frequently in sporadic malignancies. One concern is that certain mutations in the MMR machinery, which still cause Lynch syndrome, may not result in a high level of microsatellite instability in these markers. Microsatellite unstable tumors are often unstable in many microsatellite markers, but sometimes not in all of them, and instability can vary depending upon the markers tested. These mononucleotide markers used in this assay are among the most sensitive and specific [1], but may rarely miss a case not tested by another approach.

Question 5: What utility does the normal DNA provide in this assay?

Results with Interpretation Guideline

Results for MSI testing on our patient are presented in Fig. 27.1. The upper diagram shows the PCR products seen when the patient's "normal" or non-cancerous DNA was analyzed. This DNA was extracted from myometrium present on the same slide as the tumor, but greater than 5 mm away from the invasive carcinoma. The lower panel shows the PCR results from the DNA extracted from the patient's tumor. The tumor has a prominent benign lymphocytic infiltrate, so this DNA most likely reflects a mix of noncancerous DNA and DNA from the cancer cells. Comparing the PCR profiles of the two determines whether this tumor demonstrates the microsatellite instability phenotype or has stable microsatellites. The five mononucleotide markers are used to identify instability. The two pentanucleotide markers are used as tissue identification controls. The following guidelines were created for determining if a sample is MSI-high, MSI-low, or microsatellite stable (MSS) [2]: Greater than 30–40% of markers unstable: MSI-high; less than 30–40% of markers unstable: MSI-low; all markers stable: MSS. For the five markers tested in this assay, this translates into MSI-high when two or more markers are unstable,

MSI-low when only one marker is unstable, and MSS when no markers demonstrate instability.

*Question 6: Why are multiple PCR peaks seen in the patient samples when it is expected that no more than two alleles, and most often one allele (*i.e., *nearly monomorphic mononucleotide markers), should be seen for each PCR product?*
Question 7: How can we explain the three peaks in Penta C in the tumor DNA sample? Is the tumor DNA sample contaminated?

Result Interpretation

Markers NR-24, BAT-26, and MONO-27 show an obvious difference in the most prominent peak when the tumor and normal samples are compared. This, and the difference in overall pattern, is consistent with instability. NR-21 also shows its largest peak to be about 2 bp smaller than the largest peak seen in the normal sample but that change is less obvious than in the other PCR products. Because at least three of the five markers are already clearly unstable, there is no problem calling this sample MSI-high. However, BAT-25 also shows subtle changes that may be caused by, but are not diagnostic of, instability. The largest peak in the normal DNA (125) is shifted only 1 bp in the tumor DNA (124) and (125) is a very prominent peak in the tumor DNA. Also, a comparison of the two shows the tumor's BAT-25 profile to be a little broader with an increased relative peak height of some of the neighboring peaks. This difference is subtle and cannot be ascribed to instability with certainty.

In the comparisons made between tumor and normal tissue, it becomes readily apparent that the normal DNA may allow for easier detection of microsatellite instability. In most cases, the tumor sample shows an obvious two (or more) peaks or a broad irregular PCR distribution as seen with NR-24 or MONO-27. BAT-26 also shows a broad distribution consistent with microsatellite instability; however, only one prominent peak is seen (112), and this is different than the most common BAT-26 allele (116). Of note: as discussed above, the use of POP7 and a 50-cm capillary causes the most frequent allele at BAT-26, which is actually 113 bp, to appear as 116 bp. Because having the exact allele size is not important for identifying instability, this doesn't affect the ability of the test to differentiate MSI from MSS, especially when normal DNA is used.

Normal DNA also helps to identify MSI despite the noise inherent in the PCR amplification of microsatellite markers. The reason that multiple peaks are seen is the PCR process itself. Microsatellite instability is caused by the slippage of DNA when repeats are synthesized by DNA polymerase. Normally, these slips lead to a bubble in the DNA after replication, which is repaired by the DNA mismatch repair (MMR) machinery in vivo. This means that in a patient's normal cellular DNA, only one (or two) allele sizes are actually present. Because PCR is a DNA amplification process performed in vitro with no DNA repair machinery, the same DNA slippage can occur, but it is not repaired. Therefore, the presence of a distribution of alleles reflects the occasional amplification of PCR products that slipped to either one or two bases larger or smaller than the true allele (also referred to as "stutter peaks"). The correct interpretation of this is that the patient is actually only homozygous for a single allele such as 116 (113) at BAT-26, and DNA replication errors during PCR led to the wide distribution. Because of the inherent "noise" in the PCR amplification of microsatellites, it is often easier to detect the shifts caused by microsatellite instability by comparing the tumor PCR products to those of a normal or non-tumor DNA sample from the same patient.

Another reason to use a normal sample from the same patient is to detect cases in which the patient has inherited an uncommon microsatellite allele. Rarely, a patient may be heterozygous at a marker with one allele significantly smaller than the other. Using BAT-26 as an example, more than 90% of all alleles will be between 112 and 114 bp. Smaller alleles are less common, but alleles at 103 or 106 bp can be seen in up to 10% of African Americans [3, 4]. If no normal sample is used for comparison, a patient who is heterozygous for a marker (such as one who has BAT-26 alleles of 113 and 106 bp) could be misinterpreted as showing instability for that marker as opposed to the true result of being heterozygous for a rare allele.

The pentanucleotide markers, Penta C and Penta D, were originally included in the Promega MSI analysis panel for specimen identification as they are highly polymorphic markers [1, 3]. Therefore, samples from different patients are unlikely to be identical at both markers. A sample mix-up can be recognized by seeing non-identical peaks in these pentanucleotide markers, which might occur, for example, if the normal DNA and tumor DNA were from different sources

(such as normal DNA from a blood sample and tumor DNA from a surgical specimen). Penta C and D are also microsatellite repeats, in which each repeating unit is five nucleotides long, and they are more stable than mononucleotide repeats. This is evident by the lack of stutter peaks seen in the PCR products. However, like the mononucleotide repeats, they can become unstable, and the additional Penta C peak seen in the tumor sample from our patient can be explained by this instability. The shift in size is exactly 5 bp (175–170), and the wild-type alleles are still visible. Changes in the Penta markers may also be present in patients with MSS cancers. MSS cancers tend to harbor a molecular phenotype called chromosomal instability (CIN) whereby the tumor shows multiple gains and losses of chromosomes or regions of chromosomes. Penta C is located on chromosome 9p and Penta D on 21q. Loss of heterozygosity (LOH) is frequently seen at these two loci in microsatellite stable cancers (up to 50% of the time) [5]. In such situations, two Penta C or D alleles may be seen in the normal DNA sample but only one in the tumor sample, reflecting LOH and not that the tumor sample was from a patient homozygous for the Penta marker and the normal DNA control from a patient heterozygous.

Question 8: What additional testing can be offered to help determine whether this patient has Lynch syndrome or a sporadic MSI-high tumor? What if she had colorectal carcinoma?

Further Testing

Further information can be obtained from the results of antibody staining on the tumor. The patient's tumor was stained with antibodies against the mismatch repair (MMR) proteins MLH1, PMS2, MSH2, and MSH6. The tumor showed no staining for MLH1 and PMS2. Staining was seen for MSH2 and MSH6. This suggests that the molecular defect is in *MLH1* (or possibly *PMS2*). Most cases of Lynch syndrome are due to mutations in either *MLH1* or *MSH2*, with a much smaller percentage caused by mutations in *MSH6* or *PMS2*. Additionally, sporadic MSI-high tumors (those that arise sporadically and are not part of the inherited cancer syndrome known as Lynch syndrome), are almost always caused by inactivation of the *MLH1* gene by promoter hypermethylation and silencing of gene expression. The mismatch repair machinery exists as protein dimers, such as MLH1 and PMS2, and MSH2 and MSH6. Loss of one member of the dimer often results in proteolytic degradation of the other half. Because of this, mutations or silencing of *MLH1* often lead to the loss of both MLH1 and PMS2 proteins, and mutations of *MSH2* often lead to the loss of MSH2 and MSH6 proteins. However, the opposite is often not true. Loss of PMS2 by mutation does not necessarily cause loss of MLH1, and mutation in MSH6 may not cause loss of MSH2 protein expression. This is due to the formation of dimers with MMR proteins other than the ones rendered defective by mutation. Because loss of MLH1 appears to be the primary genetic defect in the tumor cells of our patient, we are still unable to reliably determine whether she most likely has Lynch syndrome or a sporadic MSI-high tumor. Testing for MLH1 methylation, if positive, would be most consistent with a sporadic MSI-high tumor, and, if negative, would be consistent with a MSI-high tumor in the context of Lynch syndrome. Such additional testing was not done on this tumor. Further testing could also include a search for pathogenic mutations in the *MLH1* and *PMS2* genes, which, if present, would be diagnostic of Lynch syndrome. However, if MSH2 and/or MSH6 protein expression were absent by immunohistochemistry, this would be highly suggestive of Lynch syndrome due to a pathogenic mutation in *MSH2* or *MSH6*. In colorectal cancer, but not in other cancers, *BRAF* mutation analysis is an additional option available for testing. Colorectal cancer with a *BRAF* mutation is extremely rare in patients affected with Lynch syndrome, whereas approximately 40–60% of sporadic MSI-high colorectal carcinomas carry a *BRAF* mutation [6–8]. Therefore, the presence of a *BRAF* mutation in a patient with an MSI-high colorectal carcinoma effectively rules out Lynch syndrome, but the absence of *BRAF* mutation is not indicative of Lynch syndrome.

Other Considerations

One of the less frequently considered issues in the course of testing a somatic (i.e., tumor) sample, is the implication for family members, such as the three children of our patient, especially if she turns out to be negative for *MLH1* methylation or for MSH2 and/or MSH6 by immunohistochemistry. In such a case,

testing for the molecular lesions in a tumor can be an *ipso facto* test for an inherited disease as well. If our patient were tested for *MLH1* methylation and negative, she would most likely have Lynch syndrome. Therefore, her children would now have a one-in-two chance of having autosomal dominant Lynch syndrome. Additionally, if she had been negative for MSH2 and/or MSH6 by immunohistochemistry, she would also most likely have Lynch syndrome, and each of her children would have a 50% chance of having inherited this cancer syndrome.

Background and Molecular Pathology

Lynch syndrome is the more common name for hereditary non-polyposis colorectal cancer (HNPCC). Numerous cancers are seen in this syndrome, with colorectal and endometrial being the most common [9]. Other tumors include those in the stomach, ovary, pancreas, ureter and renal pelvis, biliary tract, small bowel, and brain (usually glioblastoma as seen in Turcot syndrome), and sebaceous gland adenomas and keratoacanthomas as seen in Muir–Torre syndrome. Lynch syndrome is caused by an inherited defect in one of the proteins that comprise the DNA mismatch repair machinery [10]. The mismatch repair machinery is a protein complex, formed by the MLH1-PMS2 and MSH2-MSH6 protein dimers, which excises and allows for reparative DNA synthesis of single base pair mismatches and small loops or bubbles in DNA that can arise during replication.

The clinical criteria for Lynch syndrome used in the original research studies are very explicit and not very useful for screening for possible Lynch syndrome patients [11]. These criteria demanded that patients have at least three relatives with colorectal cancer or another HNPCC-associated cancer (usually endometrial or ovarian cancer), and that at least one relationship must be first degree, at least two successive generations must be affected, at least one cancer diagnosed before the age of 50, and familial adenomatous polyposis (FAP) excluded. In order to better identify patients who may have Lynch syndrome, more relaxed clinical criteria were developed to select patients for whom Lynch syndrome screening by either MSI testing and/or MMR immunohistochemistry should be performed. Because colorectal carcinoma and endometrial carcinoma are the most common malignancies seen in Lynch syndrome, most criteria include either one or the other carcinoma. For colorectal carcinoma and endometrial carcinoma, the suggested screening guidelines are shown in Tables 27.1 and 27.2, respectively [12, 13]. Screening should be done in patients with a tumor before the age of 50, those with a family history, or tumors with the pathological features associated with MSI-high less than the age of 60. However, a concern is that these criteria may miss some patients with true Lynch syndrome, who don't meet these criteria, and a recent article has found it to be cost effective to screen all patients with colorectal cancer for Lynch syndrome regardless of age, family history, or pathology [14].

Tumors from patients with the MSI-high phenotype, including those from patients with Lynch syndrome, often have a characteristic histology. Colorectal carcinoma may have, but not always, one or more of the following features: mucinous differentiation, intratumoral lymphocytes, a poorly differentiated or medullary-like histology, or peritumoral lymphoid follicles (a feature called the "Crohn's like lymphocytic reaction") [12]. The pathologic features of MSI-high endometrial carcinoma are less well established. They include dense peritumoral lymphocytes, tumor infiltrating lymphocytes, and tumor heterogeneity [15]. MSI-high tumors may manifest distinct clinical features, as well. MSI-high colorectal cancers have a better prognosis than equally staged MSS colorectal cancers; however, they respond less to 5-fluorouracil (5-FU) [16]. For endometrial carcinomas, the relationship between the MSI-high phenotype and clinical outcome is conflicting, with some studies reporting a better outcome, some a worse outcome, and some no difference [17].

Testing for MSI-high tumors, and therefore screening for Lynch Syndrome, can be done by a number of methods. The most common are immunohistochemistry for the MMR proteins (MSH2, MSH6, MLH1, and PMS2) and identification of the phenotype associated with a loss of the mismatch repair machinery, microsatellite instability. Tumors that are deficient for MMR proteins are prone to having many different types of mutations, especially single base pair changes, in addition to shifts in microsatellite size. However, shifting microsatellite size is an easy phenotype to assess in potential MMR-deficient tumors. Microsatellites are short tandem repeats of one to several nucleotides (e.g., mononucleotide: AAAAA…., dinucleotide: CACACACA…, etc.). The DNA strands containing

repeated sequences can sometimes slip during DNA replication. These slips form bubbles or loops in the DNA after replication, which are recognized by the same proteins that recognize single base pair mismatches. These proteins then excise the errant strand of DNA and allow for resynthesis of new, correctly matching DNA.

In 1998, an NCI workshop established a panel of microsatellite markers for testing that included both dinucleotide and mononucleotide markers [2]. This workshop also established the guidelines for calling MSI-high, MSI-low, and MSS. Further studies discovered that mononucleotide markers are more likely to be unstable than dinucleotide markers in MMR-deficient tumors. A panel of mononucleotides has been shown to be more sensitive than the NCI panel, and to have fewer cases of MMR-deficient tumors that are called MSI-low. Instead, they are correctly called MSI-high [1, 3, 4]. Additionally, the mononucleotide markers are more likely to be homozygous in patients as compared to the dinucleotide markers, allowing for easier analysis. Another method to screen for Lynch syndrome is to look at the expression of the MMR proteins by immunohistochemical staining. The loss of one of more of the MMR proteins correlates well with the MSI-high phenotype. The loss of a specific protein also makes that protein a candidate for the molecular lesion that led to the MSI-high tumor and possibly Lynch syndrome. However, neither one of the methods is perfect. For example, false-positive and -negative immunohistochemistry is seen with certain antibodies and fixation conditions, and not all MSH6-mutated tumors may show an MSI-high phenotype [18]. Another option suggested as a rapid screening tool is the use of a quick single point mutation test for *BRAF* mutation (c.1799 T > A, p.Val600Glu), which if present, virtually rules out Lynch syndrome [7]. However, in this case, it wouldn't be known if the *BRAF* mutation was in a MSS or MSI-high tumor, which may have added clinical value.

Most MSI-high or MMR protein-deficient tumors will not represent true Lynch syndrome. At most, about 20% of MSI-high tumors are from patients with Lynch syndrome. Although tumors lacking MSH2 and/or MSH6 protein expression most likely represent Lynch syndrome (assuming appropriate antibody reactivity), the most common abnormal immunohistochemistry result is the loss of MLH1 and PMS2, which is usually the result of sporadic *MLH1* methylation. *MLH1* methylation testing may help differentiate sporadic and hereditary tumors; however, some rare cases of Lynch syndrome may have methylation of *MLH1* as the second hit in Lynch syndrome tumorigenesis. Also, colorectal cancers with *BRAF* mutations are exceedingly rare to absent in patients with Lynch syndrome, so a *BRAF* mutation, in colorectal carcinoma only, effectively rules out Lynch syndrome. Still, wild-type *BRAF* is seen in about 50% of sporadic MSI-high colorectal carcinomas in addition to those of Lynch syndrome, and the lack of a *BRAF* mutation therefore cannot reliably differentiate between sporadic and Lynch-associated tumors [6].

Multiple Choice Questions

1. A patient with endometrial carcinoma can be evaluated for Lynch syndrome with all of the following EXCEPT: (select the one best answer)
   A. A detailed family history
   B. *BRAF* mutation testing
   C. Immunohistochemistry for MMR (mismatch repair) proteins
   D. Microsatellite instability (MSI) testing
   E. Sequencing of the genes encoding the MMR proteins
2. Each of the following histological features is more consistent with MSI-high histology in colorectal carcinoma EXCEPT: (select the one best answer)
   A. A poorly differentiated syncytial growth pattern
   B. Dirty necrosis
   C. Intratumoral lymphocytes
   D. Mucinous differentiation with signet-ring cells
   E. Peritumoral lymphoid follicles
3. Each of the following patients may benefit from MSI testing as a initial screening test for Lynch syndrome EXCEPT: (select the one best answer)
   A. A 27-year-old male with colorectal carcinoma
   B. A 53-year-old woman with endometrial carcinoma and no unusual histologic features, personal history or family history
   C. A 59-year-old woman with a colorectal carcinoma showing intratumoral lymphocytes
   D. A 65-year-old female with endometrial carcinoma and a personal history of colorectal carcinoma three years ago
   E. A 71-year-old male with colorectal carcinoma whose father had small bowel cancer at age 69 and one aunt having endometrial carcinoma at age 63

4. Each of the following is true about the MSI analysis system (Promega) with five mononucleotide markers and two pentanucleotides markers EXCEPT: (select the one best answer)
   A. Greater than 90% of the time, the mononucleotide markers are monomorphic
   B. The markers are more likely to call a tumor MSI-high or MSS (as opposed to MSI-low) than the original Bethesda panel
   C. The markers are successfully amplified from FFPE tissues due to the small size of the amplicons
   D. The pentanucleotide markers are always stable and can be used for reliable tissue identification
   E. The presence of multiple "stutter" peaks in the PCR product is the result of instability created by the PCR DNA synthesis
5. All of the following tumors are consistent with Lynch syndrome, EXCEPT: (select the one best answer)
   A. *BRAF* mutation negative, *MLH1* methylation negative, MSI-high colorectal carcinoma
   B. MLH1 immunohistochemistry negative, *BRAF* mutated colorectal carcinoma
   C. MSH2 and MSH6 immunohistochemistry negative endometrial adenocarcinoma
   D. MSI-high, *MLH1* methylation negative endometrial adenocarcinoma
   E. MSI-high, *MLH1* non-expressing endometrial adenocarcinoma in a patient with three first-degree relatives all of whom had either colorectal carcinoma or endometrial carcinoma before the age of 50

Answers to Multiple Choice Questions

1. *The correct answer is B.*

BRAF mutation testing is only useful in patients with colorectal carcinoma. In colorectal carcinoma, about 50% of sporadic MSI-high tumors have *BRAF* mutations but these are absent in the MSI-high tumors associated with Lynch syndrome. In endometrial carcinoma, both the Lynch syndrome and sporadic tumors are *BRAF* wild-type. A detailed family history can determine if a patient meets the Amsterdam criteria for Lynch syndrome. Immunohistochemistry of the MMR proteins will identify which, if any, of the MMR proteins are lost, and this can suggest or even be consistent with Lynch syndrome depending on which protein(s) is/are missing. MSI testing will reveal whether a tumor is MSS, effectively ruling out Lynch syndrome, or MSI-high, which could represent either Lynch syndrome or a sporadic MSI-high tumor. Sequencing of the genes may be useful if a patient has a strong family history and/or a relative with a known mutation.

2. *The correct answer is B.*

Dirty necrosis is a feature of the more common MSS colorectal carcinomas. The other answer choices are features that are associated with, but not completely reliable predictors of MSI-high colorectal carcinoma. Of note, the presence of signet-ring cells is one of the features of mucinous differentiation.

3. *The correct answer is B.*

The patient who does not meet any of the criteria, as specified in Tables 27.1 and 27.2, is the 53-year-old with carcinoma and no other features suggestive of either an MSI-high tumor or an inherited cancer syndrome. However, some recent studies and cost analysis suggest that these criteria may still miss some cases and testing all patients for Lynch syndrome is feasible, at least for colorectal carcinoma [14]. Patient A is less than 50. Patient C is less than 60 and has an MSI-high suggestive histology. Patient D has two metachronous Lynch syndrome-associated tumors, and Patient E has one first-degree and one second-degree relative with Lynch syndrome-associated tumors regardless of age.

4. *The correct answer is D.*

As shown in our case, the Penta markers can also be unstable, and any apparent tissue nonidentity should be interpreted in light of this. Also, LOH can be seen with these markers. Answers A, B, and E are discussed in the text. Answer C was not discussed directly in the text, but in the figure it is evident that the amplicons are all between 95 and 180 bp. The reported ranges for the MSI analysis kit are from 94 to 201 bp. DNA from FFPE tissue is often degraded, but except for the most degraded samples, DNA products less than 200 are usually amplifiable. On the other hand, amplicons greater than 400 bp are often difficult to amplify from FFPE tissue.

5. *The correct answer is B.*

A *BRAF* mutated colorectal carcinoma effectively rules out Lynch syndrome and it has been suggested

that *BRAF* alone can be used as an initial screen to rule out Lynch syndrome [7]. If a colorectal cancer or endometrial carcinoma is MSI-high and *MLH1* methylation negative, it is most likely to be from a patient with Lynch syndrome (answers A and D). Any carcinoma with the loss of MSH2 and/or MSH6 is likely to be from a patient with Lynch syndrome (answer C). The patient in answer E has an MSI-high tumor and meets the Amsterdam criteria for Lynch syndrome; therefore, she likely has a Lynch syndrome-associated mutation.

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Oligodendroglioma

28

Matija Snuderl and Jennifer L. Hunt

Clinical Background

A 40-year-old male patient complained of four weeks of a right frontal headache and right neck pain. The pain was associated with intermittent blurred vision in the right eye and three episodes of morning emesis without nausea. The patient denied dizziness, fever, diplopia, change in hearing, tinnitus, vertigo, weakness, numbness, difficulty speaking or with language, swallowing, eating, or gait. He reported no recent travel and no ill contacts. A head CT scan showed a large, primarily cystic lesion within the right frontal lobe containing areas of calcification with surrounding vasogenic edema. There was significant mass effect on the right lateral ventricle with a shift of midline structures to the left and evidence of subfalcine and descending herniation.

Question 1: What is the differential diagnosis?

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Reason for Molecular Testing

The patient underwent exploratory craniotomy and a frozen section was diagnosed as high-grade glioma. A subtotal resection of the tumor was performed. The final pathological diagnosis was anaplastic oligodendroglioma (Fig. 28.1). Further management would have potentially included reoperation to achieve gross total resection, chemotherapy, and/or radiation.

Test Ordered

Testing for the loss of chromosomal regions 1p/19q.

Question 2: Is this an appropriately ordered test?

Laboratory Test Performed

Testing for the loss of the 1p and 19q minimal deletion regions is usually performed using PCR-based loss of heterozygosity (LOH) analysis or fluorescent in situ hybridization (FISH). Array comparative genomic hybridization (aCGH) can also detect the loss, but is less commonly used in clinical practice. Cytogenetics is not recommended as the cells of oligodendroglioma are unlikely to grow well.

FISH and PCR-based methods have advantages and disadvantages. LOH analysis by PCR is technically straightforward and can yield a semiquantitative,

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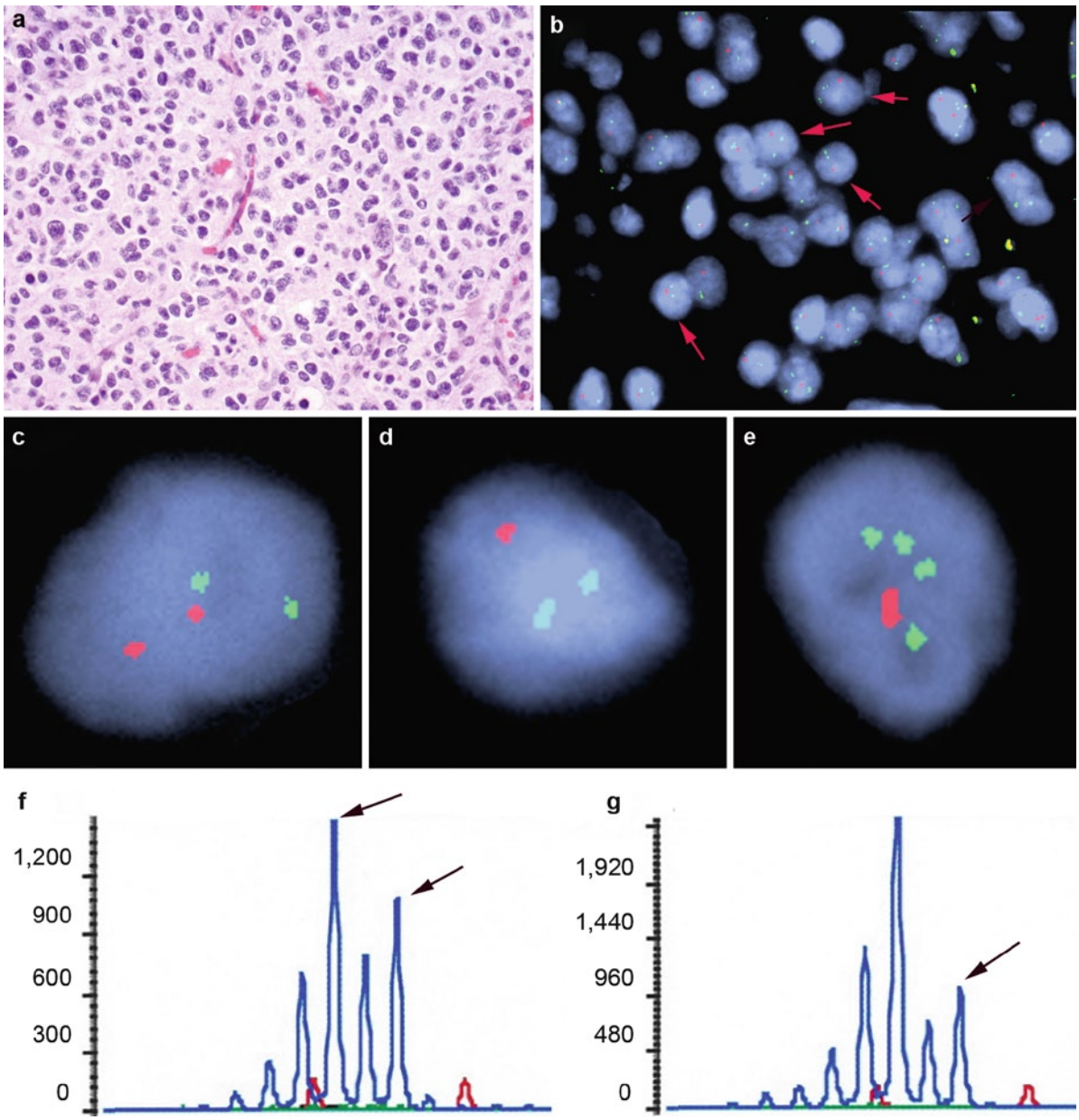

Fig. 28.1 (**a**) A hematoxylin and eosin stain of the patient's tumor revealed classical histological features of oligodendroglioma with round nuclei, perinuclear halos, and delicate capillaries (400×). (**b**) FISH results of the tumor revealed loss of chromosomal arms 1p (1p: *red probe*, 1q: *green probe*) and 19q (not shown) with nuclei (*blue*, DAPI stain) showing two green signals and one red signal (*arrows)*. Panels **c**, **d**, **e**: Examples of nuclei with (**c**) intact 1p or 19q, (**d**) loss or (**e**) relative loss, i.e.: loss with concurrent polysomy. Capillary electrophoresis of PCR products indicated a dinucleotide repeat polymorphism on 1p. The normal tissue showed preservation of both alleles (the tallest peak was used for interpretation of each allele) (**f**, *arrows*). Loss of heterozygosity for 1p presented as a shortening of the height of the second peak (**g**, *arrow*). PCR cannot distinguish between loss of 1p/19q only and loss of 1p/19q with concurrent polysomy (relative loss)

easily interpreted electropherogram result. However, this technique is optimally performed with simultaneous testing of the patient's normal (germline) DNA for comparison with the DNA obtained from the tumor. LOH analysis interpretation is also complicated by impure or suboptimal tumor samples, such as infiltrative or paucicellular tumors and those that are necrotic. Microdissection is usually needed

to enrich for the tumor. LOH only tests for allelic imbalance and while it is sensitive in detecting a loss of allele, it cannot distinguish between a simple 1p/19q loss and so-called relative loss, i.e.: loss on a background of concurrent polysomy. The FISH method is also technically straightforward and commercial probe sets are available to perform the testing. The scoring is time consuming, however, and needs to be performed by a highly trained and experienced individual. Similar to LOH analysis, interpretation is even more challenging in mixed samples with low tumor cell density. In addition to detecting overall loss of 1p and 19q, FISH studies can allow for identification of additional copies of chromosomes 1 and 19 (polysomy), which seems to provide additional prognostic information. Anaplastic oligodendrogliomas with 1p/19q loss and concurrent polysomy, i.e., relative loss, have a higher rate of recurrence and shorter progression-free survival when compared to anaplastic oligodendrogliomas with 1p/19q loss only [1].

Results with Interpretation Guideline

Testing results are summarized in Fig. 28.1.

- Interpretation guidelines for FISH studies: red signals indicate the probes of interest (1p or 19q); green signals indicate the control probes (1q or 19p).
- Interpretation guidelines for studies by PCR with capillary electrophoresis: the tallest peak is used for interpretation of each allele. Two alleles are expected and identified. Subsequently, the pattern of the normal tissue is compared to that of the tumor tissue.

Result Interpretation

There was a loss of both 1p and 19q in the tumor tissue of our patient. Loss of chromosomal arms 1p and 19q is strongly correlated with the presence of classic morphological features of oligodendroglioma [2, 3]. The patient was subsequently treated with additional chemotherapy, and follow-up CT scans showed significant decrease of the tumor volume.

Question 3: Does the test result correlate with the response to the chemotherapy?

Further Testing

Oligodendrogliomas, like all infiltrating gliomas, have a high rate of recurrence despite surgery and adjuvant therapy. These tumors typically gradually increase in grade over time. The mean time to progression from oligodendroglioma WHO Grade II to anaplastic oligodendroglioma WHO Grade III is approximately six to seven years. There is little consensus in practice about whether recurrent tumors should also be tested for the 1p/19q status [1, 4]. Recurrent oligodendrogliomas tend to maintain their original 1p/19q status, but some laboratories will test recurrent tumors, because there is high correlation between the presence of these somatic mutations and response to chemotherapy or radiation. Repeated testing also has the advantage of detecting increasing copy number of 1p and 19q, which is associated with tumor progression. Accumulation of other genetic changes such as mutation of the *PTEN* gene at 10q23, deletion of the *CDKN2A* gene at 9p21, or amplification of the *EGFR* gene at 7p12 has also been associated with tumor progression and poor outcome [5, 6].

Other Considerations

Testing for 1p/19q loss can be used for diagnostic purposes, because these somatic loss mutations are highly associated with the presence of morphological features of oligodendroglioma [2, 3]. This can be particularly important in high-grade gliomas, where an underlying oligodendroglial component is not obvious histologically. This assay can be also useful in gliomas composed of small round cells where differential diagnosis includes oligodendroglioma versus small cell glioblastoma. Because *EGFR* amplification and 1p/19q loss are virtually never present in the same tumors, simultaneous testing for *EGFR* amplification and 1p/19q loss, which are associated with glioblastoma and oligodendroglioma, respectively, can be helpful in distinguishing between these two tumors. Even in the absence of 1p/19q loss, the presence of polysomy (aneuploidy) can be helpful in histologically subtle lesions, to confirm that the tested tissue is tumor.

This type of testing in oligodendrogliomas is most important, however, for its role in predicting therapeutic response. Combined loss of 1p and 19q is highly associated with an excellent response to conventional chemotherapy and radiation therapy in these tumors

[2, 3, 7]. Loss of 1p alone is also associated with good response to adjuvant therapy. Isolated 19q loss can be seen in astrocytic tumors, where it is not associated with response to therapy. Aneuploidy or polysomy of 1p and 19q, particularly in the setting of allelic imbalance, has a prognostic role as well, given that it is seen in higher grade tumors with early recurrence [1].

Background and Molecular Pathology

Glial tumors are classified, based on their morphological resemblance to normal anatomic counterparts, into astrocytomas, oligodendrogliomas, and ependymomas. Mixed gliomas can exhibit morphological features of an oligodendroglioma and astrocytoma, and are called oligoastrocytomas. Oligodendrogliomas represent 7–10% of all gliomas and 2–3% of all adult primary central nervous system (CNS) tumors. Low-grade oligodendrogliomas (WHO grade II) have a favorable prognosis with long progression-free and overall survival. Anaplastic oligodendrogliomas (WHO grade III), in contrast, have aggressive behavior with early recurrence and progression and shorter overall survival. Loss of chromosomal arms 1p and 19q is typically seen in 80% of oligodendrogliomas, 50–60% of anaplastic oligodendrogliomas, and 30–50% of oligoastrocytomas and anaplastic oligoastrocytomas [3]. Tumors with 1p/19q co-deletion have a favorable response to chemotherapy, as well as to radiotherapy [2, 3]. Oligodendrogliomas with 1p/19q loss also have significantly better progression-free survival and overall survival. Therefore, 1p/19q represents a reliable marker of biologic behavior and therapeutic response. Interestingly, 1p/19q co-deletion is also strongly associated with location of the tumor. Oligodendrogliomas most commonly arise in the frontal lobe, less commonly in the temporal or parietal lobes and are rare in the occipital lobe. Almost 90% of oligodendroglial tumors arising in the frontal lobe have 1p/19q co-deletion while less than 10% of tumors arising in the temporal lobe show this molecular change [1–3].

Though the correlation between 1p/19q loss and response to therapy has been well known for more than a decade, the genes responsible for development of the tumor and genes responsible for the sensitivity to the therapy remain elusive and have not been discovered [5, 6]. The mechanism of 1p/19q loss is also not entirely understood. Recent evidence has shown that loss of 1p and 19q follows the formation of a balanced whole arm translocation involving chromosomes 1 and 19, with a loss of the derivative chromosome der(1;19)(p10;q10) and maintenance of the der(1;19)(q10;p10) in the nucleus [8, 9].

Multiple Choice Questions

1. Which brain tumors typically have loss of chromosomal arms 1p and 19q?
   A. Ependymoma
   B. Glioblastoma
   C. Meningioma
   D. Metastatic carcinoma
   E. Oligodendroglioma
2. When comparing PCR and FISH for assessment of 1p19q, the most significant *advantage* of FISH is:
   A. Interpretation and counting of the cells is simple
   B. It can be performed on paraffin-embedded tissue
   C. It does not require a normal sample for correlation
   D. It is more sensitive in detecting 1p/19q loss
   E. It is technically easier to perform
3. What is the main role of testing for 1p19q loss?
   A. 1p/19q loss is associated with inherited brain tumor syndromes
   B. 1p/19q loss is characteristic for primary brain tumors
   C. 1p/19q loss is diagnostic of glioblastoma
   D. 1p/19q loss is not seen in low-grade brain tumors
   E. 1p/19q loss predicts response to adjuvant therapy
4. What does it signify when aneuploidy is identified by FISH in a tested tissue?
   A. That the tested tissue is neoplastic
   B. That the tested tissue is not a brain tumor
   C. That the test has failed and needs to be repeated
   D. That the tissue was overfixed and the DNA was damaged
   E. There is no significance of aneuploidy
5. Which of the following tumors has the highest likelihood of having 1p19q loss?
   A. Frontal lobe anaplastic oligoastrocytoma, WHO grade III
   B. Frontal lobe oligodendroglioma, WHO grade II
   C. Temporal lobe anaplastic oligodendroglioma, WHO grade III
   D. Temporal lobe oligoastrocytomas, WHO grade II
   E. Temporal lobe oligodendroglioma, WHO grade II

Answers to Questions Embedded in the Text

Question 1: What is the differential diagnosis?

Based purely on imaging and clinical presentation, the differential considerations for this type of lesion should include a primary brain neoplasm, infectious etiology such as cysticercosis or Echinococcus, and possibly metastatic disease.

Question 2: Is this an appropriately ordered test?

Testing for 1p19q is considered the standard of care [5, 6] for all tumors with an oligodendroglial component and it is entirely appropriate.

Question 3: Does the test result correlate with the response to the chemotherapy?

Tumors with 1p loss, particularly when accompanied with 19q loss, have a good response to adjuvant chemotherapy and radiation therapy. The excellent response of this patient's tumor to conventional chemotherapy is exactly in line with expected results, given the loss of 1p and 19q in the tumor.

Answers to Multiple-Choice Questions

1. *The correct answer is E.*

Loss of 1p and 19q is typically seen in oligodendroglioma. In addition, mixed gliomas such as oligoastrocytoma and glioblastoma with an oligodendroglial component can also have loss of 1p and/or 19q. While some meningiomas and ependymomas show gain of chromosomal arm 1q, the 1p/19q loss is not present in these tumors. 1p/19q loss is not known to be present in carcinomas.

2. *The correct answer is C.*

FISH analysis for 1p and 19q is an in situ test and does not require normal tissue for comparison. Both assays are equally straightforward to perform and both can be done on paraffin-embedded samples. Sensitivity of PCR and FISH is similar for detecting 1p/19q loss. The FISH counting is not simple and is relatively time-consuming.

3. *The correct answer is E.*

The main role of 1p/19q loss is to predict response of the tumors to adjuvant therapy. Loss of 1p/19q is characteristic for primary brain tumors with an oligodendroglial component but not for all primary brain tumors. Although 1p/19q loss can be seen in glioblastomas with an oligodendroglial component, 1p/19q loss is not a diagnostic feature of glioblastoma. Oligodendroglioma and loss of 1p/19q are not known to be part of any inherited brain tumor syndrome.

4. *The correct answer is A.*

Aneuploidy signals that the tested tissue has an abnormal number of chromosomes, which, in the context of tumor analysis, confirms that the tested tissue is neoplastic. Aneuploidy seen by FISH is not due to overfixation or DNA damage. When the assay fails, no signals are seen in any of the sample cells. Aneuploidy can be seen in primary brain tumors as well as in other neoplasms.

5. *The correct answer is B.*

Oligodendrogliomas arising in the frontal lobe have the highest probability of 1p/19q loss. Low-grade tumors have higher likelihood of 1p19q co-deletion than high-grade tumors or mixed gliomas. Tumors arising in the temporal lobe have lower probability of 1p/19q loss and oligodendrogliomas are uncommon in other locations.

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Glioblastoma Multiforme

29

Li Chen and Neal I. Lindeman

Clinical Background

A 62-year-old man with a two month history of headaches, nausea, and vomiting presented with a seizure. Neurologic examination indicated moderate left hemiparesis, which was more pronounced in the upper extremity with sensory deficits. Magnetic resonance imaging (MRI) revealed a $2.4 \times 2.5 \times 2.6$ cm enhancing mass in the subcortical region of the right frontal lobe, adjacent to the anatomical primary motor cortex. The patient's family history was notable for a grandfather and an uncle who died of brain tumors of unknown pathology. A nearly gross total resection was performed and histologic examination showed an infiltrative and densely cellular tumor, with pleomorphic astrocytes in a fibrillary background, frequent mitosis, pseudopalisading necrosis, and microvascular proliferation, compatible with a diagnosis of glioblastoma multiforme (GBM), grade IV (WHO 2007 classification criteria). He was subsequently scheduled to receive combined radiochemotherapy, followed by adjuvant chemotherapy with temozolomide.

Question 1: Which molecular testing could help guide postoperative therapy?

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Reason for Molecular Testing

Temozolomide is an analog of mitozolomide, one of the antitumor imidazotetrazines synthesized in the 1980s. It has been shown to be significantly, although modestly, better than radiotherapy (RT) alone in a phase III clinical trial coordinated by the European Organization for Research and Treatment of Cancer (EORTC) and the National Cancer Institute of Canada [1]. Median overall survival in the chemoradiotherapy arm was 14.6 months compared with 12 months in the RT arm. More importantly, however, the percentage of patients alive at two years increased from approximately 10% to approximately 26%. The primary mechanism of action of temozolomide is the addition of methyl groups to the sixth position oxygen atoms of guanine to produce methylguanine adducts, which subsequently pair with thymidine [2]. When DNA mismatch repair enzymes attempt to excise O^6-methylguanine, they generate single- and double-strand breaks in the DNA, leading to activation of apoptotic pathways. The DNA-repair enzyme O^6-methylguanine-DNA methyltransferase (MGMT) restores the normal configuration of the nucleotide by transferring the methyl group to a cysteine residue of MGMT. As a result, MGMT provides a mechanism of resistance to temozolomide treatment. However, in gliomas and other tumors, inactivation of the *MGMT* gene has been observed, leading to a decreased ability to repair DNA and a corresponding increase in efficacy of these alkylating agents. Epigenetic silencing of *MGMT* transcription via promoter hypermethylation is the major mechanism for *MGMT* inactivation in these tumors. A 2005 trial demonstrated that there

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was a significant difference, irrespective of treatment assignment, in overall survival between patients whose tumors had *MGMT* promoter methylation and those whose tumors did not [3]. Furthermore, among patients whose tumor contained a methylated *MGMT* promoter, median survival in patients treated with temozolomide and radiotherapy was 21.7 months, as compared with 15.3 months among those who were assigned to only radiotherapy. Nevertheless, *MGMT* promoter methylation assessment is not yet part of the routine work-up of GBM specimens, because currently temozolomide is offered for newly diagnosed patients with GBM regardless of *MGMT* status. Still, knowledge of *MGMT* promoter methylation status is relevant for both prognostic and predictive considerations. Furthermore, *MGMT* promoter methylation status has been used as a stratifying factor or eligibility criterion in ongoing and accruing clinical trials.

Test Ordered

Analysis for *MGMT* promoter methylation status.

Laboratory Test Performed

A variety of different methods and protocols have been used for *MGMT* analysis in GBM. Currently there is no consensus on which specific procedure is best suited for routine clinical use. An optimal method for diagnostic *MGMT* assessment would be easy to establish, cost-effective, reproducible both within a given laboratory and between different laboratories, with a clinically relevant lower limit of detection, and a threshold for distinguishing negative or positive samples that is not subject to operator-dependent interpretation.

The current practice of using formalin-fixed paraffin-embedded (FFPE) neurosurgical specimens affects the selection of a method for *MGMT* assessment. Another challenge with analysis of GBM is extensive tumor necrosis, which may limit samples to only small fragments of viable and informative tumor tissue. The infiltrative growth pattern of gliomas leads to a high content of nonneoplastic cells (e.g., astrocytes, oligodendrocytes, microglial cells, hematogenous cells) in the biopsy specimens, further complicating molecular analysis. Nevertheless, amplification-based analysis of relatively short amplicons can be reliable and successful.

Protein-Based Assessment

Because loss of protein expression is the end result of promoter methylation, *MGMT* assessment at a protein level would seem a logical approach. This has the advantages of being relatively inexpensive and of being adaptable to almost any clinical diagnostic laboratory setting. However, caution should be exercised, because discrepancies between *MGMT* promoter methylation status and MGMT protein expression have been reported [4], most likely because other variables such as methylation dosage and methylation status of other regions of the *MGMT* gene also contribute to transcriptional control.

MGMT Enzyme Activity Assay

Although more a research procedure rather than a clinical lab assay, MGMT activity can be measured by quantitating the transfer of ^{3}H-labeled methyl groups from the O^{6} position of guanine to protein in the cell extract [5]. Only a few studies have analyzed MGMT enzyme activity in human glioma tissues. The mean MGMT activity in untreated GBM was reported at 37 ± 45 (range 0–205) fmol/mg proteins. Patients expressing MGMT activity of less than 30 fmol/mg protein in the pretreatment tumor had a significantly better therapeutic response than patients expressing MGMT above this level. Aside from technical complexity, another drawback to this approach is the need for freshly resected or frozen tumor tissue, which is often not available in the routine diagnostic setting.

Western Blotting

Very few published studies used western blotting for MGMT analysis in brain tumors. In one study, tumor tissue from 19 patients was analyzed for MGMT protein expression using western blotting [6]. Patients with low MGMT protein expression had a significantly improved progression-free survival and median overall survival compared to those with high expression. Compared with an enzymatic activity assay, the western blot immunoassay is more sensitive and requires less patient material: only 1/10 of the tumor material needed for reliable quantitation by the enzyme assay is needed for the western blot immunoassay. Nevertheless, western blotting is also limited by a requirement for unfixed tumor tissue.

Question 2: What is the value of immunohistochemical assessment of MGMT expression?

Immunohistochemistry

MGMT protein can be visualized immunohistochemically, and commercial anti-MGMT antibodies are available. The major advantage of immunohistochemistry (IHC) as compared to other methods of MGMT assessment is that it usually works reliably on FFPE specimens. Unfortunately, a high interobserver variability, even among expert neuropathologists, impairs the reproducibility of this method [7]. Most likely, this variability is caused by interobserver differences in cutoff definition for intensity of the immunostaining signal, because there is marked intratumoral heterogeneity for MGMT immunoreactivity. A high variability in distinguishing nonneoplastic cells within the tumor tissue (e.g., endothelial cells, reactive astrocytes, microglial cells/macrophages, and tumor-infiltrating lymphocytes) may also contribute to poor interobserver agreement. Furthermore, while some retrospective studies on small patient series have reported significant associations of immunohistochemically assessed MGMT expression with patient outcome in glioma [4], such an association could not be found by others [7].

RNA-Based Assessment

MGMT mRNA levels can be successfully determined in fresh or frozen surgical specimens by real-time RT-PCR [8]. The requirement for unfixed tissue may be circumvented by in situ hybridization or in situ RT-PCR [9]. Still, fixation with a fixative such as 10% buffered formalin will cross-link proteins to themselves as well as to RNA, and will reduce the availability of the target mRNA to the cDNA probe. The technical challenges in optimizing the in situ RNA-based methods to prevent RNA degradation and achieve robust and consistent results may limit their feasibility for widespread use in the routine clinical setting.

DNA-Based Assessment

Different from direct measurement of *MGMT* expression, either at mRNA or protein level, DNA-based assessment aims at predicting overall promoter methylation, which is associated with silencing of the gene, by interrogating a subset of the CpG dinucleotides in the *MGMT* promoter for their methylation status.

Question 3: Which technologies are commonly used to evaluate MGMT status?

Methylation-Specific PCR

Methylation-specific PCR (MSP) is now the most widely employed method for detection of *MGMT* promoter methylation status and this was the assay performed in our patient. The principle for the discrimination of unmethylated from methylated sequences is based on a chemical modification of DNA by sodium bisulfite (Fig. 29.1a) [10]. In this reaction, DNA is first denatured to create single-stranded DNA, which is then treated with sodium bisulfite to deaminate cytosine through formation of a 5,6-dihydrocytosine-6-sulfonate intermediate at acidic pH; by contrast, 5-methylcytosine remains essentially nonreactive. Alkalinization with a strong base then causes the transformation of the intermediate product into uracil. The net effect is a substitution of uracils in place of unmethylated cytosines, while methylated cytosines remain. This difference in sequence between methylated and unmethylated DNA can then be detected by downstream applications. For example, PCR primer pairs can be designed to be "methylated-specific" by including sequences complementing only unconverted 5-methylcytosines, or conversely "unmethylated-specific," complementing uracils converted from unmethylated cytosines (Fig. 29.1b). Methylation is determined by the ability of the methylated-specific primer to achieve amplification, similar to allele-specific PCR.

Bisulfite conversion is the most critical step in the assay. Incomplete conversion of non-methylated cytosine is a major concern and it results in a false overestimate of methylation levels. It has been noted that inadequate conversion may occur in up to 10% of MSP analyses, and is particularly common when DNA quality or quantity is poor. Such conditions are common in the clinical setting, because samples are typically obtained from FFPE tumor tissue specimens. Optimal conversion of cytosine to uracil can be achieved by treating single-stranded DNA with sodium bisulfite (pH 5.0, final concentration 2.5–3 M) for 16 hours at 50°C. However, the long incubation, high temperature, high molar concentration of sodium bisulfite, and the extreme change in pH may cause degradation and fragmentation of up to 80% of the DNA molecules [11]. To further control for incomplete modification of unmethylated DNA, primers should recognize a region

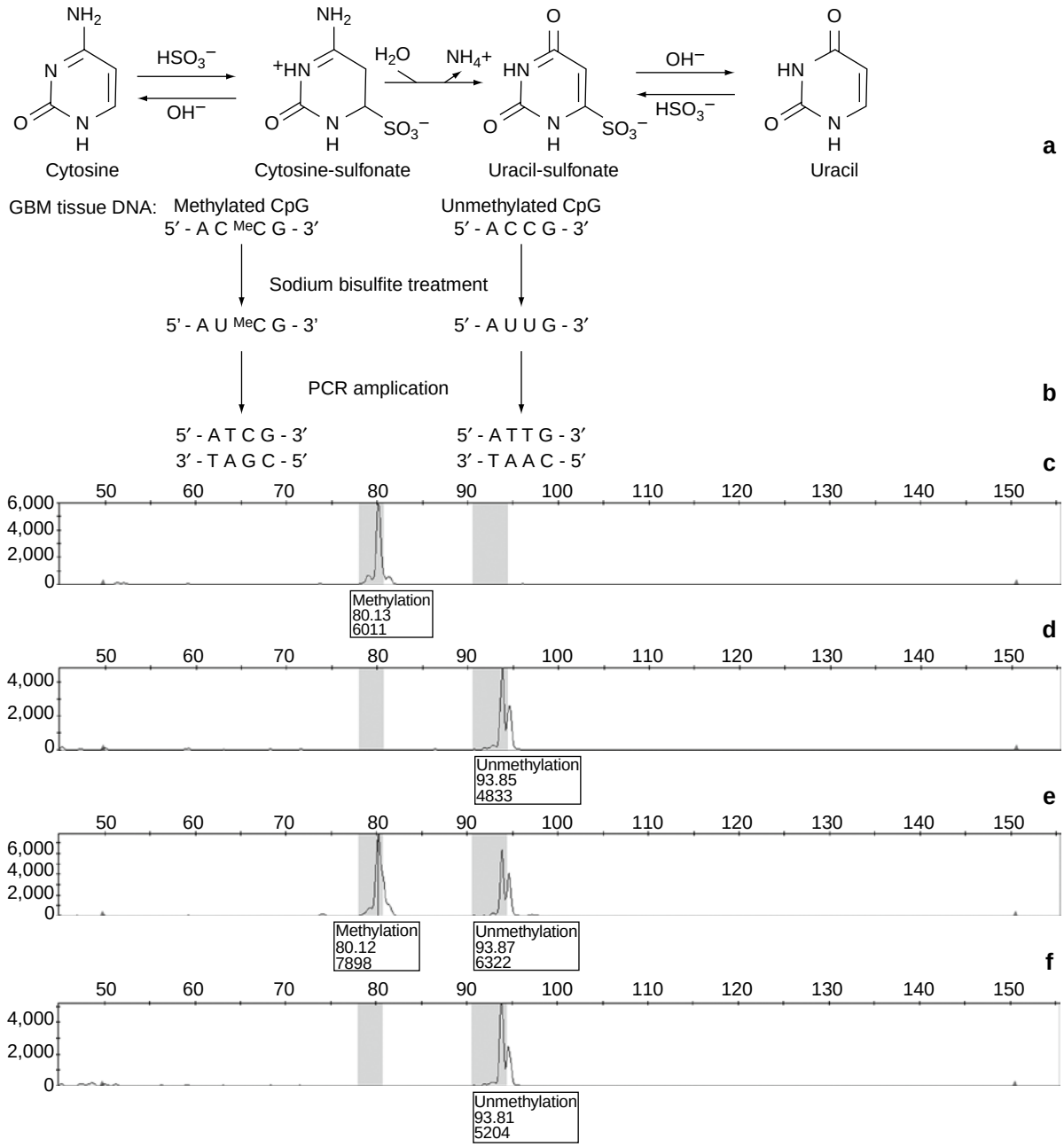

Fig. 29.1 *MGMT* methylation-specific PCR assay. (**a**) Sodium bisulfite conversion. (**b**) Methylation-specific PCR. (**c**) The capillary electrophoresis tracing from completely methylated DNA treated with excess SssI methyltransferase (positive control). (**d**) CpGenome™ universal unmethylated DNA (negative control, from Millipore, Billerica, MA). (**e**) DNA extracted from this 62-year-old patient's GBM tumor tissue. (**f**) DNA from another patient with primary GBM

containing some cytosines that are not part of CpG dinucleotides. In addition, for maximum discriminative power between methylated and unmethylated DNA and to increase the specificity of the primer annealing, primers should contain at least one CpG site at the very 3′ end [12]. Including multiple CpG sites will give the primer better specificity than one CpG site, but if these sites are not equally methylated [13], it is unpredictable as to whether the primer will bind to the DNA or not.

Quantitative MSP

Quantitative MSP (qMSP) combines the advantages of MSP (high sensitivity, applicability to any CpGs) and real-time PCR (rapidity, small quantity of starting DNA). Good concordance between conventional MSP and real-time qMSP has been described [14]. Compared with conventional MSP, the cutoff value of which is defined by the visual presence or absence of the PCR product, a quantitative measurement of the copy number of the methylated *MGMT* promoter in qMSR is achieved by normalization to an internal control gene.

Bisulfite Sequencing

Bisulfite sequencing is currently regarded as the gold standard for the analysis of DNA methylation profiles, because it provides single base pair resolution and quantitative methylation information. For this assay, PCR amplicons containing the bisulfite-treated *MGMT* promoter are ligated into a cloning vector and individual clones are sequenced [13]. This method is widely used in biomedical basic research, but it is too expensive and complex for routine clinical application.

Multiplex Ligation-Dependent Probe Amplification (MLPA)

In contrast to MSP, MS-MLPA does not require the troublesome step of bisulfite conversion and can be used to evaluate methylation status of multiple CpG dinucleotides simultaneously. Methylation-specific MLPA is a variant of the MLPA technique in which methylation-specific probes contain a methylation-sensitive restriction site [15]. To establish the amount of methylated sequences, the sample DNA is divided into two aliquots after hybridization; one aliquot is subjected to a single ligation step, while ligation is combined with the methylation-sensitive digestion in the other aliquot. Hybrids of probes and unmethylated sample DNA are digested by the restriction enzyme, whereas methylated DNA-probe hybrids are protected against digestion. Subsequent PCR, therefore, exponentially amplifies either total DNA or the methylated fraction only. This technique has also shown a high agreement with MSP [16]. The semiquantitative aspect of MS-MLPA may prove to be of value if the ratio of unmethylated/methylated cells is predictive of the duration of response to alkylating agents.

Other DNA-Based Methods

Other DNA-based methods that have been used for *MGMT* analysis include methylation-specific pyrosequencing, which allows analysis of several CpG positions simultaneously [13]; combined bisulfite restriction analysis (COBRA), which takes advantage of restriction enzymes that differentiate between methylated and unmethylated sequences [13]; methylation-sensitive high-resolution melting, which exploits the melting behavior of the amplicons derived from methylated and unmethylated sequences [17]; and microarray based technologies [18]. So far, data on these methods are limited and further studies exploring their feasibility for use in the clinical setting are needed.

In summary, DNA-based methods for *MGMT* analysis are more promising for translation into the clinical setting than RNA- or protein-based methods. To date, MSP is the only test that has repeatedly been shown to be of predictive or prognostic value in clinical trials. However, given the large variation of 30–60% *MGMT* methylation of GBM reported in the literature, there is a strong need for systematic comparisons and validation of intra- and interlaboratory reproducibility and clinical performance of different methods for *MGMT* assessment, to identify and standardize the best method for clinical application.

Results with Interpretation Guideline

MSP was performed following bisulfite modification. The PCR products were analyzed by capillary gel electrophoresis to determine whether the sample was methylated or unmethylated (Fig. 29.1c–f). It is not uncommon to see amplification of both methylated and unmethylated *MGMT* promoter sequences in the same specimen, which may represent tumor cell heterogeneity with mixtures of hypermethylated and unmethylated *MGMT* promoters or the presence of nonneoplastic cells such as lymphocytes, vascular endothelial cells, and macrophages/microglial cells.

Result Interpretation

The result from our patient is depicted in Fig. 29.1e. The PCR, using methylation-specific primers for the *MGMT* promoter, generated an amplification product

equivalent in size to that of the methylated control (Fig. 29.1c). In addition, primers specific to the unmethylated *MGMT* promoter sequence generated an amplification product similar in size to that of the unmethylated control (Fig. 29.1d). The correct interpretation of these results is that the analyzed region of the *MGMT* promoter is methylated. As an example of a different result, another patient (Fig. 29.1f) clearly demonstrated absence of *MGMT* promoter hypermethylation by the lack of products from PCR with methylation-specific primers.

Further Testing

The epidermal growth factor receptor (EGFR) mediated signaling pathway has been shown to contribute to tumorigenesis and tumor progression of various human epithelial cancers. Gene amplification of *EGFR* is found in 40% of GBM's, often associated with structural variants [e.g., *EGFR* variant III, the most common *EGFR* mutation in glioblastoma, results from an in-frame deletion of exons 2–7]. *EGFR* amplification has been related to decreased overall survival and resistance of glioblastoma cells toward radiation and chemotherapy [19]. With the advent of small-molecule tyrosine kinase inhibitors and monoclonal antibodies targeting EGFR, evaluation of *EGFR*-amplification status will be of increasing interest. Currently, fluorescence in situ hybridization (FISH) is the standard testing method for this genetic alteration. Chromogenic in situ hybridization (CISH) is an alternative assay because the concordance of CISH and FISH has been reported to be above 90% [20]. In CISH, the *EGFR* gene is detected by a digoxigenin-labeled *EGFR* probe and peroxidase reaction. In contrast to FISH, which is relatively expensive and necessitates the use of a fluorescence microscope, CISH can be tested using equipment already available in a typical anatomic pathology laboratory that routinely performs immunohistochemical stains, and can be evaluated by a standard light microscope. In addition, CISH allows for the visualization of morphologic details in the tissue sections. Another advantage of CISH is its durability – the hybridization signal does not fade, unlike FISH, for which the signal intensity decreases over time.

Other Considerations

Approximately one third of primary glioblastomas harbor amplified alpha subtype receptor sequences of platelet-derived growth factor (*PDGFRA*), and vascular endothelial growth factor receptor (*VEGFR*) [21]. Like activation of EGFR, the binding of ligands to PDGFR results in receptor dimerization and activation of several signal transduction pathways including Src family kinases, phosphoinositide-3 kinase (PI3K), phospholipase C-gamma, and mitogen-activated protein kinase. Vascular proliferation, or neoangiogenesis, is a distinct histopathological characteristic of GBM and is correlated with prognosis. VEGF is a key factor involved in the angiogenic process that can elicit several responses such as endothelial cell proliferation, extracellular matrix degradation, cell migration, and expression of other proangiogenic factors. One of the intracellular second messengers activated by EGFR and PDGFR is PI3K, which converts phosphatidynositol-4,5-bisphosphate (PIP2) to phosphatidylinositol(3,4,5)-trisphosphate (PIP3). Gain-of function mutations in the PI3K catalytic subunit PIK3CA were found in 11% of GBM cases, leading to constitutive kinase activity [22]. The major negative regulator for PIP3 is the phosphate and tensin homologue (PTEN) protein. *PTEN* is mutated in 15–40% of GBMs, likely contributing to the overactivity of this pathway [23].

A wide variety of targeted agents are being studied in the preclinical setting and in clinical trials. The overall experience thus far has been that monotherapy of all types has shown limited efficacy. Therefore, a multiple target approach, concomitantly aimed at different signal transduction pathways, might be a favorable concept. Indeed, with attempts to comprehensively profile GBM by high-throughput efforts such as The Cancer Genome Atlas (TCGA) project [24], we are approaching a complete dissection of the genetic lesions involved in GBM.

Background and Molecular Pathology

DNA methylation is a covalent chemical modification occurring at cytosine residues in CpG dinucleotides (p denotes physical linkage via a phosphodiester bond) catalyzed by DNA methyltransferases. CpG

islands are short CG-rich DNA stretches, typically of 300–3,000 bp, found preferentially in the promoter region of genes. Most of these CpG islands are associated with housekeeping genes and are normally unmethylated. In cancer, the methylation landscape is profoundly distorted. Human tumors undergo a global overall loss of DNA methylation, but also acquire hypermethylation at specific promoters. It is thought that methylated DNA sequences are bound by methyl-CpG-binding proteins, such as MeCP2 and MBD2, and make complexes including histone deacetylase or methyltransferase, leading to condensed chromatin. Inactivation by methylation of genes such as tumor suppressor genes, DNA-repair genes, and pro-apoptotic genes offers a strong selective advantage to the tumor and contributes to the initiation and progression of human malignancies.

The *MGMT* gene is located on chromosomal band 10q26 and has a TATA-less, CAT-less promoter [25]. The region with maximal activity lies 5′of the gene from −953 to +202 bp (transcription initiation site as +1), and consists of a minimal promoter (−69 to +19 bp), an enhancer (+143 to +202 bp) to which MGMT enhancer-binding protein (MEBP) binds, and several transcription factor-binding sites, such as SP1 and AP1. The CpG island is located between −552 and +289 bp and includes 97 CpGs. Two highly methylated regions in the island have been identified on the basis of luciferase reporter assays carrying different regions of the methylated promoter. One was upstream of exon 1, including the minimal promoter, and the other was downstream, including the enhancer. The latter segment seems to be more critical for the loss of MGMT gene expression upon methylation. Hence, most methylation-specific tests are designed to interrogate this region.

Multiple Choice Questions

1. What chemical group transferred to target DNA is the principal mechanism responsible for the cytotoxicity of temozolomide to malignant cells?
   A. Carbonyl
   B. Methyl
   C. Nitro
   D. Phosphate
   E. Sulfonyl
2. What are the potential consequences of *MGMT* promoter hypermethylation?
   A. Better response to temozolomide
   B. Decreased ability to repair DNA damage
   C. Inactivation of *MGMT*
   D. Overall survival benefit
   E. All of above
3. Which of the following represents the DNA modification caused by sodium bisulfite treatment?
   A. Adenine → Guanine
   B. Cytosine → Thymine
   C. Cytosine → Uracil
   D. Methylated cytosine → Uracil
   E. Thymine → Uracil
4. Which of the following may result in a pattern of partial methylation in a GBM specimen?
   A. Incomplete conversion
   B. Presence of nonneoplastic cells
   C. Tumor heterogeneity
   D. All of the above
   E. None of the above
5. Which of the following DNA-based assessments does not rely on bisulfite conversion?
   A. Bisulfite sequencing
   B. Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA)
   C. Methylation-specific PCR
   D. Quantitative MSP
   E. None of the above

Answers to Multiple Choice Questions

1. *The correct answer is B.*

Temozolomide, a 3-methyl derivative of mitozolomide, transfers the methyl group to DNA. Among the lesions produced in DNA after treatment of cells with temozolomide, the most common is methylation at the N^7 position of guanine, followed by methylation at the O^3 position of adenine and the O^6 position of guanine.

2. *The correct answer is E.*

The *MGMT* gene encodes a DNA-repair protein that removes alkyl groups from the O^6 position of guanine. High levels of *MGMT* expression in cancer cells can reduce the effectiveness of chemotherapy. Epigenetic silencing of *MGMT* by methylation of promoter CpG islands will decrease expression and, thereby, DNA repair. *MGMT* promoter methylation

status has been shown to be a favorable prognostic and predictive factor for patients with GBM.

3. *The correct answer is C.*

Bisulfite modification is a principal tool for analyzing DNA methylation. Sodium bisulfite deaminates cytosine into uracil, but does not affect 5-methylcytosine.

4. *The correct answer is D.*

It is not uncommon to see amplification of both methylated and unmethylated *MGMT* promoter sequences in the same specimen, which may represent tumor cell heterogeneity with mixtures of hypermethylated and unmethylated *MGMT* promoters or the presence of nonneoplastic cells such as lymphocytes, vascular endothelial cells, and macrophages/microglial cells. In addition, incomplete conversion of non-methylated cytosine may appear as "methylated" DNA.

5. *The correct answer is B.*

MS-MLPA is a variant of the MLPA technique in which copy number detection is combined with the use of a methylation-sensitive restriction enzyme. The MS-MLPA protocol is very similar to the standard MLPA method, except that each MS-MLPA reaction generates two samples: one undigested sample for copy number detection and one digested sample for methylation detection. In contrast to MSP and bisulfite sequencing, MS-MLPA does not require the troublesome step of bisulfite conversion.

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Carcinoma of Unknown Primary

30

Hidehiro Takei and Federico A. Monzon

Clinical Background

Case 1

A 74-year-old woman presented with seizures and change in mental status. An MRI of the brain revealed a single ring-enhancing 1.6 × 2.1 × 2.1 cm mass with surrounding prominent edema in the deep white matter of the left posterior parietal region. The radiological differential diagnosis was a high grade glioma versus a metastatic malignancy. She had been debilitated all of her life due to brain damage during birth, with related hearing loss and mental retardation. There was no significant family history. On admission, her vital signs were unremarkable and no palpable masses were identified in the body, including her breasts. The laboratory data were unremarkable. She underwent craniotomy for gross total excision of the tumor. Histologic examination showed a metastatic, poorly differentiated adenocarcinoma with massive necrosis. Immunohistochemically, the tumor cells were diffusely and strongly positive for cytokeratin 7 (CK-7) (basic cytokeratin found on specific epithelia, such as breast, lung, and gastric cancers) and mammaglobin (breast marker); focally positive for p63 and CA125 (urothelial markers), CD10 and PAX-2 (renal cell markers), and gross cystic disease fluid protein (GCDFP)-15 (breast marker); and negative for CK-20 (colonic and urothelial marker), CDX-2 (colonic marker), CA19-9 (pancreatic marker), TTF-1 (thyroid and lung marker), GFAP (glial marker), S-100 protein (neural and melanoma marker), RCC (renal cell marker), and WT-1 (ovarian marker). As a metastatic evaluation, a CT scan of the thorax, abdomen, and pelvis was performed, and revealed no suspected primary or secondary malignancies. The patient did not cooperate for mammography. By bilateral breast ultrasonography, a 1.5 cm solid mass was detected in her left breast. Her family decided against further invasive procedures for diagnostic purposes.

Case 2

A 67-year-old man was found to have multiple liver masses and intra-abdominal nodules by abdominal CT scan. His past medical history was significant for tuberculous orchitis. Laboratory data on admission demonstrated an elevated serum prostatic specific antigen (PSA) level and normal liver function tests, with the exception of mildly elevated alkaline phosphatase. Fine needle aspiration biopsy was performed on the liver lesions, and histologically indicated a poorly differentiated adenocarcinoma with massive necrosis. Immunohistochemically, the tumor cells were positive for CK-7 and carcinoembryonic antigen (CEA, colonic/ovarian/gallbladder marker), and negative for CK-20, CDX-2, Hep Par-1 (liver

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marker), and prostate-specific antigen (PSA). As a metastatic evaluation, a whole-body CT scanning was performed and revealed no probable primary site of this malignancy other than the liver. With a presumptive diagnosis of cholangiocarcinoma with multiple metastases, the patient was treated with chemotherapy. Approximately six months later, an ultrasound-guided core needle biopsy of the liver masses was performed for post-therapeutic evaluation.

In summary, both cases were characterized by a metastatic, poorly differentiated adenocarcinoma of unknown primary.

Question 1: How can molecular testing help in cases of unknown/uncertain primary cancers?

Reason for Molecular Testing

The primary purpose of molecular testing in both cases was to identify the primary site of the metastatic malignancy, in order to confirm the results of the radiologic and pathologic investigations. Identification of the primary site would allow the treating physician to administer an appropriate therapy or intervention.

Patients with unknown/uncertain primary cancer (UPC) or cancer of unknown primary (CUP) have, in general, a poor prognosis, with median survival ranging from six to 10 months in clinical studies of unselected CUP patients and two to three months in other studies [1]. Treatment guidelines are currently based on categorizing CUP patients into favorable and unfavorable groups based on clinical information [2, 3]. The treatment recommended for each classification is, for the most part, based on the most likely primary for each patient. This explains why patients in the favorable groups have better outcomes [4, 5]. In fact, longer survival has been observed in UPC patients in whom the primary source of cancer is ultimately identified [6]. It is clear then, that successful identification of the tissue of origin (TOO) has a significant impact on patient prognosis and management.

For case 1, the patient's hearing loss and mental retardation did not allow the treating physician to obtain detailed clinical information, which would have assisted in refining the diagnosis. For case 2, although the immunohistochemical profile and radiological findings supported an intrahepatic bile duct origin (i.e., cholangiocarcinoma), the initial biopsy did not exhibit a typical histology and the immunohistochemical studies were non-conclusive. For both cases, the treating physicians requested more supportive evidence of the primary site to help guide their management.

Test Ordered

To determine the molecular profiles of the tumors, the following gene expression assays were ordered.

Case 1: Pathwork Tissue of Origin Test (Pathwork Diagnostics, Redwood City, California).

Case 2: ProOnc TumorSource (Prometheus Laboratories Inc., San Diego, California).

Laboratory Test Performed

The Pathwork Tissue of Origin Test and the ProOnc TumorSource are molecular tests that analyze the gene expression pattern of mRNAs (Pathwork) and microRNAs (ProOnc) of tumor specimens. Prior to performing these tests, an adequate specimen that is representative of the tumor (with a limited amount of non-tumoral tissue) needs to be selected by a pathologist. Both of these tests can be performed in formalin-fixed paraffin-embedded (FFPE) tissue and are currently only performed in reference laboratories [7].

In the Pathwork Tissue of Origin Test, the extracted RNA is reverse transcribed and converted into complementary DNA (cDNA). After second strand synthesis, the cDNA is amplified to cRNA and labeled with biotin. The cRNA is then hybridized to a microarray. The gene expression patterns are compared between the patient's tumor and a database of gene expression patterns from 15 known tumors covered by the test. The degree of similarity with each tissue type (i.e., the similarity score) is reported with the tissue. The highest similarity score reflects the most likely site of origin.

In the ProOnc TumorSource test, quantitative reverse transcription PCR (qRT-PCR) is performed to measure the expression level of 48 miRNAs. Using a combination of a proprietary binary decision tree and a *k*-nearest neighbors (k-NN) algorithm, the tissue of origin is predicted from 25 different tumor types (from 17 tissues). The test yields a high confidence prediction when both the

Table 30.1 Tissue of origin results for case 1: Pathwork Tissue of Origin Test

Data Quality: Acceptable

TISSUE	Similarity SCORE	Low 0 5	High 100
Breast	74.4		✦
Ovarian	14.9		✦
Non-small cell lung	3.7	✦	
Kidney	3.5	✦	
Colorectal	1.2	✦	
Pancreas	0.5	✦	
Thyroid	0.4	✦	
Soft tissue sarcoma	0.3	✦	
Prostate	0.2	✦	
Bladder	0.2	✦	
Gastric	0.2	✦	
Testcular germ cell	0.2	✦	
Hepatocellular	0.2	✦	
Non-Hodgkin's lymphoma	0.1	✦	
Melanoma	0.1	✦	

decision tree and the k-NN algorithms agree and a low confidence prediction when each predicts a different site of origin [8].

Results with Interpretation Guideline

Case 1

Tissue of origin results showed the highest similarity score to be 74.4 for breast tissue (Table 30.1). The next highest similarity score was ovary with a score of 14.9. The similarity for all other tissue types was below 5. For this test, the highest similarity score defines the most likely site of origin [9]. Importantly, similarity scores below 20 showed low positive predictive value in the validation of the FFPE version [9]. Any tissue type with a similarity score less than or equal to 5 has a 99.8% probability of not being the correct tissue of origin. Thus, the results for this case are consistent with a breast primary.

Case 2

The ProOnc TumorSource test yielded two different predicted sites of origin in each of the test's algorithms. The k-NN algorithm predicted the tissue of origin as colon adenocarcinoma, while the binary tree classification algorithm predicted stomach or esophagus. In this situation, the k-NN algorithm's result is reported as the most likely site of origin. According to the report, based on validation studies (unpublished), the overall sensitivity of the test for detection of colon adenocarcinomas is 88.9%. However, the stated positive predictive value (PPV) of this result is 44.4%.

Result Interpretation

Case 1

The Pathwork Tissue of Origin Test, run on a surgical specimen obtained from the brain mass, revealed that the highest Similarity Score was 74.4 (for breast), and that the next highest score was 14.9 (for ovary), which is unlikely for the origin. Because Similarity Scores less than or equal to 5 indicate a greater than 99% probability of that tissue type not being the correct tissue of origin, origin from 13 other tissue types with scores ranging from 3.7 to 0.1 could be effectively excluded by this test.

Case 2

The ProOnc TumorSource test, performed on a core needle biopsy specimen of the liver, predicted that colonic adenocarcinoma was the most likely primary tumor, as determined by the k-NN algorithm. Because the result of the k-NN algorithm and that of the binary tree classification did not agree in this case, the result of the latter was reported as the second most likely tissue of origin and histological type: stomach or esophagus, adenocarcinoma. (In cases where both results agree, only one origin is reported.)

Question 2: What is the clinical significance of the results from a molecular test for tissue of origin?

Further Testing

For case 1, no further testing was performed.

For case 2, based on the results of ProOnc TumorSource, which indicated a likely colorectal origin, *KRAS* mutation analysis (for codons 12 and 13) was performed by unidirectional sequencing using a pyrosequencing method. No *KRAS* mutation was detected. Of note is that absence of a *KRAS* mutation does not exclude a colorectal primary, given that 60–70% of colorectal adenocarcinomas bear wild-type *KRAS* sequence. In the context of this case, *KRAS* mutational analysis was performed to determine if the patient was eligible for colon-cancer targeted therapy with anti-EGFR antibodies such as cetuximab and panitumumab [10].

Other Considerations

In case 1, a breast primary was strongly supported by the clinical history, the ultrasound findings, the immunohistochemical profile, as well as the result of the Pathwork Tissue of Origin Test. This information combined enabled the treating physician to make therapeutic decision even without further confirming the pathology diagnosis on a breast biopsy, which was denied by the patient's family.

In case 2, based on the result of metastatic colonic adenocarcinoma rendered by the ProOnc TumorSource test as well as the absence of a *KRAS* mutation, it was concluded that this patient could benefit from anti-EGFR therapy.

Question 3: What is the reliability of the currently available molecular tests for tissue of origin?

Background and Molecular Pathology

Metastatic cancer of unknown primary (CUP), also known as unknown or uncertain primary cancer (UPC), is defined as a histologically proven metastatic malignant tumor whose primary site cannot be identified during pretreatment evaluation, and which represents a challenging heterogeneous collection of malignancies that share a unique clinical behavior. CUP accounts for 3–5% of all new cancer cases and is one of the 10 most frequent cancer diagnoses [11]. In CUP autopsy series, lung and pancreas are the most common primary sites of origin [12]. Histologically, approximately 50% of CUPs are well to moderately differentiated adenocarcinomas, 30% are undifferentiated or poorly differentiated carcinomas, 15% are squamous cell carcinomas, and the remaining 5% are undifferentiated neoplasms [4]. This diagnosis requires extensive work-up including cytohistopathologic studies, immunohistochemistry (IHC) panels, serum tumor markers, and modern imaging technology that can help identify the site of primary tumor. However, even with these investigations, the primary site is identified in fewer than 30% of patients. Moreover, in 20–50% of those patients, the site of origin is not identified even after postmortem examination [13].

Patients diagnosed with a CUP have a dismal prognosis with a median survival of about six to nine months. Conversely, a definitive diagnosis of the primary tumor site provides information that can be used to select specific therapy, resulting in potential improvement of survival, and is informative for other family members to assess the risk when the tumor has a hereditary etiology. A meta-analysis study revealed that IHC investigation provided the correct primary sites of metastatic tumors in 65.6% of cases [14]. Recently, gene expression assays have been developed to identify primary site of origin in patients with CUP/UPC. Molecular profiling of CUP for the primary site identification can overcome the limitations of IHC and can be used for the quality assurance of this and other diagnostic techniques [15]. In the future, such analysis could potentially provide personalized treatment alternatives through the identification of gene expression patterns associated with therapy response profiles.

Three different molecular gene expression tests are currently clinically available for tumor origin determination in the United States: (1) a qRT-PCR mRNA assay to measure 92 mRNA transcripts (bioTheranostics, CancerTYPE ID®), (2) a microarray-based assay to measure 1,550 mRNA transcripts (Pathwork Diagnostics, Tissue of Origin Test), and (3) a qRT-PCR assay to measure 48 miRNA mature transcripts (Prometheus, ProOnc TumorSource).

The CancerTYPE ID® test (bioTheranostics, San Diego, CA) was developed from gene expression patterns derived from 466 frozen tumors (75% primary and 25% metastatic) [16]. It uses 87 classification genes and five reference genes to generate a gene expression profile that was evaluated with 119 FFPE tumor samples representing 30 tumor classes and

showed an overall accuracy of 82% [16]. Although published data demonstrate test performance in 30 tumor classes, the test is reported to have the ability to distinguish up to 54 different tumor types [17].

The Pathwork Tissue of Origin Test (Pathwork Diagnostics, Redwood City, CA) is a 2000-gene proprietary microarray (PathChip) manufactured by Affymetrix (Santa Clara, California) and runs on Affymetrix's FDA-approved clinical instrumentation. The molecular similarity of the tumor specimen is compared to the expression patterns of a panel of 15 known tissue types covered by the test, and the test's proprietary algorithm reports a similarity score, ranging from 0 (very low similarity) to 100 (very high similarity), for each of the tissue types evaluated by the test. The similarity scores for all 15 tissues sum to 100. The test was developed from gene expression patterns from 2,039 tumors comprising 15 tissue types and 60 different morphologies (90% of all solid tumors) [18]. In the FFPE version, the test interprets the expression of 2000 genes by applying normalization and classification algorithms to gene expression data from a microarray. According to a blinded, multicenter validation study of this assay, the assay has an overall sensitivity of 87.8% (agreement between the test result and the reference diagnosis) (95% confidence interval [CI], 84.7–90.4%) and an overall specificity of 99.4% (95% CI: 98.3–99.9%) [19]. The test showed 84.5% agreement in the subgroup of metastatic tumors. Validation of the FFPE version of the test with 462 FFPE tumor specimens indicated a positive percent agreement of 88.5%, and a negative percent agreement of 99.1% [9].

The ProOnc TumorSource test (Prometheus Laboratories Inc., San Diego, California) is a qRT-PCR assay measuring 48 miRNAs. It classifies 25 different tumor types corresponding to 17 distinct tissues and organs. miRNAs are small, nonprotein-coding sequences of RNA (20–25 nucleotides in length) that are critically important in many biological and pathological processes and modulate the expression of other genes by binding to mRNA [20]. Interestingly, in contrast to mRNAs, miRNAs remain largely intact (i.e., are not significantly affected by fixation, paraffin embedding, and storage time) and keep reliable expression levels in FFPE tissue, which is one of the advantages of using miRNA classifiers [20]. This test uses a dual algorithm approach (i.e., binary decision tree and k-NN analyses) for the determination of the tissue origin, with 67% of cases having concordant results [8]. In other words, one third of cases (including our case 2) return two different predicted origins for the tumor. For this assay, 84–90% sensitivity and 97–99% specificity have been reported when both algorithms agree [21]. However, as described above, the PPV for the colon cancer prediction in our case (with both algorithms having discordant results) was only 44.4%.

Gene expression tests are very promising for the identification of a primary site for CUP patients. However, there is still discussion about the clinical utility of these assays. One frequent criticism is that most studies on tissue of origin tests have established performance on samples from tissues of a known type which raises the question whether the biology of the tissues used for validation is reflective of the biology of CUP tumors [13]. It is important to note that IHC panels used in routine diagnosis of cancer have, for the most part, also been validated with tissues of known type [22, 23]. Another concern is the lack of studies that unequivocally demonstrate that the use of these molecular tests does translate into patient benefit, both in terms of clinical outcomes and cost. It seems clear, however, that patients in whom a tissue of origin is identified using current diagnostic approaches fare better than patients who remain with an unknown primary [6]. Thus, it is reasonable to expect that, if tissue of origin identification can be achieved by molecular profiling, this would lead to better therapeutic selection, which in turn could decrease the use of costly, ineffective therapies and could improve patient outcomes. According to one study, patients treated on the basis of a molecular profile of colorectal origin had better outcomes than those treated with conventional CUP management [11]. Furthermore, another study using unselected CUP cases illustrated that CUP patients who were treated based on molecular profiles exhibited the expected outcomes for their identified tumor type [24]. Hence, it is likely that patient management guided by results from molecular tissue of origin tests could be reflected in better patient outcomes and reduced costs.

When using molecular assays to identify the tissue of origin, a careful interpretation of the reported result taking into account the clinical context of the case is of the utmost importance. After all, the real tissue of origin for a given CUP case might not be part of the tissue types included in the test panel. It is not yet clear what type of result is generated by each assay when that situation occurs. For example, cytology fluids from samples with abundant inflammatory infiltrate can show high similarity to lymphomas (unpublished data). This underscores the importance of thoroughly understanding the limitations of these

tests and to integrate molecular profiling results along with the pathologic and clinical context of the individual patient.

Multiple Choice Questions

1. Which one of the following is a *false* statement regarding cancer of unknown primary (CUP)?
   A. Adenocarcinoma is the most common histologic type of tumor
   B. Cases of CUP all show similar prognosis and response to therapy
   C. CUP treatment guidelines are based on categorizing CUP patients into favorable and unfavorable groups, derived from clinical information
   D. In a strict definition, this diagnosis should be rendered after thorough clinicoradiological and pathologic investigations
   E. Lung and pancreas are reported to be the most common primaries of CUP according to autopsy series
2. In which one of the following clinical situations would a gene expression profiling test typically *not* be useful?
   A. When determining the histologic type (e.g., adenocarcinoma, squamous cell carcinoma) of tumor
   B. When determining if a tumor is a metastasis originating from a different site
   C. When the diagnosis cannot be confirmed with conventional methods (e.g., immunohistochemistry).
   D. When the diagnosis is CUP
   E. When the diagnosis of hereditary cancers may have implications for the family members
3. Which one of the following is *false* for microRNA (miRNA)?
   A. miRNA function is to regulate gene expression
   B. miRNA is a small, single-stranded RNA molecule, about 20–25 nucleotides in length
   C. miRNA is expressed in highly tissue-specific patterns
   D. miRNA is transcribed from DNA and then translated into protein
   E. miRNA is well preserved in formalin-fixed paraffin-embedded (FFPE) tissue
4. Which one of the following is *true* for the Pathwork Tissue of Origin Test ?
   A. A total of 48 known tumor types are covered by the test
   B. Cell block sections (FFPE) can be tested
   C. It evaluates the gene expression of 92 genes
   D. It is a miRNA qRT-PCR assay
   E. The test result is based on two different algorithms to predict the site of origin for the tumor
5. Which one of the following is *true* for ProOnc TumorSource ?
   A. Frozen tissue is required for the test
   B. It is a gene microarray-based assay
   C. It is a qRT-PCR assay that measures mRNA transcripts
   D. It requires 48 miRNAs to identify the tissue of origin based on miRNA expression levels
   E. The two applied algorithms agree >90% of the time

Answers:

1. *The correct answer is B.*

Adenocarcinoma is the most common histologic type of CUP. Lung and pancreas are the most common primaries according to autopsy series, whereas microarray platforms demonstrate breast and colon as the most common sites of origin with pancreas and lung in <25% of cases [13]. Several favorable and unfavorable subsets of CUP have been identified, each with different treatment guidelines and prognosis. Treatment groups are mostly defined by the most likely source of tumor, based on the patient's demographic and clinical characteristics.

2. *The correct answer is A.*

In general, determining the histologic types of tumors should be done by conventional cytohistological examination, often with immunohistochemistry. However, new molecular tests based on miRNA expression can now be used to differentiate squamous cell carcinomas from other types of non-small cell lung carcinoma [25]. This distinction has therapeutic implications for lung cancers.

3. *The correct answer is D.*

miRNAs are encoded by genes that are transcribed from DNA but not translated into protein (i.e., noncoding RNA). miRNAs are not significantly affected by fixation, paraffin embedding, or storage time. Thus, FFPE tissue can be routinely be used for the evaluation of miRNA expression in tissues.

4. *The correct answer is B.*

The Pathwork Tissue of Origin Test is a microarray-based gene expression assay, measuring the expression pattern of 1,550 mRNAs that is able to differentiate between 15 different tissues of origin. A single, proprietary algorithm is used to identify the tissue of origin with most similarity to the tested sample. FFPE tissue, including cytology cell block sections, can be used for this test.

5. *The correct answer is D.*

The ProOnc TumorSource is a qRT-PCR assay for 48 miRNAs that uses two independent algorithms to predict the tissue of origin. The k-NN and binary tree classification algorithms agree in approximately two thirds of cases. FFPE tissue, including cytology cell block sections, can be used in this test.

Note in Proof

The ProOnc TumorSource test is not longer offered by Prometheus Laboratories. As of November 2010, this test is available as the miRView mets test from Rosetta Genomics (Philadelphia, PA).

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Part IV
Infectious Diseases

Human Immunodeficiency Virus

31

Ruan T. Ramjit and Angela M. Caliendo

Clinical Background

A 34-year-old Caucasian female who moved to the United States from Europe 15 years ago presented to her primary care physician for a routine physical exam. She had no new symptoms or complaints and indicated that nothing in her medical history had changed except that she traveled to Africa for a period of two months about two years ago. The physician reviewed her chart and saw a note written just before her trip in which he recommended malaria prophylaxis that the patient faithfully took. During her trip, the patient visited West and Central Africa and admitted having some indiscriminate sexual relations while there. She was concerned because a friend, who accompanied her on the trip, recently tested positive for human immunodeficiency virus (HIV-1) and the patient now felt it was necessary that she be tested as well. The remainder of her medical history and physical exam were normal. The pertinent laboratory values immediately obtained were a WBC of 7,000/μL with a normal differential and a CD4+ T-cell count of 1100 cells/mm^3 (normal is approximately 500–1500 cells/mm^3). A few days later, the patient received a call from her physician's office with additional laboratory results indicating she had a reactive HIV-1/HIV-2 enzyme immunoassay (EIA) result. Confirmatory HIV-1 Western blot testing was performed and showed bands for p24, the HIV-1 virus capsid antigen, and gp41, the HIV-1 transmembrane envelope glycoprotein. The patient was then referred to the infectious disease clinic for further follow up.

Question 1: What is the differential diagnosis to be considered in a patient who presents with the described history (think about what you may already know about the different HIV viral species and genetic subtypes)?
Question 2: What are the next most relevant tests that the infectious disease specialist should order to further classify this patient's disease?

Reason for Molecular Testing

The differential diagnosis to be considered in a patient who is asymptomatic and has a history of travel to West and Central Africa includes not only HIV-1 Group M (Main), but also HIV-1 Group O (Outlier), HIV-1 Group N (Non-M and Non-O), and HIV-2 infections. Group M has managed to spread all over the world while Group O, Group N, and HIV-2 are predominantly confined to certain regions of Africa.

The first step in evaluating a patient for HIV infection is to perform a screening test. Screening tests for HIV are typically serological tests and consist of HIV antibody testing or HIV-1/HIV-2 EIA with a reflex to

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confirmatory Western blot testing, as was done in this patient. If the results of the HIV-1 antibody Western blot were negative or indeterminate, reflex testing to Western blot testing for HIV-2 would have been performed. Based on these initial laboratory data, the patient most likely has a new diagnosis of HIV-1 infection and due to her travel history the possibility of infection with a Group O virus should be considered. Therefore, the next phase in the clinical assessment and determination of antiretroviral drug therapy in this patient is to order molecular testing for HIV-1 RNA viral load and HIV-1 genotyping.

Test Ordered

Molecular testing was performed to determine the patient's HIV-1 RNA viral load. Two real-time RT-PCR tests have been FDA approved for this clinical application, offering several advantages over conventional viral load assays, including a broad linear range, extensive automation, and decreased risk of carryover contamination. These tests are the RealTime TaqMan HIV-1 assay (RealTime HIV-1; Abbott Molecular, Des Plaines, IL) and the COBAS Ampliprep/COBAS TaqMan HIV-1 test (Ampliprep; Roche Molecular Diagnostics, Indianapolis, IN). Both tests quantify HIV-1 RNA in human plasma samples and are approved for use in combination with the clinical presentation and additional laboratory markers of HIV-1 disease progression, such as the CD4+ T-cell count, to aid in clinical management. Further, the tests can be used to monitor the effects of antiretroviral therapy by examining changes in plasma HIV-1 RNA levels during the course of treatment.

Question 3: Why do you think the infectious disease physician also ordered HIV-1 genotyping on a patient who has not yet been placed on antiretroviral treatment?

Resistance to antiretroviral drugs is a significant limitation to successful treatment of HIV-1. The International AIDS Society-USA Panel 2008 recommends that whenever possible, resistance testing is performed at the time of initial HIV-1 diagnosis as part of the comprehensive patient evaluation [1]. Person-to-person transmission of drug resistant viruses has increased in frequency over the years and genotype testing in treatment naïve patients is considered beneficial in choosing a patient appropriate antiretroviral drug regimen [1]. FDA-cleared genotype resistance tests include the TRUGENE HIV-1 genotyping kit (TRUGENE; Siemens Healthcare Diagnostics, Tarrytown, NY) to be used in conjunction with the OpenGene DNA sequencing system (OpenGene; Siemens Healthcare Diagnostics, Tarrytown, NY) and the ViroSeq HIV-1 Genotyping System (ViroSeq; Abbott Molecular, Des Plaines, IL). These tests provide a method to obtain DNA sequence of the protease and most of the reverse transcriptase coding regions of HIV-1 viral RNA. A report is generated illustrating the identified mutations within these coding regions and an interpretation of results for specific antiretroviral agents. This information can then be used by the clinician to determine a starting antiretroviral drug regimen or alterations to a regimen for a patient who is already on antiretroviral therapy.

Laboratory Test Performed

Real-time HIV-1 viral load tests are based on three major processes: (1) nucleic acid extraction to isolate HIV-1 RNA, (2) reverse transcription (RT-PCR) of target RNA to generate complementary DNA (cDNA), and (3) real-time amplification and quantitative detection. The Ampliprep and the RealTime HIV-1 tests are both widely available platforms with different targets and performance characteristics. The target for the Ampliprep test is the HIV-1 *gag* gene which is detected by cleavage of a target-specific dual-labeled oligonucleotide probe. This test was designed to quantify all group M viruses, group N viruses, and many circulating recombinant forms (CRFs) [2, 3] over the linear range of 48–10,000,000 copies/mL (1.68–7 $\log_{10}$ copies/mL). Version 2 of the Ampliprep assay was recently approved by the FDA. It amplifies a portion of both the long terminal repeat (LTR) region and the *gag* gene which allows quantification of Group O virus and improved quantification of CRFs. The lower limit of quantification for this assay is 20 copies/mL. The RealTime HIV-1 test, quantifies Group O virus in addition to group M viruses, group N viruses, and many CRFs. The non real-time assays have not been optimized for Group O virus and will under-quantify HIV-1 RNA levels [4]. The RealTime HIV-1 test utilizes the HIV-1 integrase gene as the target and has a linear range of 40–10,000,000 copies/mL (1.6–7 $\log_{10}$ copies/mL).

There are other viral load assays that are FDA-cleared for HIV-1 viral load detection from clinical specimens. These tests are the VERSANT HIV-1 RNA 3.0 (based on branched chain DNA signal amplification), and two RT-PCR tests, the Amplicor HIV-1 Monitor version 1.5 and the COBAS Amplicor HIV-1 Monitor version 1.5. None of the currently FDA-approved viral load tests detect or quantify HIV-2.

The next test that typically follows HIV-1 viral load testing is HIV-1 genotyping. The FDA-cleared platforms use automated sequencing technology to determine the sequence of nucleotides in the viral genome and compare that sequence to wild-type virus. The initial steps include extraction of HIV-1 viral RNA from plasma and RT-PCR to amplify viral RNA sequences which code for a portion of the patient's reverse transcriptase and protease genes, the targets of many antiretroviral drugs. Once the nucleotide sequence of interest is obtained, it is compared to the wild-type sequence by alignment and editing on genetic analysis software. A drug resistance report is then generated linking the identified mutations to specific antiretroviral drugs.

Question 4: What are some limitations of the available genotyping tests?

Genotyping assays will only yield results if the plasma used for testing contains at least 500 HIV-1 RNA copies/mL. Though it may be possible to obtain results with a lower viral load by using high speed centrifugation to concentrate specimens with a viral load < 500 copies/mL, this process may also concentrate interfering substances and inhibitors. Because performing genotyping tests is labor intensive and expensive, laboratories should establish the lower viral limit that ensures reliable results. Only part of the entire HIV-1 genome is amplified and mutations associated with resistance to fusion inhibitors, integrase inhibitors, and CCR5 inhibitors are not detected with the FDA-cleared platforms. Other issues include the possibility of cross contamination, as sequencing requires manipulation of amplified product and the requirement that the resistant viral mutant constitute 20–30% of the viral quasispecies to be detected [5].

Question 5: What other methods can be used to determine antiretroviral drug resistance in a HIV-1 positive patient?

Another way to measure antiretroviral drug resistance is through the use of phenotypic assays. In phenotypic assays, the ability of HIV-1 to grow in the presence of various concentrations of a given antiretroviral agent is measured. The amount of drug required to inhibit virus replication by 50% or by 90% is determined and given as a 50% or a 90% inhibitory concentration (IC_{50} or IC_{90}). The IC_{50} or IC_{90} obtained with the patient sample is compared to a control wild-type virus, and the result is reported as a fold difference. Like the genotyping assays, results can only be obtained if the plasma used for testing contains at least 500 HIV-1 RNA copies/mL. In some cases, a resistance mutation may be detectable by genotypic tests before a phenotypic change has occurred so the results of these tests do not always correlate.

Lastly, the virtual phenotype can be used as an alternative to phenotypic testing. The virtual phenotype is an interpretation of genotyping results evaluated with the aid of a large database of samples with paired genotypic and phenotypic data to predict resistance. A patient's virtual phenotype is determined by entering the genotype into the database and finding the closest matching phenotypic results. This test can be a useful tool for clinicians when resources for phenotypic resistance testing are limited and the patient has had extensive exposure to antiretroviral drugs.

Results with Interpretation Guideline

The following report was generated from the laboratory after testing the patient's plasma specimen (Table 31.1). Testing was performed on a platform able to quantify Group O HIV-1 virus and CRFs. The linear range of reportable results for the assay used is 40–10,000,000 copies/mL (1.6–7 $\log_{10}$ copies/mL). Due to the results of the first sample (Sample 1), a second specimen (Sample 2) was sent to the laboratory for confirmation, one week after receipt of the first sample. As described above, genotype testing has certain requirements in order to attain reliable results.

Table 31.1 The patient's result summary

Test identification	Result
Sample 1: HIV-1 viral load quant	<40 copies/mL
Sample 1: HIV-1 viral load quant (repeat)	<40 copies/mL
Sample 2: HIV-1 viral load quant	<40 copies/mL
Sample 2: HIV-1 viral load quant (repeat)	<40 copies/mL
HIV-1 genotype	Cannot be performed
HIV-1 antibody test and Western blot	Positive

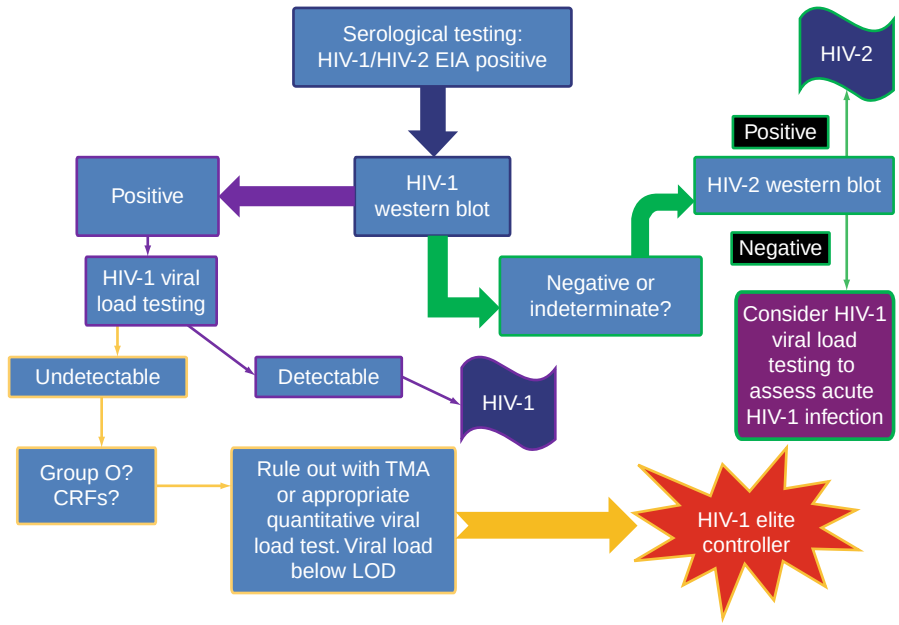

Fig. 31.1 A differential diagnostic algorithm for HIV

Question 6: Why do you think the initial viral load tests on each sample were repeated, and why could HIV-1 genotyping not be performed?

Result Interpretation

In a patient with a new diagnosis of HIV-1 and who is also treatment naïve, it is unusual to have undetectable viral load values. As a result, the laboratory repeated testing on both of the patient's samples to verify the values. In addition, repeat serological testing was performed to rule out pre-analytic error or any further clinical consideration of infection with HIV-2. Each time the patient's samples were run, the viral load results were below the limit of detection (LOD) of the assay. Depending on the operating procedures of individual laboratories, results can be reported as <40 copies/mL (<1.6 $\log_{10}$ copies/mL) or as undetectable. It is also important to note that the assay used to run the patient's samples is able to quantify Group O virus and CRFs. Because of this capability, it is unlikely that the patient is infected with this form of HIV-1. HIV-1 genotype testing could not be performed because the available assays will only yield results if the plasma used for testing contains at least 500 HIV-1 RNA copies/mL.

Question 7: You and the infectious disease clinician analyze the data together. What could be a new consideration in the diagnostic process of this untreated patient with a surprisingly low viral load and a positive HIV-1 EIA confirmed by Western blot?

Further Testing

The described clinical and laboratory scenario is not an uncommon challenge facing infectious disease clinicians and molecular laboratory diagnosticians alike. A differential diagnostic algorithm is illustrated in Fig. 31.1. The first step is to perform a screening test which is typically done by serology and consists of HIV-1/HIV-2 EIA or antibody tests. These tests are confirmed by Western blot procedures by an algorithm that corresponds with Centers for Disease Control (CDC) recommendations. Group O virus and most CRFs can be ruled out by quantitative viral load assays such as the RealTime HIV-1 test, the Ampliprep version 2 test, or by a qualitative method that utilizes transcription-mediated amplification (TMA) technology (APTIMA HIV-1 RNA Qualitative Assay; Gen-Probe Inc., San Diego, CA). Once this algorithm has been followed, the astute clinician will be ready to contemplate the possibility that their patient may be an HIV-1 elite controller.

A small number of HIV-1 infected patients maintain high CD4+ T-cell counts and low viral loads

(<50 copies/mL) in the absence of antiretroviral therapy and despite prolonged infection [6, 7]. These patients are generally referred to as "HIV-1 controllers" and can be subdivided into the elite controllers (ECs) and long-term nonprogressors (LTNPs) by differences in their viral load levels. Much remains to be learned about this patient population and these patients should continue to be followed with routine quantitative viral load to monitor whether their HIV-1 viral loads increase to a level that would yield reliable results. Identifying changes in plasma viremia is useful to determine when to initiate therapy, to monitor response to therapy, and to predict time to progression to acquired immunodeficiency syndrome (AIDS). When therapy begins, patients should be tested within two to eight weeks to assess drug efficacy, and then every three to four months to assess durability of response, with the goal of achieving viral loads below the LOD the assay [8].

Other Considerations

In order for viral load tests to be used effectively, both clinicians and molecular diagnosticians must understand what change in viral load represents a clinically significant change in viral replication. In order to address this issue, information on both the biological variation of the virus and the analytical performance of the test is required. In untreated individuals, the amount of virus in the plasma is relatively stable over time with a biological variation of approximately 0.3 $\log_{10}$ [9]. In general, the intra-assay variation ranges from 0.1 to 0.2 $\log_{10}$ copies/mL when testing multiple replicates in the same run, although the variation is greater near the limit of the detection of the test [10, 11]. Based on these data, changes in HIV-1 viral load must exceed 0.5 $\log_{10}$ copies/mL (threefold) to represent biologically relevant changes in viral replication. Reporting viral load values as $\log_{10}$ copies/mL can be very helpful in preventing clinicians from over interpreting small changes in viral load, this is particularly important for patients with low viral load values.

Interpretation of HIV-1 genotyping results is intricate, requiring an understanding of the mutations associated with each drug, the interactions of resistance mutations, and the genetics of cross-resistance. Most systems use a rules-based approach, using interpretation algorithms established by a group of experts and based on the type of mutations or combination of mutations that are associated with resistance to specific drugs. Depending on the mutations detected, an automated report will indicate for each drug, in each of the antiretroviral categories, whether virus contained in the sample shows no evidence of resistance, resistance, possible resistance, or if there is insufficient evidence to place the virus in any one of those three categories. This system provides easy-to-interpret information for clinicians, and with access to online databases, the most current information is typically readily available.

Recently, a fourth generation HIV-1/2 antibody test was approved by the FDA which detects both HIV-1/2 antibodies as well as the p24 antigen. The detection of the p24 antigen narrows the window between exposure to HIV-1 and seroconversion, thus allowing the detection of some individuals with acute HIV-1 infection. The detection of the p24 antigen complicates confirmatory testing. A positive screen associated with a negative or indeterminate Western blot may represent an acute infection (detection of the p24 antigen only), or a false positive screening test. Detection of HIV-1 RNA would confirm acute infection and a negative HIV-1 RNA would support a false positive test. The decision to perform RNA testing is based on the patient history and risk factors. If HIV-1 RNA testing is performed, a separate specimen should be collected to minimize the risk of contamination between samples.

Background and Molecular Pathology

HIV/AIDS is regarded to be one of the most significant infectious diseases worldwide. According to data from the UNAIDS Global Summary, it is estimated that in 2008 there were 33.4 million people living with HIV and that 2.7 million of these were newly infected (2009 AIDS Epidemic Update/Global Summary, UNAIDS).

HIV is classified into two viral species, HIV-1 and HIV-2. Both viruses are members of the genus Lentivirus within the family Retroviridae. HIV-1, which is responsible for the majority of the AIDS pandemic, is further subdivided into three genetic groups designated M (Major), O (Outlier), and N (Non-M, Non-O). These genetic groups are based on sequence diversity within the HIV-1 *gag* and *env* genes. HIV-1 group M is categorized into nine subtypes (A–D, F–H, J, and K). Presently, subtype C is more predominant

globally, although subtype B is the major subtype in the United States, Europe, and Australia. Certain HIV-1 viral isolates appear to be recombinant, containing sequences from more than one subtype. These are known as circulating recombinant forms (CRFs). Group O viruses are rarely isolated and mostly found in people from Cameroon, Gabon, and Equatorial Guinea. In contrast to HIV-1, HIV-2 typically follows a less pathogenic course and is limited to a few countries in West Africa [12].

HIV viruses are enveloped positive-sense RNA viruses. The HIV-1 genome contains the *gag*, *pol*, and *env* genes which, respectively, encode structural proteins, viral enzymes, and envelope glycoproteins. Replication begins with attachment of virus to the target cell via interaction of gp120, the external portion of the HIV-1 viral envelope protein, and the CD4+ T-cell receptor [13]. This results in gp120 conformational changes allowing the virus to interact with other cellular co-receptor sites, CXCR4 or CCR5. The interaction with CXCR4 occurs primarily with T-cell tropic, syncytium inducing viruses [14] while interaction with the β-chemokine receptor CCR5 is involved in macrophage-tropic non-syncytium inducing HIVs [15]. Once fusion to the host cell occurs, HIV-1 RNA is released along with one of the most important enzymes to viral replication, the reverse transcriptase (RT). The RT can function as an RNA-dependent DNA polymerase to synthesize cDNA, as an RNase H to degrade RNA from the cDNA–RNA complex, and as a DNA-dependent DNA polymerase which duplicates the cDNA strand. The reverse transcribed genome is then associated with several viral proteins, and transported into the nucleus. Once the DNA copy becomes integrated into the host cell via integrase, it is called a provirus, and can serve as an additional template for viral RNA. At the end of the replication cycle, the viral particle assembles and buds through the plasma membrane.

The dynamics between viral and host factors are known to impact the clinical course of HIV-1 disease. Generally, HIV disease progression is characterized by a gradual loss of CD4+ T-cells and cellular immunity with a concurrent increase in plasma viral load resulting in the development of AIDS. In patients who do not receive antiretroviral therapy, progression to AIDS typically occurs within eight to 10 years. While variable progression rates are seen among HIV-1 infected patients, there is a subset of individuals who experience completely asymptomatic phases of more than 15 years. These individuals manage to maintain an otherwise healthy immune system, normal CD4+ T-cell counts, undetectable viral loads, and are not usually treated throughout the course of HIV-1 infection [7, 16, 17]. Termed the natural or elite controllers, this small division of patients, estimated to comprise <1% of the total HIV population in the world, maintains a viral load at <50 copies/mL whereas the LTNPs are defined as those who maintain a viral load <5000 copies/mL [7]. Much about their ability to maintain control over the virus is unknown and it remains unclear whether this control will last indefinitely. One of the most important host genetic factors reported to affect virus infection rates is a 32 base pair deletion in the CCR5 gene. An individual homozygous for this deletion is resistant to HIV-1 infection [18]. In addition to mutations in chemokine receptor (CCR) genes, some HLA class I alleles have been associated with lower steady state viral levels and slow disease progression [19].

The standard of care in the treatment of HIV-1 patients is a combination of the highly active antiretroviral drugs, which are classified based on their viral targets: nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors (PI), fusion inhibitors, integrase inhibitors, and CCR5 entry inhibitors. Current guidelines (DHHS Panel on Antiretroviral Guidelines, http://AIDSinfo.nih.gov) recommend an initial regimen of two NRTIs and either a NNRTI or a PI. However, if therapy with a CCR5 inhibitor, such as maraviroc, or vicriviroc is being considered in a patient's treatment regimen, tropism testing must be performed to determine whether the patient's virus uses predominantly CCR5 (R5-tropic) or CXCR4 (X4-tropic) or both (dual/mixed or D/M-tropic) as a co-receptor for entry. Maraviroc is only effective against CCR5 tropic virus. After beginning appropriate therapy, there is typically a 2 $\log_{10}$ or greater decrease in viral load within two to three months. The goal is to achieve a viral load level below the limit of detection of the most sensitive assays (40–50 copies/mL). Furthermore, clinicians must determine whether there is suboptimal viral load suppression early in the treatment course. This is important to assess whether there are factors affecting adherence to therapy and whether the antiretroviral regimen should be modified.

Multiple Choice Questions

1. What is the target for the Ampliprep HIV-1 version 1 test?
   A. HIV-1 *env* gene
   B. HIV-1 *gag* gene
   C. HIV-1 *pol* gene
   D. p24 antigen
   E. VIF protein
2. Where is the predominant geographic location of HIV-2?
   A. South Africa
   B. Southeast Asia
   C. United States
   D. West Africa
   E. Western Australia
3. Which of the following statements regarding HIV-1 genotyping is false?
   A. Current FDA-cleared assays generally use sequencing technology to compare the patient's sequence with the wild-type
   B. Genotyping can be performed by the TRUGENE and ViroSeq assays
   C. Genotyping reports provide information on antiretroviral drug resistance
   D. Patients who are treatment naïve should have genotyping tests performed
   E. Performing genotyping tests is the only way to determine a patient's antiretroviral drug resistance profile
4. Which of the following is *not* a characteristic of elite controllers?
   A. They constitute <1% of the global HIV infected population
   B. They have a normal CD4+ T-cell count
   C. They maintain a viral load greater than 5000 copies/mL
   D. They typically maintain a viral load less than 50 copies/mL
   E. They will likely have a positive HIV-1 antibody screen
5. When monitoring patients on antiretroviral therapy, what is considered a significant change in viral load for HIV-1 patients?
   A. 0.2 $\log_{10}$
   B. 0.3 $\log_{10}$
   C. 0.4 $\log_{10}$
   D. 0.5 $\log_{10}$
   E. 1.0 $\log_{10}$

Answers to Multiple Choice Questions

1. *The correct answer is B.*
2. *The correct answer is D.*
3. *The correct answer is E.*
4. *The correct answer is C.*
5. *The correct answer is D.*

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Recommended Reading/Suggested Links

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Panel on antiretroviral guidelines for adults and adolescents. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. Department of Health and Human Services (2009), pp 1–161. Available at http://www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf. Accessed 21 June 2010

Cytomegalovirus

32

Michael S. Forman and Alexandra Valsamakis

Clinical Background

A 69-year-old man with chronic renal failure due to hypertension and type 2 diabetes mellitus received a kidney allograft from a deceased donor (Fig. 32.1, week 0). The donor and recipient had no detectable Cytomegalovirus (CMV) IgG. The patient was discharged one week after transplant with an immunosuppressive regimen that included thymoglobulin (begun intraoperatively), tacrolimus, mycophenolate mofetil, and prednisone. The patient was readmitted with pulmonary emboli and deep venous thromboses after complaining of dyspnea. During his hospitalization, he complained of abdominal pain (Fig. 32.1, week 5). The presumed diagnosis was CMV hepatitis, based on elevated liver enzymes and plasma CMV DNA of 5.1 $\log_{10}$ copies/mL by real-time PCR (Fig. 32.1). Ganciclovir was administered intravenously to treat the CMV infection. After two weeks of treatment, the hepatitis appeared to be resolving, as demonstrated by diminished AST levels. However, the viremia remained high (Fig. 32.1, week 7, >5.0 $\log_{10}$ copies/mL). The lack of virologic response raised a concern for the emergence of a ganciclovir-resistant strain, but direct sequencing of viral DNA isolated from plasma detected only wild-type virus. The patient was discharged on intravenous ganciclovir and was transitioned to oral valganciclovir when his viral load reached 3.7 $\log_{10}$ copies/mL (Fig. 32.1, week 9). His dose was reduced by half approximately one week later, due to the side effect of diarrhea. His viral load continued to decline but was still detectable (Fig. 32.1, week 11). During routine follow-up one month later (Fig. 32.1, week 15), the level of CMV viremia was noted to have increased tenfold, although no biochemical signs of hepatitis were observed.

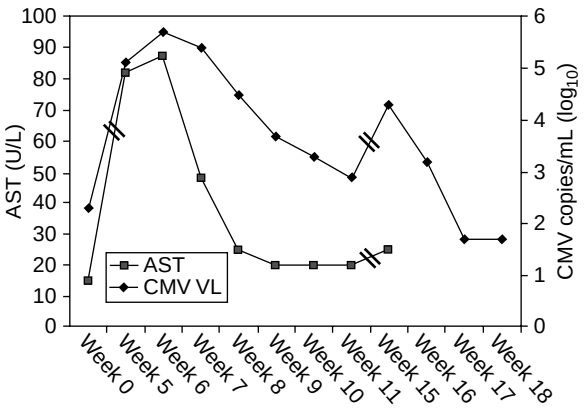

Fig. 32.1 Posttransplant aspartate aminotransferase (AST) and Cytomegalovirus viral load (CMV VL) in plasma. Transplantation was performed during week 0. Abdominal pain was noted in week 5, during hospitalization for pulmonary embolism. Short parallel lines indicate discontinuous time periods

Question 1: Which etiologies could account for the recrudescence in viral load?
Question 2: Which molecular test would be helpful in diagnosing the etiology of recrudescence?

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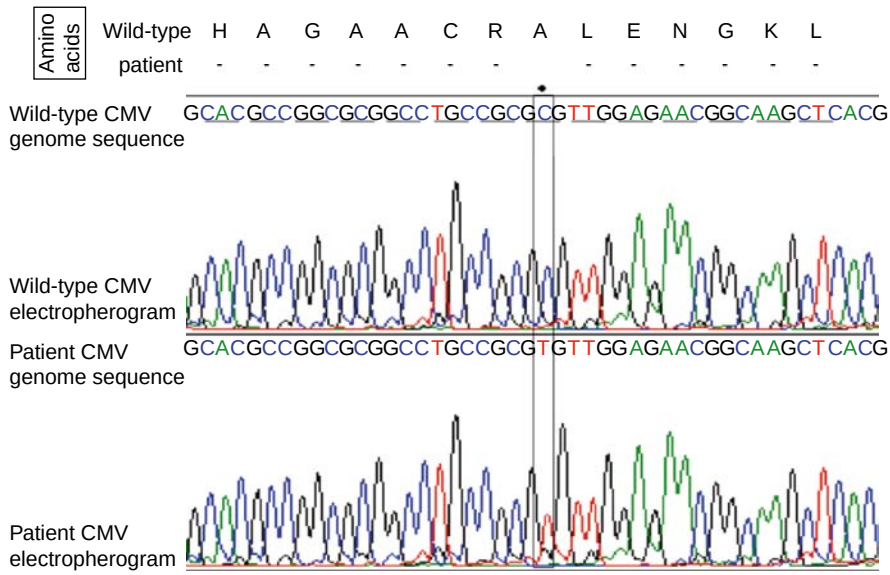

Fig. 32.2 Electropherograms of wild-type and patient CMV sequence data

Reason for Molecular Testing

Resurgence of viremia after an initial treatment response could be due to noncompliance with medications or to the emergence of a drug-resistant virus. One method for distinguishing between these etiologies is direct sequence determination to detect mutations that confer ganciclovir resistance in viral genes required for drug activity. Mutations that confer resistance occur in either of two genes, UL97 and UL54.

Test Ordered

The clinician ordered direct sequencing of the UL97 gene because mutations in UL97 occur more commonly than mutations in UL54.

Laboratory Test Performed

The laboratory-developed test was designed to amplify the region within UL97 where drug resistance mutations are localized. The UL97 gene is translated into a 707 amino acid protein. Mutations in a region of approximately 700 nucleotides (1292–1998, corresponding to amino acids 430–666) confer drug resistance. The assay consisted of nested PCR, gel electrophoresis to check for correctly sized amplicon, cycle sequencing, and sequence analysis [1]. Laboratory experience suggests that a viral load of at least 1000 copies/mL is required for successful target amplification in the initial nested PCR reaction.

Question 3: What nucleotide change was detected (Fig. 32.2)?
Question 4: What is the predicted effect of this mutation on the amino acid sequence of UL97?

Results with Interpretation Guideline

Sequences obtained from forward and reverse primed reactions were assembled and a consensus sequence was built using sequence assembly software. The consensus sequence was aligned to a reference strain, which in this case was CMV Towne sequence. ClustalW was used to align patient and reference sequence. The nucleotide sequence was converted to amino acids. The amino acid change was identified by visual comparison with reference strain amino acid sequence. Comparison of wild-type and mutant sequence plots demonstrated a C > T substitution of nucleotide 1781 (numbering according to coding sequence, but HGVS nomenclature is not yet used for CMV) that resulted in Ala594Val (Fig. 32.2).

Result Interpretation

Ganciclovir triphosphate is an acyclic 2′-deoxyguanosine analog that acts as a "suicide substrate" for CMV DNA-dependent DNA polymerase, encoded by the UL54 (*pol)* gene. The triphosphate form of the drug is produced by an initial phosphorylation mediated by the UL97-encoded viral kinase. Ganciclovir monophosphate is subsequently di- and tri-phosphorylated by cellular kinases. UL97 is a serine threonine kinase found in the nucleus of infected cells and within virions. Its function during replication is unknown. Mutations conferring ganciclovir resistance are found primarily at amino acids 460, 520, and 591–607. Amino acid 460 lies within the catalytic phospho-transfer domain; region 591–607 is hypothesized to be the ganciclovir-binding domain [2]. The mutation detected in this patient, resulting in Ala594Val, is one of the more common ganciclovir resistance mutations [3].

Mutations that affect antiviral resistance are defined biologically. Genes with site-specific mutations are introduced into wild-type virus. The effect of each mutation is determined by assessing the extent of replication inhibition in the presence of increasing drug concentrations and calculating the concentration required for 50% inhibition (termed "IC_{50}"). Several mutations have been defined in this manner [2, 4]. Mutations that increase IC_{50}s greater than fivefold compared to wild-type virus are termed major mutations. A594V is a major mutation because it increases the IC_{50} approximately eightfold [3]. Sequence changes other than those found to confer antiviral resistance can be found in clinical samples and are thought to be genetic polymorphisms without any functional consequence [5].

Question 5: What, if any, change in management should be instituted after detecting A594V in this patient?
Question 6: What, if any, change in treatment should be considered in a patient who does not appear to be responding virologically but who carries no demonstrable drug resistance mutations?

Further Testing

Further infectious disease testing was not indicated for this patient.

Other Considerations

It is recommended that patients demonstrated to have major resistance mutations within UL97 be switched to foscarnet, a potent inhibitor of herpesvirus DNA polymerases [6]. Foscarnet does not require phosphorylation by UL97 kinase for bioactivity. In this patient, intravenous foscarnet was begun after the A594V result was reported and he responded rapidly (Fig. 32.1, weeks 16–18). Cidofovir, another inhibitor of herpesvirus DNA polymerases, can also be used but is not recommended as a first-line agent to treat ganciclovir resistance because some commonly observed polymerase (UL54 gene) mutations confer cross-resistance between cidofovir and ganciclovir. It is also less desirable due to its nephrotoxicity. Cidofovir can be considered if the disease is mild and no polymerase mutations are detected [6].

Antiviral resistance is often suspected in individuals who have completed a prophylactic ganciclovir regimen after an organ transplant and who have a prolonged high level of, or increasing, viremia despite several weeks of ganciclovir treatment. Often, no genotypic evidence of antiviral resistance is detected. In these instances, reduction of immunosuppression has been recommended to allow antiviral immune responses to supplement ganciclovir inhibitory activity [6]. If this is ineffective, ganciclovir treatment intensification through dosage augmentation can be implemented when no ganciclovir resistance mutations are detected and the risk of severe disease is low. Empiric therapy with foscarnet has been advocated when the risk of severe disease is high (such as in lung transplant recipients and CMV seronegative recipients of an allograft from a seropositive donor) [6].

Background and Molecular Pathology

CMV infection in solid organ transplant recipients can be asymptomatic or can cause a variety of manifestations ranging from distinct disease syndromes directly related to viral replication to processes associated with, but indirectly caused by infection. Recognized CMV diseases are organ-specific presentations reflecting localized replication such as gastrointestinal disease (occurring anywhere from the esophagus to the colon, with associated symptoms), pneumonitis, hepatitis,

pancreatitis, and urinary tract infections (ranging from nephritis to cystitis) as well as "CMV syndrome," a constellation of fever, anorexia, and malaise often accompanied by leucopenia and thrombocytopenia. Retinitis and central nervous system disease are uncommon. Organ-specific disease tends to occur more often, although not exclusively in the allograft (hepatitis in liver transplant patients, pneumonitis in lung transplant patients, pancreatitis in kidney or pancreas transplantation). Kidney allograft recipients such as our patient appear to be an exception; while urinary tract presentations occur more commonly in these individuals than in recipients of other allografts, the most common CMV diseases observed in renal transplant patients are CMV syndrome and gastrointestinal disease [7]. CMV infection has also been implicated as a risk factor for increased susceptibility to other infections, allograft rejection and/or dysfunction, and for decreased long-term survival [8]. These effects are thought to be indirect and most likely due to CMV-mediated immunomodulation.

In the infected host, CMV replication is contained by the cellular immune response and immune memory serves to control CMV replication and to prevent disease. Predictably therefore, the highest rates of disease among solid organ transplant patients (40–60% of patients with disease) are observed in seronegative recipients of allografts from seropositive donors, designated "D+/R−" [9]. These patients have no pre-existing immunity and develop primary infections originating from the allograft. Disease risk is lower in seropositive recipients of allografts from seropositive donors (D+/R+). Again, predictably, intensification of immune suppression, particularly the use of antilymphocyte antibodies such as thymoglobulin or OKT3, in the treatment of rejection in these patients, increases the risk of disease three- to fourfold [9].

CMV disease in a seronegative recipient of an allograft from a seronegative donor (D−/R−), as occurred in the patient above, is rare [8, 10]. Infection could have occurred due to any of the following uncommon events: peritransplant exposure to secretions of an infected individual who was shedding virus; transplantation of an allograft from a donor in the early stages of primary infection when virus was replicating and IgG was not yet produced; or transplantation of an allograft from a latently infected seronegative donor (CMV DNA has been detected in peripheral blood mononuclear cells in a small proportion of CMV IgG-negative individuals) [11]. Blood products were not administered in the peritransplant period. Therefore, transfusion-transmitted infection could not be implicated.

One of the mainstays of CMV management in solid organ transplant patients is prevention of disease through antiviral treatment, to inhibit viral replication. This approach is useful because the relationship between CMV concentration in peripheral compartments (blood, urine) and the probability of disease is sigmoidal; disease probability increases steeply above a certain viral load threshold, which depends on assay, disease, and transplant [12, 13]. Two preventive strategies are in use worldwide: pre-emptive treatment of viremic individuals and antiviral prophylaxis of all patients at risk of disease. Most CMV disease occurs in the first three months after transplant. In the pre-emptive paradigm, patients are monitored weekly for viremia during this period and treatment is initiated when a threshold predictive of disease is reached, with the aim of preventing its onset. In the prophylaxis paradigm, patients at elevated risk of disease (D+/R− and any seropositive recipients) are treated with antivirals in the immediate post-transplant period. The duration of prophylaxis is dependent on disease risk [6].

Relatively few studies directly comparing prophylaxis versus pre-emptive treatment have been performed. However, studies conducted thus far confirm the equivalent efficacy for disease prevention and suggest that prophylaxis offers additional benefits of mitigating some of the indirect effects of CMV disease, by producing decreased allograft rejection rates and improved allograft survival [14]. Prophylaxis is therefore becoming the preferred approach to disease prevention.

Late onset disease, defined as disease after discontinuation of antiviral prophylaxis, is becoming more problematic as this strategy is increasingly adopted. Rates of 12% within three months and 17% within nine months of drug discontinuation were reported in the clinical trial of the now commonly used drug valganciclovir, the L-valyl ester prodrug form of ganciclovir with high bioavailability [15]. Similar rates were reported for oral ganciclovir [15].

Ganciclovir-resistant CMV disease has also become problematic due to the adoption of prophylaxis. For CMV, resistant viruses emerge in the presence of drug during sustained viral replication (usually months), which can occur in the absence

of pre-existing immunity (most commonly in D+/R− allograft recipients or rarely in D−/R− patients as in our case), immune suppression intensification to treat rejection, or inadequate antiviral dosing. Among solid organ transplants, rates of approximately 1–10% have been reported [16–19]. Ganciclovir resistance has been associated with D+/R− serostatus, intensity of immunosuppression, and prolonged drug exposure (five months versus three months), as one would predict given the prophylaxis population and the selection mechanism for drug-resistant viruses [16, 17]. Ganciclovir-resistant CMV disease can have severe morbidity and mortality [16, 17, 19]. Thus, the diagnosis of ganciclovir-resistant CMV is increasingly being considered, particularly among prophylaxed individuals with late onset viremia or disease, in whom no virologic response is observed after 10–14 days of adequately dosed therapy. Although this situation typically creates a good deal of anxiety among providers, they can be advised that selection of ganciclovir-resistant mutant viruses usually requires months of treatment and that other strategies, such as decreasing immunosuppression or intensifying ganciclovir dosing, can be attempted, particularly among individuals with no genetic evidence of resistance mutations.

Molecular tests have become the mainstay for detecting CMV viremia, disease, and drug resistance. Quantitative analysis of blood (whole blood or plasma) has largely replaced the antigenemia assay, a technically cumbersome semiquantitative method in which isolated neutrophils containing the CMV protein pp65 are identified by immunostaining. An international standard for use in the calibration of quantitative nucleic acid tests is under development. Until it is available, patients should be followed with a single assay because quantitative results can vary considerably if quantification is based on different calibrators. Antiviral resistance through phenotyping (determining the IC_{50} of an isolate growing in the presence of drug) has now been replaced by direct sequencing due, largely, to faster time-to-result and increasing implementation of direct sequencing by molecular laboratories for other clinical uses. The results of genotyping should be interpreted using publications that list UL97 mutations known to confer resistance [2, 4, 20]. Difficulties in genotype interpretation can arise when sequence changes not known to cause resistance and not established as polymorphisms are detected. In these instances, clinical data including the likelihood of resistance given the duration of therapy and the risk for severe outcome should be considered in the decision to maintain or empirically change antiviral therapy.

Occasionally, requests for UL54 (*pol*) sequence determination are received. UL54 mutations that confer antiviral resistance are encoded in a broad region (codons 300–1000). These mutations are usually observed in individuals who have undergone prolonged treatment with ganciclovir and have pre-existing UL97 mutations or who have been treated with other polymerase-active drugs such as cidofovir or foscarnet. As a consequence, direct sequencing of UL54 requires a greater number of primers and more sequencing reactions than UL97 [21]. Additionally, viral loads should be fairly robust in order to successfully generate the required 2100 nucleotide amplicon in the initial nested PCR. Given these issues, it is fortunate that the need for UL54 sequence determination is uncommon.

Multiple Choice Questions

1. Ganciclovir inhibits CMV replication by disrupting:
   A. A cellular kinase required for viral polymerase activity
   B. A cellular transcription factor required for viral polymerase activity
   C. A viral kinase required for viral polymerase activity
   D. Chain elongation by viral polymerase
   E. Template recognition by viral polymerase
2. In solid organ transplant patients, ganciclovir resistance in CMV infection occurs most commonly due to:
   A. Contaminated intraoperative blood products
   B. Transmission of resistant strains from community contacts
   C. Transmission of resistant strains from the donor organ
   D. Treatment with inappropriately high drug doses for several weeks
   E. Viremia after months of treatment
3. Mutations conferring ganciclovir resistance during treatment of CMV infections are most commonly localized:
   A. In a limited region of the UL97 gene
   B. In several nucleotide “hotspots” of the UL54 gene
   C. In the UL97 promoter
   D. Throughout the UL54 gene
   E. Throughout the UL97 gene

4. Ganciclovir resistance is emerging as a significant problem in solid organ transplant patients with which pretransplant CMV serologic profile?
   A. Donor negative/recipient negative
   B. Donor negative/recipient positive
   C. Donor positive/recipient negative
   D. Donor positive/recipient positive
   E. None; CMV serologic profiles are not a risk factor for ganciclovir resistance
5. The first-line drug recommended for treatment of ganciclovir-resistant CMV infection is:
   A. Acyclovir
   B. Cidofovir
   C. Famciclovir
   D. Foscarnet
   E. Tenofovir

Answers to Multiple Choice Questions

1. *The correct answer is D.*

Ganciclovir is an acyclic 2′-deoxyguanosine prodrug that is initially phosphorylated by the UL97-encoded viral kinase. Diphosphate and triphosphate moieties are added by cellular kinases. Ganciclovir triphosphate acts as a suicide substrate for viral DNA-dependent DNA polymerase (encoded by UL54), inhibiting chain elongation.

2. *The correct answer is E.*

CMV resistance to ganciclovir typically occurs after months of treatment and coincident viremia is the result of selection of mutant viruses that can replicate in the presence of antiviral drugs. Resistance can arise when patients are treated with inappropriately low drug doses; inappropriately high doses are more likely to produce drug side effects such as pancytopenia. In the absence of antiviral drugs, resistant viruses are less fit than wild-type strains; therefore these viruses do not circulate in the community and should not be present in untreated donors.

3. *The correct answer is A.*

Ganciclovir resistance is most commonly due to mutations in the viral kinase UL97. Mutations in UL54 are less frequently observed and are usually preceded by UL97 mutations. Mutations conferring ganciclovir resistance are found in an approximately 700 nucleotide region of UL97. UL54 antiviral resistance mutations are dispersed throughout a 2100 nucleotide region of the gene.

4. *The correct answer is C.*

Solid organ transplant recipients with no prior immunity to CMV who receive an allograft from a seropositive donor have the highest risk of CMV disease and subsequent morbidity and mortality. Ganciclovir prophylaxis, with treatment for three to six months depending on disease risk, is being increasingly implemented. Ganciclovir resistance is emerging as a significant management issue in this drug-experienced population.

5. *The correct answer is D.*

Foscarnet is preferred as first-line treatment of ganciclovir-resistant CMV because some polymerase (UL54) mutant viruses are cross-resistant to ganciclovir and cidofovir. Cidofovir can be used if no polymerase mutations have been detected. Acyclovir has been used as prophylaxis against CMV, but not in treatment of infection. Famciclovir is active only against herpes simplex viruses and varicella zoster virus.

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Human Papillomavirus

33

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Clinical Background

The patient was a 24-year-old female who presented for her annual gynecologic exam. Her last pap smear was one year ago and was normal. She had been sexually active in the past. She requested a refill of the birth control pills that she takes. She reported regular menstrual cycles of approximately 28 days without complaints or problems and no prior sexually transmitted infections or pregnancies. The patient's past medical history did not include hospitalizations. Her immunizations were up to date, including receiving the series of three human papillomavirus (HPV) vaccinations two years prior to her presentation.

The general physical examination was unremarkable with normal vital signs and no abnormal findings. The pelvic exam demonstrated normal external genitalia without erythema or lesions. Bartholin, urethral, and Skene glands were normal. Lesions or abnormal discharge were not observed during the vaginal examination. The cervix was without lesions or friability. Bimanual examination identified no cervical motion tenderness or pain. No palpable uterine or adnexal masses were appreciated. A cervical swab specimen was collected with the cervical spatula and placed in SurePath™ liquid-based cytology preservative for cytologic examination. Slides were prepared from the specimen and reviewed by the pathologist. The cytology was graded as atypical squamous cells of undetermined significance or ASC-US. Thus, the assessment was of a healthy appearing 24-year-old female patient with ASC-US cytology.

Question 1: What does a cytology result of ASC-US mean?

Reason for Molecular Testing

Worldwide, cervical squamous cell carcinoma continues to cause significant morbidity and mortality with nearly half a million new diagnoses every year [1]. Cytologic evaluation of cervical cells collected with a swab or brush can help to identify cervical carcinoma and its precursor lesions. The Bethesda System for Cytologic Classification categorizes squamous precursor lesions into low or high-grade squamous intraepithelial lesions (LSIL and HSIL, respectively) [2]. These have a lower (LSIL) and higher (HSIL) potential for the presence of high-grade lesions or cervical carcinoma. Histologically, biopsies of cervical lesions are classified as mild dysplasia or cervical intraepithelial neoplasia (CIN) 1, moderate dysplasia or CIN2, and severe dysplasia or

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carcinoma in situ or CIN3. Frequently, the cytologic evaluation demonstrates mildly atypical cells that do not meet criteria for LSIL and are referred to as atypical squamous cells of undetermined significance (ASC-US).

Cervical squamous cell carcinoma has been shown to be caused by infection with the human papillomavirus (HPV). HPV infects squamous epithelium, subverting normal cell growth and has the potential to cause squamous cell carcinoma and adenocarcinoma [3]. High-risk (HR) HPV types associated with development of squamous cell carcinoma of the anogenital region include types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. HPV types 16 and 18 are the most prevalent high-risk types and are the most common types identified in invasive cervical carcinoma. Results from the ALTS (ASC-US and LSIL Triage Study) trial in the late 1990s demonstrated that testing for high-risk HPV DNA performed better than repeat cytologic evaluation to identify women at risk for high-grade (CIN2 or greater) cervical disease [4]. This and other studies have led to the current Consensus Guidelines for the Management of Women with Abnormal Cervical Cancer Screening Tests established in 2006. These guidelines recommend that women 20 years or older with ASC-US pap results be tested for the presence of high-risk HPV DNA to determine their risk for cervical dysplasia or neoplasia [5]. Women who test positive for high-risk HPV DNA should undergo further clinical and pathologic examination with colposcopy and biopsy of suspicious lesions, while those testing negative for HPV DNA can be followed according to routine practice. The guidelines also recommend that women 30 years or older with normal cytology results should be screened for high-risk HPV DNA. Women with positive high-risk HPV test results should have HPV genotyping performed. If HPV types 16 or 18 are present, then the patient should be referred for immediate colposcopy. It is recommended that those women with a high-risk HPV that is not type 16 or 18 should receive repeat cytologic evaluation or a second HPV test one year later, while those with HPV-negative results can have less frequent exams, approximately every three years.

Question 2: Which test methods have been FDA approved for clinical HPV testing?

Test Ordered

Molecular testing for high-risk HPV detection was ordered.

Laboratory Test Performed

The patient's cervical cytology specimen was processed for HPV molecular detection with the Cervista™ HPV HR test (Hologic, Inc.) using Invader signal amplification technology.

Results with Interpretation Guideline

Cervista™ HPV HR test results demonstrated a FAM (fluorophore) fold over zero (FOZ) of 1.19 for the A reaction, 6.81 for the B reaction, and 2.17 for the C reaction (See the description of the assay in the Background and Molecular Pathology section). The FAM FOZ ratio was 5.71 (Fig. 33.1).

The FAM FOZ Ratio threshold has to be greater than or equal to 1.525 and at least one of the FAM FOZ values greater than 2.0 to call a specimen positive for HPV DNA.

Question 3: What is your interpretation of these results in the context of this patient?

Result Interpretation

The FAM FOZ ratio was 5.71 and both FAM FOZ for the B and C reactions were greater than 2.0. Thus, the result was interpreted as positive for high-risk HPV DNA.

Further Testing

Positive results for ASC-US and high-risk HPV indicated that the patient required colposcopic examination and biopsy of any suspicious lesions observed, to be submitted for review by a pathologist. This patient was identified to have CIN1.

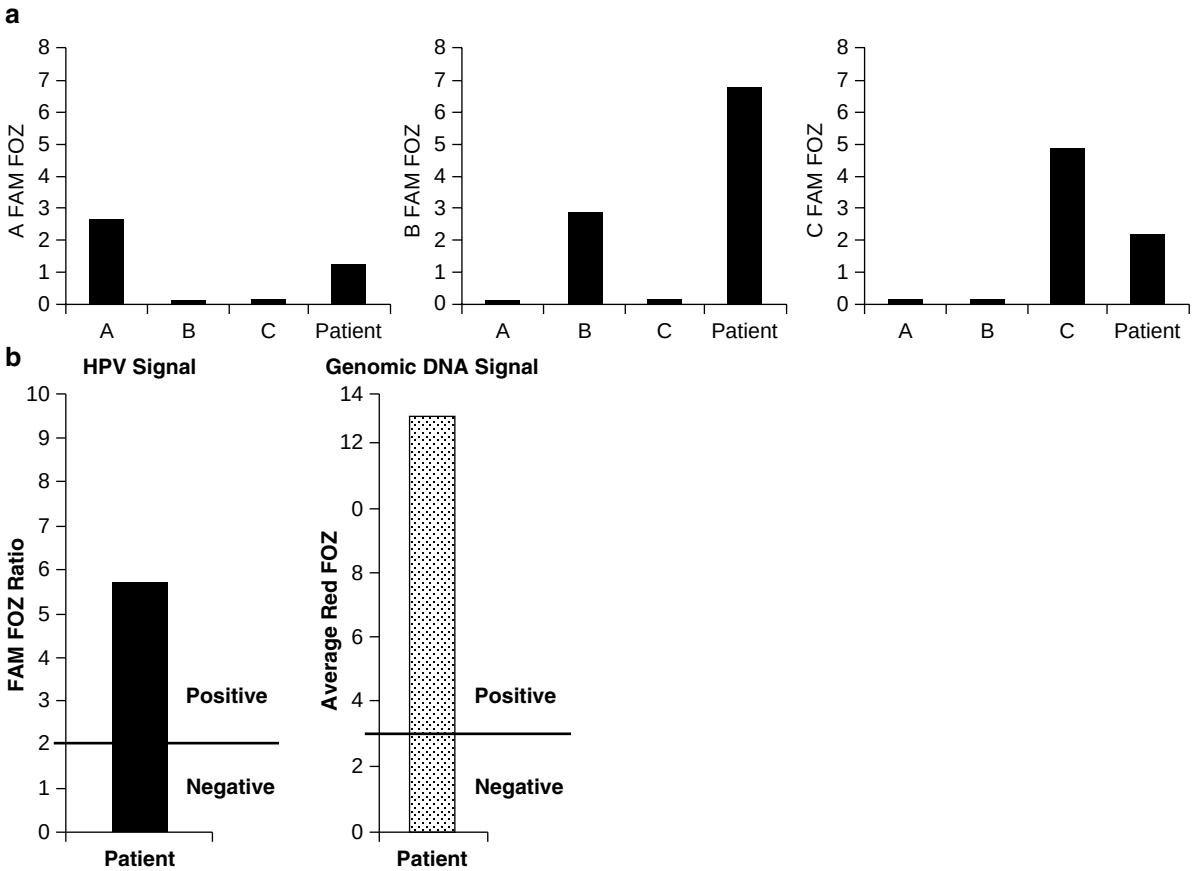

Fig. 33.1 Results of Cervista™ HPV HR testing. (**a**) The three bar graphs demonstrate the FAM FOZ results for each of the three HPV oligonucleotide reactions A, B, and C. Each positive control (A, B, and C) and the patient are labeled on the x-axis with the calculated FAM FOZ represented by the *bars*. (**b**) The graph on the *left* represents the patient's FAM FOZ ratio which is calculated by dividing the highest FAM FOZ value from any one of the three HPV oligonucleotide reactions by the lowest FAM FOZ value of the three. The graph on the *right* represents the control for DNA signal amplification as an average of the Red FOZ values from the signal amplification of the human histone 2β gene in the three reaction mixtures A, B, and C

Background and Molecular Pathology

The gold standard for the identification of cervical carcinoma in situ and its precursor lesions has been histologic analysis of a cervical biopsy specimen. However, this is an invasive form of testing with the potential to cause damage to the cervix. Cytologic evaluation of a swab or brush specimen collected from the squamocolumnar junction of the cervix has proven a good means of identifying potentially devastating disease while being much less invasive. Cytology has a very high specificity and thus a high positive predictive value, but suffers from poor sensitivity [6, 7]. HPV DNA testing has significantly better sensitivity than cytology for high-grade CIN2, or greater, cervical disease [8]. Two assays have been approved by the Federal Drug Administration (FDA) for the detection of HPV DNA and one for genotype identification of HPV 16 and 18: the Digene HPV Hybrid Capture® 2 (Qiagen, Inc.), Cervista HPV HR (Hologic, Inc.) and Cervista HPV-16 and 18 Genotyping tests. All three tests are approved for use with ThinPrep™ PreservCyt liquid-based cytology media (Hologic, Inc.), but not with the other commonly used liquid-based cytology media, Surepath™ (Becton Dickinson, Inc.). Laboratories using SurePath™ liquid-based cytology media have to independently validate the assay, which changes assay classification to a Laboratory-Developed Test (LDT).

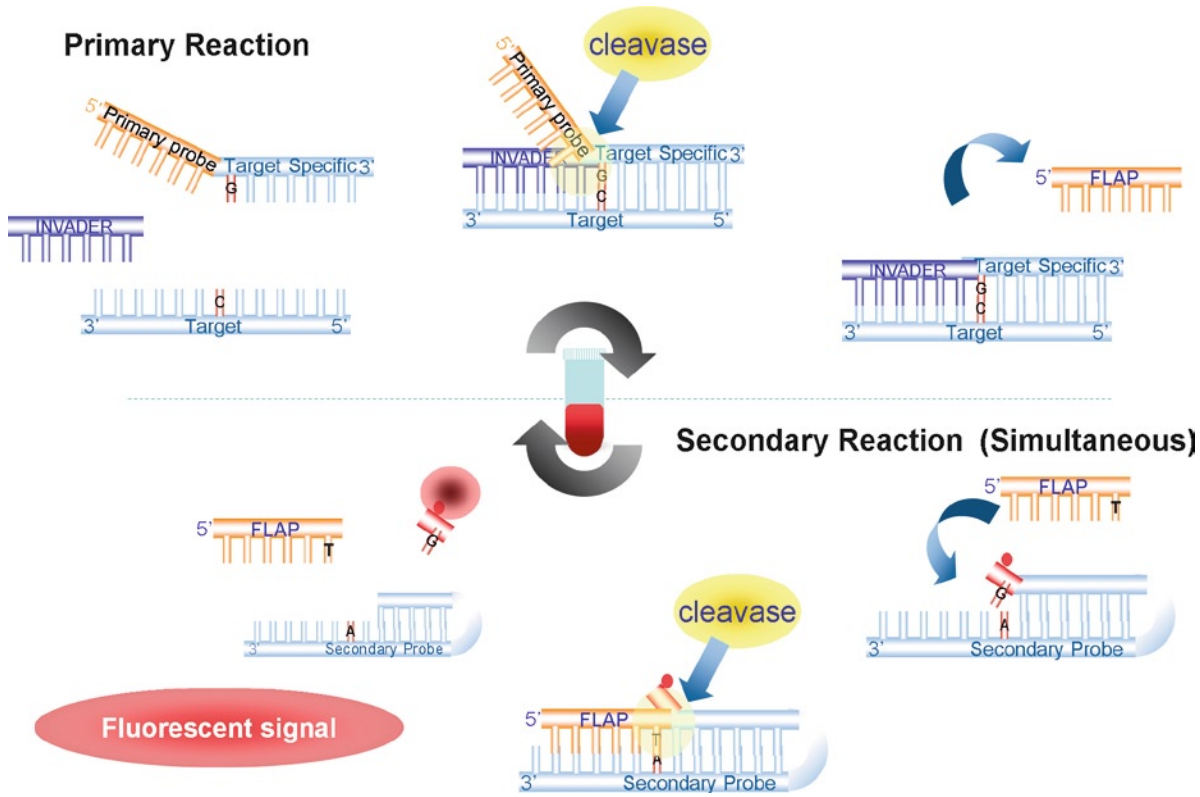

Fig. 33.2 Illustration of the Cervista™ HPV HR invader methodology. The primary reaction includes a Primary probe oligonucleotide and an Invader probe oligonucleotide (*dark blue*) that are complementary to the target DNA sequence; there is one set of Primary and Invader probes for detection of human histone (*HH*) 2B gene DNA and three different sets for high-risk HPV detection. After binding the target, Cleavase enzyme recognizes the complex of the target, primary probe, and invader probe and hydrolyzes the DNA back bone of the primary probe. This releases the Flap sequence and the complex falls apart so that fresh primary probe can bind. The Flap sequence is engineered to be complementary to the stem structure of a Secondary probe oligonucleotide. In the secondary reaction, Flap oligonucleotides bind to the appropriate Secondary probe; one labeled with red fluorescent dye that is complementary to the Flap oligonucleotide from the HH2B reaction and another secondary probe labeled with 6′FAM or green fluorescence that is complementary to the Flap oligonucleotide from the HPV-specific reactions. The engineered sequences for the Secondary probes form an internal hairpin loop structure that positions the fluorescent reporter dye (highlighted in red at the end of the secondary probe) next to its quencher dye preventing fluorescence transmission. The Flap acts like an Invader probe binding to the stem sequence of the secondary probe and creating a similar DNA complex that is recognized by the Cleavase enzyme, hydrolyzing the secondary probe and releasing the fluorescent dye. Red fluorescence indicates the presence of DNA in the sample and that signal amplification was successful. The 6′FAM or *green* fluorescence indicates the presence of HPV DNA

The Cervista™ HPV HR test is a signal amplification method of detection. Fluorescent signal is generated from three reactions for all specimens and controls (Fig. 33.2). Each reaction contains a combination of oligonucleotide probes complementary to the HPV DNA or to the control DNA, together with Cleavase enzyme which hydrolyzes the fluorescently labeled HPV or control DNA probes so that the appropriate fluorophores are released if the target DNA is present. The FAM (6-Carboxyfluorescein) fluorophore is present on the HPV-specific probes while the RED (Redmond Red dye) fluorophore is present on the probes for the human histone-2b gene as an internal control in each of the three separate multiplex probe reactions, A, B, and C. The HR HPV types cannot all be detected in a single reaction because of their sequence diversity but testing can be reduced to these three multiplexed reactions which are essentially grouped by their phylogenetic relationships. A signal to noise value referred to as a fold over zero (FOZ), which represents the signal from the sample or control measured against the similar signal measured from a no-DNA-target-control reaction for both the RED (RED FOZ) and FAM (FAM FOZ) signals is generated for each of the three reactions. The FAM FOZ values represent whether the specimen is positive for HPV DNA while the RED FOZ values demonstrate whether sufficient DNA was present for testing. When any FAM FOZ value is greater than one

(i.e., greater than the background), this value potentially represents signal from the presence of HPV DNA. A FAM FOZ ratio is calculated by dividing the highest FAM FOZ value from any one of the three reaction mixtures by the lowest FAM FOZ value of the three as an additional determination for whether a specimen is positive. Specific threshold values for the RED FOZ, each reaction's FAM FOZ, and the FAM FOZ ratio were determined during assay development and set specifically for delineating between positive and negative results as described above.

The Cervista™ HPV-16 and 18 Genotyping Test uses the Invader probe and cleavase signal amplification method, similar to the prior description. HPV-16 is the most common and HPV-18 is the second most common HPV type identified in cervical cancer. One of the reasons that genotype testing is recommended is because these two viruses have been shown to have a higher probability of progression to high-grade cervical disease (CIN3 or greater) than do other HR HPV types [9].

The Hybrid Capture® 2 (HC2) test uses RNA probe hybridization to the HPV DNA, antibody capture of the duplex DNA:RNA hybrid molecules, and detection with chemiluminescent signal amplification using a 96-well microtiter plate format. The test uses a pool of RNA probes, spanning the entire HPV genome, that are specific for 13 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) in a single reaction well. There is no identification of the specific type. The captured DNA:RNA hybrids are bound by alkaline phosphatase-conjugated antibodies and the cleavage of the chemiluminescent substrate emits light that is measured by a luminometer. The intensity of the light emitted denotes the presence or absence of target DNA in the specimen and is represented by the calculated value of a relative light unit (RLU). RLU signal threshold values have been set for determining positive results in the assay. The HC2 tested has been used in several large studies, and reproducibly demonstrates high sensitivity of 93–96% but has been reported to have the potential of false-positive results due to cross-reaction with low-risk HPV types [10].

Two vaccine formulations using the viral capsid proteins have been developed for administration to adolescent and young adult females nine to 26 years of age to prevent the development of cervical cancer: Gardasil (Merck, Inc.) and Cervarix (Glaxo-Smith-Kline, Inc.). Both vaccines protect only against high-risk types 16 and 18 although Gardasil also contains low-risk HPV 6 and 11 capsid proteins. In 2010, the FDA also approved the use of Gardasil to vaccinate males nine through 26 years of age for the prevention of genital warts caused by HPV types 6 and 11. Some potentially beneficial cross-reactivity with other related high-risk types has been postulated [11]. These vaccines have been shown to be highly efficacious with protection for CIN2 or greater approaching 100%. However, rare cases of CIN1 have been reported in women who received the vaccines in the FDA clinical trials [12]. Because the vaccines are targeted against a limited number of high-risk HPV types, any of the other high-risk HPV types could be responsible for abnormal test results. Also, the vaccines are designed to prevent acquisition of infection with HPV 16 and HPV 18 and not to treat infections present at the time of vaccination [13]. In most situations where lesions are detected early in the follow-up of a vaccinated sexually active woman, these lesions are likely caused by infections present before vaccination.

Multiple Choice Questions

1. Which high-risk HPV type accounts for the highest percentage of disease of the cervix?
   A. 6
   B. 11
   C. 16
   D. 18
   E. 31
2. Transmission of high-risk HPV which can lead to cervical cancer occurs by which of the following?
   A. Contact by infected epithelium with mucous membranes, as occurs with sexual activity
   B. Hand shaking or contact with individuals who have plantar warts or warts on their hands
   C. Poor sanitation and fecal–oral route
   D. Respiratory droplet transmission from coughing or sneezing
   E. Urinary contamination and poor sanitation
3. The recommendations from the 2006 Consensus Guidelines suggest all of the following *EXCEPT*:
   A. All women 30 years and older should be screened for high-risk (HR) HPV DNA testing from their cytology specimen
   B. HPV-16 and HPV-18 genotyping is not recommended as the initial screening test for women 30 years and older
   C. HPV-16 and HPV-18 genotyping should be used for women 30 years and older with HR HPV DNA to determine whether to perform colposcopy and biopsy of suspicious lesions or wait 12 months for repeat cytology testing

D. HR HPV DNA testing should be included in evaluating a patient with atypical glandular cells of undetermined significance (AGUS)
E. Women with atypical squamous cells of undetermined significance (ASC-US) cytologic results of any age should have HR HPV DNA testing on that cytology specimen

4. The only FDA-approved methods currently available for detection of high-risk human papillomavirus are based on which methods?
   A. Branched-chain DNA signal amplification and real-time PCR
   B. Hybrid capture signal amplification using antibodies to RNA/DNA heteroduplexes and real-time PCR
   C. Invader/cleavase signal amplification and Hybrid capture using antibodies to RNA/DNA heteroduplexes
   D. Real-time PCR and Invader/Cleavase signal amplification
   E. Transcription-mediated target amplification and Hybrid Capture using antibodies to RNA/DNA heteroduplexes
5. Which sentence most appropriately describes the Digene (Qiagen, Inc) Hybrid Capture II assay?
   A. It has become available in 2009 for detection of 14 high-risk types and genotyping of HPV types 16 and 18
   B. It is a signal amplification method using RNA probes and antibodies specific for RNA:DNA duplex hybrids
   C. The assay uses PCR amplification to detect 37 high-risk HPV types
   D. The captured DNA:DNA hybrids are detected by antibodies conjugated to alkaline phosphatase
   E. The performance parameters include better specificity than cytology for detection of CIN2 or greater disease

Answers to Multiple Choice Questions

1. *The correct answer is C.*

HPV-16 is the most common type found in cervical cancer and cervical dysplasia, accounting for more than 50% of high-risk types identified. HPV-18 is the second most common high-risk type and HPV-31 is in the top five types identified. Low-risk types HPV-6 and 11 are associated with the development of genital warts.

2. *The correct answer is A.*

High-risk HPVs are only found in the superficial epithelium and mucous membranes of the genital tract and oral cavity. They are primarily spread by sexual contact. Benign HPV types, such as plantar or hand warts do not cause cervical cancer. The other three routes of transmission are not associated with HPV transmission.

3. *The correct answer is E.*

ASC-US cytology results should not be tested for HPV DNA in women less than 20 years of age. All the other foils are correct.

4. *The correct answer is C.*

Invader/cleavase signal amplification and Hybrid capture using antibodies to RNA/DNA heteroduplexes are the only FDA-approved methods currently available for detection of high-risk human papillomavirus.

5. *The correct answer is B.*

Answer A refers to the Cervista HPV assays. There are currently 14 HPV types that are considered high risk, not 37 as in answer C. Answer D is not correct. High-risk HPV DNA testing (answer E) has been shown to demonstrate greater *sensitivity* than cytology for detection of CIN2 (or greater) disease.

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Chlamydia trachomatis

34

Ruan T. Ramjit and Angela M. Caliendo

Clinical Background

A 29-year-old male with a history of HIV-1 infection since 2002 was being treated with antiretroviral therapy until 18 months ago when he decided to discontinue all of his medications. He presented to the emergency department describing a five month duration of intermittent left lower quadrant abdominal pain which became worse after eating. The pain had increased in severity in the weeks leading up to his admission and had become so excruciating that the patient was unable to have a bowel movement over the last several days. He denied fever, chills, or night sweats but indicated he had lost 10–15 pounds within the last two months. Review of his chart revealed prior perianal human papilloma virus (HPV) and syphilis infections. The pertinent laboratory values were a CD4 cell count of 250 cells/mm^3 and a HIV-1 viral load of 110,000 copies/mL. A CT scan was performed which showed moderate ascites in the pelvis, perirectal and inguinal lymphadenopathy, and thickening of the rectal wall consistent with proctitis. A flexible sigmoidoscopy (Fig. 34.1) and a biopsy (Fig. 34.2) were also performed.

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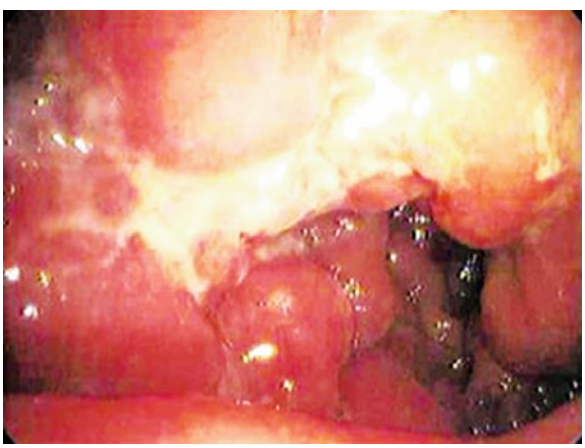

Fig. 34.1 Flexible sigmoidoscopy of the rectal area revealed circumferential ulceration and edema including a protuberant mass-like lesion covered with purulent exudate

Question 1: Based on the above clinical and pathologic information, what is your differential diagnosis?

Reason for Molecular Testing

The differential diagnosis of proctitis to be considered in HIV-1-positive men who have sex with men (MSM) includes multiple infectious diseases such as *Neisseria gonorrhoeae*, *Chlamydia trachomatis* serovars D through K (genital tract disease) and serovars L1 through L3 (lymphogranuloma venereum), herpes simplex virus (HSV), HPV, and primary syphilis

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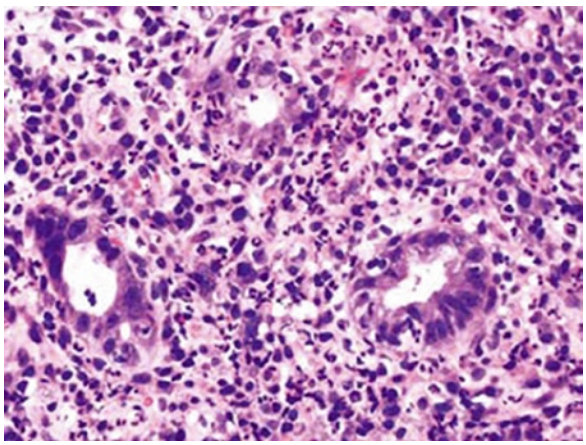

Fig. 34.2 Biopsy showed a dense mixed inflammatory infiltrate in the lamina propria consisting of neutrophils, lymphocytes, histiocytes, and plasma cells with crypt abscess formation. No viral inclusions were seen

(*Treponema pallidum*). As part of the workup for the cause of proctitis in this patient, molecular testing for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* was ordered on a rectal swab.

Test Ordered

The laboratory test available for the detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* was a molecular assay that used strand displacement amplification (SDA). This type of technology allows for the direct, qualitative detection of *Chlamydia trachomatis* or *Neisseria gonorrhoeae* DNA from endocervical swabs, male urethral swabs, and urine specimens from male or female patients. In addition, specimens may be obtained from symptomatic or asymptomatic males or females. The reagent pack for this test has the option of including a separate amplification control (AC) for PCR inhibition testing.

Laboratory Test Performed

Before discussing the common clinically available molecular tests for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infections, it is important to note a few points about other laboratory techniques. First, serologic tests for chlamydial infections have little diagnostic value. Antibodies to chlamydial infections usually persist after the infection resolves and are not indicative of active disease. Further, these tests lack specificity and cannot differentiate between the *Chlamydia trachomatis* serovars that cause urethritis and cervicitis from the L1 to L3 serovars that cause lymphogranuloma venereum (LGV). As it relates to this clinical case, serologic testing was also not validated for patients presenting with possible LGV proctitis. Finally, routine culture has largely been replaced by molecular methods for identification of these pathogens and one of the most frequently used assays is performed by SDA.

For this patient, testing for *C. trachomatis* (CT) and *N. gonorrhoeae* (GC or NG) was performed by SDA. The target for CT in this test is the cryptic plasmid. In SDA, a double-stranded DNA target is denatured and allowed to hybridize two primers, one known as a "bumper primer", and another primer containing the single-stranded restriction enzyme sequence 5′ to a target binding region to be amplified [1]. With the addition of DNA polymerase and dNTPs, simultaneous extension of both primers occurs, producing exponential target amplification. Real-time detection of the products takes place by restriction enzyme cleavage and binding of a fluorescent probe to the single-stranded products [1].

Performance of the laboratory test has a relatively simple work flow designed to allow for multiple analytic runs within a single shift and for a relatively short turnaround time. To conduct the test, the SDA reagents are received dried into two separate disposable microwell strips. The processed sample, which rehydrates the dried reagents, is added to the priming microwell which contains the amplification primers, the fluorescent detector probe, and other reagents necessary for amplification. Because amplification does not occur in the priming microwells, no amplicon contamination is possible at this stage. After incubation, the reaction mixture is transferred to the amplification microwell which contains a DNA polymerase and a restriction enzyme necessary for SDA. If the reagent pack containing the amplification control is used for monitoring reaction inhibition, then each sample plus the control is tested in three separate microwells (one each for GC, CT, and AC). The amplification microwells are sealed to prevent contamination and are incubated in a thermally controlled fluorescent reader which monitors the reaction for the generation of amplicon. The qualitative presence or absence of CT or GC is determined by

Table 34.1 Patient's result panel

Patient test panel	MOTA score	Interpretation
Chlamydia trachomatis (CT)	34,815	?
Neisseria gonorrhoeae (GC)	155	?
Amplification control (AC)	20,002	?

comparing MOTA (Method Other Than Acceleration) scores for the patient sample to predetermined cutoff values. The MOTA score is a value used to assess the scale of the signal produced due to the reaction and does not indicate the level of organism in the sample.

Besides the assay described above, there are other FDA-cleared tests for the detection of CT and NG from clinical specimens. These tests are the APTIMA CT assay (based on target capture and transcription-mediated amplification), the PACE 2 CT probe competition assay, HC2 CT ID (hybrid capture technology), the Abbott Real-time CT/NG test (real-time PCR), and two conventional PCR amplification assays, the AMPLICOR CT/NG test and the COBAS AMPLICOR CT/NG test. Each of these methods utilizes a variety of specimen types including cervical and vaginal swabs, urethral swabs, and urine from both asymptomatic and symptomatic individuals. Not all assays are approved for both conditions, and the current assays are not FDA-cleared for oral, rectal, respiratory, or conjunctival specimens.

Results with Interpretation Guideline

Table 34.1 reflects a panel of the patient's results from the rectal swab. Table 34.2 can be used to interpret the values.

Question 2: Based on the data given and the specimen type provided to the molecular diagnostic laboratory, what is the most appropriate way to report the patient's results?

Result Interpretation

The patient tested positive for *C. trachomatis* DNA depicted by a MOTA score of ≥10,000. The MOTA score for *N. gonorrhoeae* (<2,000) illustrates a negative result. Review of the values for the amplification control showed that it was elevated above the specified MOTA score cutoff and that there was no inhibition of SDA in the sample. Following institutional laboratory testing, the remaining specimen was sent to the Centers for Disease Control and Prevention (CDC) for sequencing of the outer membrane protein A (ompA) gene and the result was serovar L2b, consistent with the diagnosis of lymphogranuloma venereum proctitis.

Further Testing

Patients diagnosed with LGV proctitis are generally placed on antibiotic regimens of longer duration, compared with the time period used to treat anogenital infections caused by non-LGV *Chlamydia* serovars [2]. The current recommendation is a minimum 21-day course of doxycycline for LGV proctitis versus a seven day course for proctitis caused by non-LGV *Chlamydia* infections [3]. This treatment regimen relates to guidelines from the CDC and to efforts aimed at evaluating the microbial cure rate in LGV proctitis by screening rectal swab specimens collected during and after treatment [2, 3]. It was found that the detection of *Chlamydia* RNA, implying remaining infectious organisms, could persist for prolonged periods (up to 16 days) in patients suffering from LGV proctitis thereby lending further support to the 21-day treatment duration [2]. In patients who did not become reinfected, the extended course of doxycycline was successful at clearing the infection. As a result, additional molecular testing by the assay described in this clinical case is not likely to be beneficial to the patient

Table 34.2 CT/GC/AC MOTA score interpretation values

CT or GC MOTA score	AC MOTA score	Result	Interpretation
≥ 10,000	Any	Positive	*C. trachomatis* DNA and/or *N. gonorrhoeae* DNA detected by SDA
2,000–9,999	Any	Low positive	*C. trachomatis* and/or *N. gonorrhoeae* likely. Supplemental testing may be helpful to verify the presence of either of these organisms
<2,000	≥1,000	Negative	*C. trachomatis* or *N. gonorrhoeae* DNA not detected by SDA
<2,000	<1,000	Indeterminate	Amplification control inhibited. Repeat test on next run. If inhibited again, specimen is reported as inhibited

unless he were to become reinfected. This is because DNA for *C. trachomatis* and *N. gonorrhoeae* can persist in the absence of viable organisms and infectivity or viability cannot be inferred from the results.

Other Considerations

This clinical case presents the scenario of running a molecular test on a clinical sample for which the assay was not FDA cleared. In order to appropriately report the patient's results, the laboratory must perform a validation study on rectal specimens. In this situation, the assay was validated using specimens from an outbreak of rectal proctitis caused by *C. trachomatis* in our HIV-1 infected patient population. Adequate specimens were collected to determine the sensitivity and specificity of the assay and all positive results were confirmed by DNA sequencing.

Question 3: Do you think the available molecular tests for CT have the ability to distinguish between the CT serovars that cause LGV and those that do not?

It is important to recognize that though the available molecular tests for *C. trachomatis* can detect all serovars of CT, they cannot distinguish between the L1 through L3 serovars causing LGV and other serovars of CT or variant strains. An example of this occurred in 2006 in Sweden when a variant strain of CT was identified with a 377 base pair deletion in the cryptic plasmid, the target for several of the available CT tests [4, 5]. This deletion led to false negative results with some, but not all, of the tests that target the cryptic plasmid. Assays that target other regions of the organism were not affected. The ability of a test to detect this variant is crucial when choosing a test, particularly if this variant is commonly found in the specific geographic area from which the laboratory receives specimens for testing.

Question 4: Based on what you have learned and what you know about laboratory testing, what do you think could be causes of false positive and false negative results when performing molecular testing for CT?

Another concern for laboratories is the generation of a false positive result which can occur from carryover contamination of amplified product and cross-contamination during specimen collection, transport, or processing. False positive results in a low-prevalence population can significantly reduce the predictive value of a positive result. For example, although the specificity of nucleic acid testing for GC or CT generally ranges from 98% to 99%, the positive predictive value may be as low as 60–70% in a population with a low prevalence.

A false negative result due to amplification inhibition is a consideration for both GC and CT testing and has been reported for both cervical swabs and urine specimens [4, 6, 7]. Inhibition rates vary depending on the amplification method and are partly related to the nucleic acid extraction method used [8]. For tests that use a crude lysate, like the assay described in our clinical case, inhibition rates tend to be higher than those seen with alternative approaches such as the APTIMA CT test which uses the target capture method to purify nucleic acid. When performing testing on a crude lysate, an internal control or amplification control should be included to assess inhibition of amplification. A specimen cannot be reported as negative for GC or CT unless there was amplification of the internal control.

Background and Molecular Pathology

Lymphogranuloma venereum (LGV) is a sexually transmitted disease caused by *Chlamydia trachomatis* serovars L1, L2, and L3. In contrast to serovars A-K which are mostly confined to the mucosal epithelial surfaces of the genital tract or eye, the serovars of LGV infect predominantly monocytes and macrophages allowing passage to lymph nodes to cause disseminated infection [9].

The disease typically manifests a chronic course with clinical presentations ranging from genital ulcers, infected inguinal lymph nodes (buboes), and proctitis/proctocolitis. The course of infection can be separated into three stages. In the primary stage, inoculation occurs. Inoculation may involve the external genitalia or in the case of proctitis, the perianal region. After about three to 30 days, a small, painless papule appears which may ulcerate [9]. This lesion is typically self-limiting and may go completely unnoticed by the patient. Some weeks after the appearance of the primary lesion, the inguinal lymph nodes, anus, or rectum become involved and this signifies the secondary stage [9]. Enlarged, painful inguinal lymph nodes are usually firm and biopsy reveals discrete

areas of necrosis surrounded by proliferating epithelioid cells and endothelial cells [9]. Proctitis due to LGV is more common in men and women who practice unprotected receptive anal intercourse and usually presents with rectal pain, constipation, and bleeding often with pronounced systemic symptoms of fever, chills, and weight loss [9]. Sigmoidoscopy of patients with symptoms of LGV proctitis reveals hyperemic, friable mucosa with areas of ulceration covered by mucopurulent or frankly purulent exudate [10]. If untreated, LGV-infected patients can progress to the tertiary stage of infection which leads to scarring, fibrosis, and the formation of strictures or fistulae in those with rectal involvement [9, 10].

In 2003, though previously considered a rare disease entity in developed nations, LGV became increasingly reported in MSM in both Europe and the United States of America [10]. In an outbreak from The Netherlands, the patients presented with proctitis rather than genital ulceration or the typical inguinal buboes, characteristic of LGV from endemic regions [11]. The majority of these men with LGV were also HIV-positive [11]. Due to the initially less obvious clinical features, it was speculated that the LGV strains associated with the European outbreak, causing proctitis, represented a new emerging infection. Whereas it is known that the serovars involved in LGV are L1, L2, and L3, DNA sequencing of the outer membrane protein A (ompA) gene of *Chlamydia trachomatis* allowed identification of the isolates down to a new variant of LGV serovar L2, known as L2b [12].

Multiple Choice Questions

1. The strain of LGV found in the European outbreak of rectal proctitis was due to which of the following serovar variants?
   A. L1b
   B. L2a
   C. L2b
   D. L2c
   E. L3b
2. Which gene is sequenced to determine the LGV serovar variations?
   A. Cryptic plasmid DNA
   B. Outer membrane protein A gene
   C. Reticulate body DNA
   D. Sequencing does not need to be performed; serologic antibodies are used
   E. None of the above
3. A molecular test has only been FDA-cleared for endocervical swabs, urethral swabs, and male or female urine specimens. Your infectious disease clinicians request that you offer testing on rectal swabs. Which of the following statements is the most correct with regard to reporting the results?
   A. Inform the clinicians that testing the rectal swab is not possible
   B. Inform them you will call them with the results, but not put the result in the medical record
   C. Just test the rectal swab, no further laboratory validation is needed
   D. Laboratory validation of rectal swab specimens would be required before clinical testing could be offered
   E. You cannot perform a validation if the assay was not FDA-cleared for that specimen type
4. You have just received a new molecular assay in your lab. The package insert indicates the results are reported by Method Other Than Acceleration (MOTA) score. Which of the following statements is true of the MOTA score?
   A. The assay provides both a quantitative and qualitative result
   B. The assay will not provide any results; you will have to determine the cutoff for each sample
   C. The assay will provide a qualitative result
   D. The assay will provide a quantitative result
   E. The MOTA score must be confirmed by DNA sequencing before reporting the patient's results
5. When is an internal control needed for a *C. trachomatis* molecular test?
   A. An internal control is never required
   B. Not required for cervical or urine specimens
   C. Required regardless of the extraction or test method
   D. Should be used in the APTIMA CT assay
   E. Should be used when testing a crude lysate

Answers to Multiple Choice Questions

1. *The correct answer is C.*
2. *The correct answer is B.*
3. *The correct answer is D.*
4. *The correct answer is C.*
5. *The correct answer is E.*

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Additional Reading

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MRSA

35

Donna M. Wolk

Clinical Background

The patient, a 16-year-old male, who was a previously healthy high school athlete, was admitted to the intensive care unit via the emergency department (ED) with a four day history of fever, chills, and occasional rigor, myalgia, and productive cough, now with increasing dyspnea and hemoptysis over the past 24 hours. His temperature was 38.1°C, blood pressure was 133/87 mm Hg, pulse 104 beats per minute, and his respiratory rate 16 per minute. He was well developed and well nourished, but in respiratory distress, with tachycardia and coarse rhonchi bilaterally at the bases of both lungs. Six weeks prior to presentation, the patient was hospitalized for knee surgery due to an injury he sustained in a North American basketball tournament. At the time of surgery the patient was screened for methicillin-resistant *Staphylococcus aureus* (MRSA) with an FDA-approved MRSA polymerase chain reaction (PCR) assay, as part of a hospital-wide active surveillance program, aimed at prevention of hospital transmission of infections. At that time, the surveillance test result was reported as "MRSA Detected." There was no personal or family history of diabetes mellitus and no immunosuppression. The patient denied HIV risk factors and denied the use of illicit drugs. He had no recent history of skin infection or chronic dermatitis. A review of systems was noncontributory. The patient had no significant medical history and lived with his parents and his six year-old sister, who had been sick with a "cold with dry cough" one week before. There was no history of medication allergies, and he had received no antibiotics within two weeks prior to the time of his presentation. Except for the basketball tournament, held in Canada, he had no recent travel or animal exposure and no known exposure to tuberculosis. His physical examination was otherwise unremarkable. Because the time frame for his respiratory symptoms was in the midst of influenza season, a nasopharyngeal swab was submitted for rapid influenza reverse-transcription PCR (RT-PCR), a sputum sample was submitted for Gram's stain and bacterial culture, and two sets of blood cultures were obtained prior to starting the patient's antiviral and antibiotic therapy. The surgery site showed no evidence of infection and was not cultured from the ED. In the ED, empiric therapy with oseltamivir (75 mg twice per day), vancomycin (1 g every 12 h), and ceftriaxone (2 g every 24 h) was initiated. The patient was admitted to the hospital with an initial diagnosis of viral pneumonia.

Reason for Molecular Testing

The spread of drug-resistant bacteria, such as MRSA, is a major concern for healthcare and for communities in which aggressive infections have caused deaths in both hospitalized patients and otherwise healthy individuals [1]. A known colonizer, MRSA is often harbored in the human nares, skin, throat, and in mucosa of the vagina and rectum. Colonized patients (carriers) have a high likelihood of developing and transmitting infections.

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The National Health and Safety Network (NHNS) estimated that in the USA, hospitalized patients acquire two million hospital-acquired infections (HAIs) each year, causing 90,000 deaths and $4.5 billion in excess healthcare costs; a large percentage of HAIs are due to MRSA [2]. The increasing prevalence and spread of MRSA is a worldwide pandemic. Infections have a negative economic impact due to prolonged and more costly hospital stays, and are associated with elevated morbidity and mortality [1, 3]. The Centers for Disease Control (CDC) issued guidelines to limit the spread of HAIs, which include options for hand hygiene, environmental cleaning, and active surveillance to identify MRSA reservoirs so that contact precautions may be initiated.

In the late 1990s, MRSA infections were reported among previously healthy individuals in the community who lacked the usual healthcare-associated risk factors. Since then, outbreaks of community-associated MRSA (CA-MRSA) have been reported in multiple diverse populations including prison inmates, athletic teams, military personnel, and domestic households [1]. By 2005, MRSA was identified as the predominant cause of skin and soft tissue infections in patients presenting to the ED [4].

For these and more reasons, a tremendous amount of effort and resources have been recently focused on the development of several rapid molecular screening tests for MRSA [5].

In response to recent public awareness, routine infection prevention practices, such as active surveillance programs, have been established by many accredited healthcare facilities. Most active surveillance programs rely on rapid detection methods [5], such as chromogenic agar or real-time polymerase chain reaction to support these efforts, which can significantly reduce the number of HAIs caused by MRSA [6, 7].

It is prudent to base the selection of laboratory methods for active surveillance on the reported literature and to customize efforts for each hospital setting, based on the needs and the resources in that setting [8]. Decisions regarding resource utilization must be made by each hospital based on local issues such as number of isolation beds available, the current MRSA transmission rate, the acumen of laboratory staff, hospital staffing, space, and capital funds.

Historically, agar susceptibility methods and latex confirmation of methicillin resistance relied on detection of products derived from expression of the *mec*A gene. The *mecA* gene is the structural gene responsible for production of an altered Penicillin Binding Protein, PBP2a, which maintains staphylococcal cell wall integrity because of its low affinity for β-lactam antibiotics. The gene is present within a mobile genetic region known as the staphylococcal cassette chromosome *mec* (SCC*mec*). When the SCC*mec* cassette is inserted into the open reading frame (*orfX*) gene of *S. aureus*, it becomes the primary genetic basis for methicillin resistance. Other methicillin resistance mechanisms are known, but *mecA* is the most common [9]. As exemplified in this case, molecular detection of MRSA by real-time PCR is becoming more commonplace in active surveillance programs and supports rapid identification of MRSA carriers.

PCR provides a sensitive method to identify MRSA carrier status and is often categorized as an "improved gold standard" when compared to direct routine culture. Of note, improved gold standard methods often have a lower calculated positive predictive value (PPV), which is calculated by dividing the number of true positives by the sum of true positives plus false positives. In other words, due to their enhanced ability to identify microbes in low densities, PCR methods can produce "PCR-positive, culture-negative" results. In contrast, they exhibit a very high negative predictive value (NPV), i.e., the number of true negatives divided by the sum of true negatives plus false negatives, a performance characteristic that is suitable for laboratory "screening" methods. Consistent with the expectations of an improved gold standard method, MRSA PCR assay results can be MRSA positive, despite a culture negative status, for 5–10% of specimens tested [10, 11].

Test Ordered

Upon admission, another nares specimen was collected and used for testing via MRSA PCR as part of the active surveillance program. Cepheid's Xpert™ MRSA assay was performed. Sputum cultures were also performed as well as two sets of blood cultures, and a reverse transcriptase PCR for influenza.

Laboratory Test Performed

Besides routine bacterial cultures for sputum and blood, two molecular tests were performed, the influenza A RT-PCR and the MRSA PCR; the latter is the focus of the chapter and will be discussed in detail.

Two PCR methods are currently FDA-cleared for detection of MRSA from nares specimens. In the order of their FDA clearance, they are: (1) the BD GeneOhm™ MRSA (BDGO MRSA) assay (BD Diagnostics, San Diego, CA), performed on the Cepheid SmartCycler and (2) the Xpert™ MRSA (Cepheid, Sunnyvale, CA), performed on the GeneXpert Dx system. Both assays offer equivalent performance and an advantage in the method's speed over agar-based cultures with PCR cycling times of 2 hours or less. Despite the fact that the targets of each assay are operationally distinct, both the Xpert MRSA and BDGO assays target SCC*mec* insertion sequences and regions within the *orfX* gene. Both PCR assays are known to have diminished performance due to genetic sequence variations in certain geographic regions such as Canada and Europe [12, 13]. Genetic diversity is due to sequence variations in the SCC*mec* element.

The Xpert™ MRSA Assay is a closed system real-time PCR assay, for detection of the SCC*mec*-*orf*X junction [14]. The assay targets the SCC*mec* cassettes, Type I-V, with primers and probes targeting a proprietary sequence signaling the presence of the SCC*mec* cassette inserted into the *S. aureus* chromosome. With an achievable turnaround time of less than 1 hour, it is currently the most rapid of all the commercial MRSA PCR methods. The Xpert MRSA assay performance compares to that of the BDGO MRSA assay; no statistical performance differences were observed between the Xpert and BDGO MRSA assays when compared to culture methods [11]. An advantage is the assay's ease of use, as it can be performed as a moderate instead of high complexity method per Clinical Laboratory Improvement Act (CLIA) classification. Thus, the Xpert method is amenable to a variety of healthcare settings that range from the clinical laboratory to off-site point-of-care testing and can be performed on-demand by laboratory technologists or technicians.

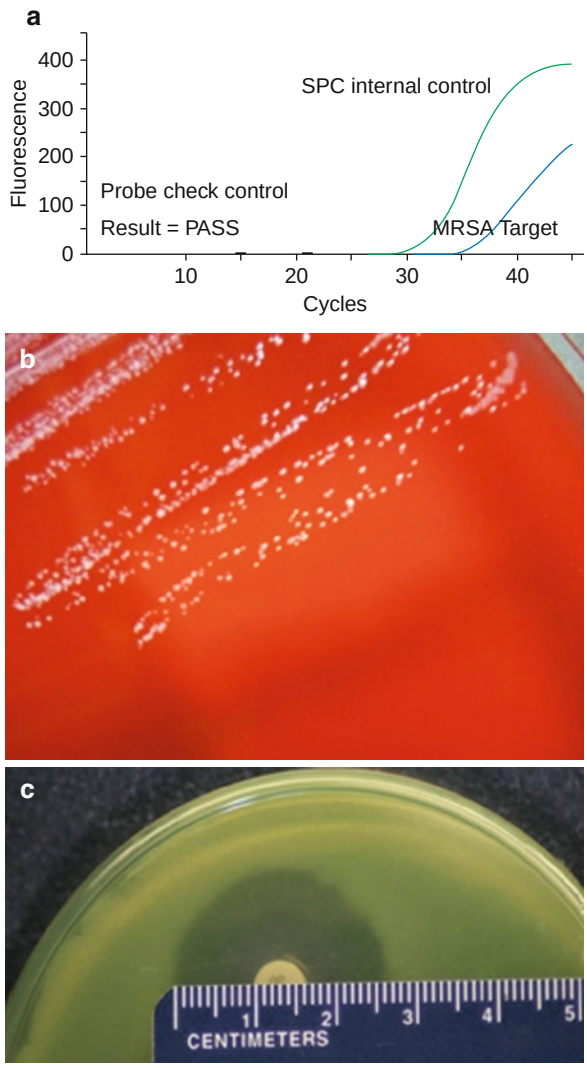

Fig. 35.1 Patient results from PCR from nares and agar subculture of a colony from the sputum culture. **(a)** Cepheid Xpert MRSA result from nares specimen with a crossing threshold (Ct) = 35.5 is depicted in *blue*. A Specimen Processing Control (SPC) is depicted in *green*. The probe check control = PASS. **(b)** Culture results. Blood Agar, colony subcultured from sputum culture. **(c)** Cefoxitin disk test from colony from sputum culture, zone size of 26 mm (Note: QC was acceptable)

Results with Interpretation Guideline

GeneXpert Dx System Interpretation

Results are derived from measured fluorescent signals (Fig. 35.1a). Typically, the software algorithms make the interpretation of the results; however, the actual cycle threshold (Ct) results are depicted here to enable independent result interpretation. Besides the PCR primers and probes for the MRSA target, there are two controls integrated into the Xpert PCR assay cartridge: (1) The sample processing control (SPC), which contains spores of *Bacillus globigii*, is used as a surrogate to verify that adequate microbe lysis has occurred and to verify that specimen processing was adequate. Additionally, this control detects specimen-associated inhibition of the real-time PCR assay. The SPC should

be positive in a negative sample and can be negative or positive in a positive sample, depending on the relative abundance of MRSA target. The SPC passes if it meets the validated acceptance criteria, producing a discernable amplification curve of sufficient Ct value. (2) The probe check control (PCC) is measured before the start of the PCR reaction, when the GeneXpert® Dx System measures the fluorescence signal from the probes alone to monitor bead rehydration, reaction-tube filling, probe integrity, and fluorophore stability. The PCC passes if it meets the assigned acceptance criteria, designated in the software. Possible Xpert MRSA Assay results are as follows:

MRSA POSITIVE: The MRSA target (SCC*mec* insertion site) has a Ct within the valid range (Ct of 36 or less) and an endpoint above the minimum setting (as defined by proprietary instrument software). The SPC may be positive, or negative, because MRSA from the sample can out-compete the SPC. PCC results must pass.

MRSA NEGATIVE: MRSA target DNA is not detected (the patient is presumed not to be colonized with MRSA), SPC meets acceptance criteria with a Ct within the valid range and an endpoint above the endpoint minimum setting. All PCC results must pass.

INVALID: The MRSA target result is negative and the SPC Ct is not within the valid range with the endpoint below the minimum setting. Presence or absence of MRSA cannot be determined and the SPC does not meet acceptance criteria, indicating that the sample was not properly processed, or that PCR is inhibited. All PCC results pass.

ERROR: At least one of the PCC results fails. Presence or absence of MRSA cannot be determined because the PCC failed, probably due to the fact that the reaction tube was filled improperly, a probe integrity problem was detected, or because the maximum pressure limits were exceeded.

In our patient, the PCC was valid. The SPC and MRSA target are depicted in left and right amplification plots, respectively: the MRSA target – SCC*mec* insertion site – has an amplification Ct less than 36 and an endpoint above the software-determined minimum setting; the SPCs target was amplified.

Influenza RT-PCR Interpretation

From the nasal swab, testing was positive for influenza A by RT-PCR.

Blood Agar Interpretation

After 32 hours of blood culture incubation, both sets of blood culture bottles flagged positive and stains revealed Gram-positive cocci in clusters (Fig. 35.1b, c). White, opaque, weakly β-hemolytic colonies were observed and were consistent with *S. aureus* colonies by phenotype and by biochemical testing. Susceptibility testing, performed by testing with a 30 μg cefoxitin disk, was consistent with methicillin-susceptible *S. aureus* (MSSA) with the zone of inhibition >22 mm (isolates are consistent with MRSA when the zone of inhibition is ≤22 mm), according to Clinical Laboratory Standards Institute (CLSI) guidelines set forth for agar disk diffusion. Similar colonies were identified as the predominant organism from the patient's sputum culture.

Result Interpretation

Question 1: Is the MRSA PCR valid according to the provided interpretation guideline?
Question 2: What is your interpretation of the patient's results?
In the GeneXpert MRSA Assay, the target (the SCC*mec* insertion site) has a Ct within the valid range and an endpoint above the minimum setting. The SPC is also positive and all PCC results passed. The assay result is valid and indicates the presence of MRSA.

The blood and sputum cultures subsequently yielded methicillin-sensitive *S. aureus* (MSSA) with an oxacillin minimum inhibitory concentration (MIC) of <0.5 μg/mL. The cefoxitin zone size was 26 mm, indicating that the isolate was susceptible to oxacillin (MSSA). A penicillin-binding protein 2′ (PBP2′) latex agglutination assay performed on colonies of the isolate was negative, consistent with MSSA. MRSA was not cultivated by routine culture techniques in sputum or blood, which leads to a possible discrepancy with surveillance testing by PCR, and an antibiotic treatment conundrum for this patient's physician.

Question 3: How do you explain this discrepancy between PCR and agar culture?

Several issues could have contributed to this discrepancy, a potentially false-positive PCR result. First, and most obvious, is the possibility that PCR is more sensitive than culture. PCR is on par or more sensitive than broth-enriched culture and far more sensitive than

direct plating, even when chromogenic agar is used [5], and especially when compared to blood agar culture.

PCR-positive, culture-negative results could also be consistent with nongenetic circumstances. For instance, poor sampling or handling of the sputum may limit bacterial recovery in culture. In addition, low bacterial densities in the nares samples can render the culture negative and the PCR positive, or vice versa, because low bacterial densities, typical with emerging subpopulations, may cause both methods to produce sporadic positive or negative results under the parameters described by the statistical phenomenon known as the Poisson effect. Finally, there is documented evidence that respiratory specimens, among others, are prone to false-negative results, which can be corrected by the use of selective agar [15] to confirm the true presence of MRSA present in low density.

Microbial growth characteristics can also be responsible for PCR-positive, culture-negative results. Reasons for the discrepancy can include staphylococcal strains that grow slowly, those that require the presence of blood for growth, and those that grow only in anaerobic environments. In these cases, PCR would identify these fastidious species but the strains would not grow without extraordinary measures for cultivation. Furthermore, small colony variants of MRSA are a subset of fastidious strains and are becoming more commonly isolated, growing slowly on blood-based agar and sometimes on chromogenic agar, although they may not exhibit the typical colony color change on certain varieties of agar [16]. In addition, high salt concentrations such as those found in mannitol-salt agar and other high salt media can prevent or delay the growth of MRSA [17].

In addition to the microbial reasons for the discrepant results, an alternative scenario is possible. "Empty cassette variants" of MRSA can exist, when the *mec*A gene is lost from the bacterial chromosome but the insertion site remains [5]. Deletions within the SCC*mec* region of MRSA strains result in the absence of a functional *mec*A gene, but these "empty cassette variants" cause PCR-positive, culture-negative results because current commercial PCR assays target genetic regions upstream from the *mec*A gene (Fig. 35.2).

In some potentially false-positive PCRs, *S. aureus* (MSSA) can be cultivated and for some isolates the insertion site can be detected by DNA sequencing. There is controversy over whether or not these variants should be considered for eligible contact isolation precautions. Such results prove that the patient once harbored MRSA,

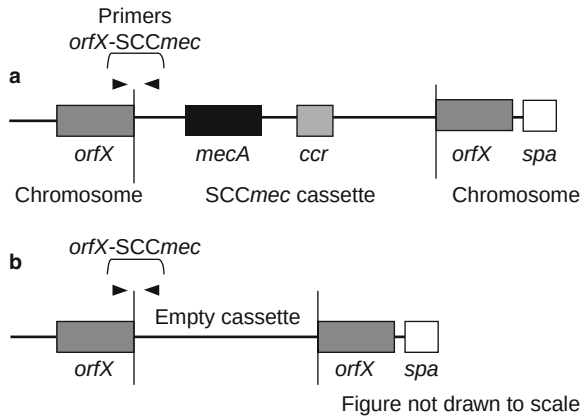

Fig. 35.2 MRSA genomes. **(a)** Cartoon depicting an intact genome of MRSA, including an intact SCC*mec* cassette with an *orf*X gene and insertion site and a *mec*A gene. MRSA primers from the first generation of commercial MRSA PCR are depicted as the *orfX*-SCC*mec* primers. The *mec*A gene, the *spa* gene (staphylococcus protein A), and the *ccr* gene (a recombinase gene responsible for insertion of the SCC*mec* cassette) are depicted to illustrate their relationship to the other genetic targets. The *mec*A and *spa* genes are targets for Cepheid's second-generation MRSA assay, which also detects and confirms MSSA, and do not result in false-positive MRSA results when empty cassette variants are encountered. **(b)** Cartoon depicting an "empty cassette" variant, in which the *orfX* insertion site is present but the *mec*A gene has been excised. These variants would be phenotypically methicillin sensitive (MSSA)

and individual risk factors may increase risk for reacquiring MRSA. This risk must be balanced with the ethical and financial considerations that surround potentially unnecessary patient isolation days and treatment with vancomycin, should an infection arise. Variant strains will be detected by the current versions of the GeneXpert MRSA and BDGO MRSA tests for the nares. These empty cassette variants constitute an ever larger percentage of nasal MRSA and can be successfully treated with a third-generation cephalosporin instead of vancomycin, if no MRSA is found to be present. The prevalence of empty cassette variants has been shown to vary by geographical region, and currently appears to be more common outside the USA, where PCR assays are known to yield unacceptable results and a high number of false-positive results [12, 13].

Further Testing

Question 4: Would you recommend additional laboratory testing to address the discrepancy?

In our patient, the discrepancy was identified because of a quality assurance audit and additional testing was pursued because of the patient's risk of harboring a community-acquired MRSA, which can cause secondary bacterial pneumonia, post influenza A infection. Several courses of action may resolve the discrepancy. First, special growth conditions or extended incubation may be required. The most expedient way to troubleshoot this scenario may be to subculture the sputum to a chromogenic agar, such as MRSA*Select* to identify MRSA that may be present in small numbers and overlooked on the Blood agar plate. For MRSA*Select* agar interpretation, agar plates, examined after 18–28 hours of incubation, are used. After 35°C incubation in room air, MRSA will appear as small pink colonies and non-MRSA organisms are inhibited or appear as white or colorless colonies. In our patient, subculture of the sputum and blood culture bottle to MRSA*Select* agar revealed rare pink colonies after 24 hours of incubation. Alternatively, extended incubation of the blood culture agar may reveal growth of slow-growing small colony variants of *S. aureus*, which are prone to be methicillin resistant. Finally, if the MSSA were indeed an SCCmec variant, DNA sequencing of the MSSA colony would be able to resolve the true nature of the isolate, although such testing may not be cost-beneficial on a routine basis.

Question 5: Would you place the patient in contact isolation and treat the patient for MRSA?

When the isolate is truly identified as MRSA, albeit missed by culture on blood agar, the patient should be placed in isolation. Our patient had a sports affiliation, a young age, prior contact with a hospital, and travel – all risk factors associated with the SCC*mec* variant and MRSA.

Besides the issue of contact precautions and preventive isolation, there are the issues of prognosis and therapy. This patient was seriously ill, and presented with an influenza-like illness, most likely complicated by a secondary bacterial pneumonia. Resolution of the discrepancy was imperative for optimal patient care. For MRSA infections, like this one, vancomycin therapy would be required. Without this patient's known risk factors for community-acquired MRSA, management for pneumonia would include empiric antibiotic therapy, which would not necessarily include vancomycin.

Although limited epidemiologic data are available, MRSA currently appears to be an infrequent cause of community-acquired pneumonia. Nevertheless, severe and sometimes fatal methicillin-resistant *Staphylococcus aureus* community-acquired pneumonia associated with postinfluenza-like illnesses have been reported in teens [18]. The most recent American Thoracic Society (ATS) and Infectious Diseases Society of America joint community acquired pneumonia (CAP) guidelines recommend that, if community-associated MRSA (CA-MRSA) is a consideration, vancomycin or linezolid therapy should be added [19]. Combination therapy may be used, because vancomycin penetrates poorly into pulmonary tissue and lung epithelial lining fluid [20].

Background and Molecular Pathology

Patients with MRSA have presented with a variety of radiographic abnormalities including interstitial, single lobar, and multilobar infiltrates or cavitary lesions. The lack of clinical, laboratory, and radiographic findings that clearly distinguish MRSA pneumonia from other respiratory infections poses a challenge when choosing empiric therapy for a patient presenting with CAP. Despite the increasing prevalence and severity of MRSA, most physicians are not thinking of this diagnosis, which is illustrated by the fact that, in the 2006–2007 season, only 43% of patients with CAP received empiric MRSA treatment. A recent history of documented or suspected MRSA skin infection in the patient, or close contact with MRSA risk groups may be helpful in identifying individuals at risk [21].

While it is useful to rapidly screen potential MRSA carriers, and, in this case, the PCR result was correct, users of "single-locus" PCR assays for MRSA should be aware of the possibility of false-positive reactions. Some MRSA PCR assays detect a single locus, which includes the right extremity of the staphylococcal cassette chromosome genetic element (SCC*mec*) downstream of the *mecA* gene and a part of the adjacent *S. aureus*-specific *orfX* gene. These tests are based on the assumption that detection of this part of the SCC*mec* region is synonymous with the presence of the *mecA* gene, due to the detection of the insertion site. Positive reactions are restricted to *S. aureus* species. This approach is sound for most MRSA isolates, but not flawless; it can be subject to interpretive complications, such as those described previously for SCC*mec* variants.

Because the results of MRSA PCR are used to guide the placement of colonized patients into "contact precautions," there is debate about the impact of positive PCR results from corresponding culture-negative samples. There is no consensus regarding the most prudent action. A cross-sectional analysis of isolates from different geographic regions may provide an overall view of assay performance, but there is no substitute for local assessment of the PCR system. Verification of the assay prior to implementation, and periodic monitoring for local emergence of the SCC*mec* variants by dual testing of a proportion of samples with both PCR and culture may be warranted in some geographic regions.

While there is continued debate about which rapid MRSA detection methods provide optimal support for MRSA screening programs, it is clear that the laboratory plays a key role in hospital screening and infection prevention efforts. Broth-enriched culture methods and molecular assays generally offer the highest sensitivity. Added PCR costs are mitigated by the flexibility of workforce and staffing options for testing because the molecular assay is classified as moderately complex. Overall cost assessment of any laboratory method should include not only laboratory costs, but also the overall costs of MRSA HAIs, isolation room cohorting practices, bed availability, bed transfer practices, the local MRSA transmission rate, and prevalence of SCC*mec* variants in the hospital service area.

Laboratory costs must be assessed in light of overall hospital resources, and potential overall savings. Most importantly, the true value of laboratory testing must be determined by the ability of the laboratory to support the other local infection prevention practices and to reduce MRSA transmission and its associated morbidity, mortality, antibiotic costs, and length of stay.

Specific guidance continues to accumulate for MRSA-related practices in healthcare and the community. In 2003, active surveillance was recommended by national guidelines put forth by the Society for Healthcare Epidemiology of America (SHEA) [22]. In 2005 and 2006, the CDC's Healthcare Infection Control Practices Advisory Committee (HICPAC) issued recommendations focused on reporting and management of Multidrug-Resistant Organisms in Healthcare Settings [23]. These recommendations detail approaches for reduction of MRSA infections in healthcare facilities [24]. The Association for Professionals in Infection Control (APIC) reports that many states require some form of mandatory MRSA reporting. On September 26, 2008, the state of California passed legislation that requires hospitals to increase their infection prevention efforts and to report their infection rates for posting to the public by 2011. Looking forward to the future, key legislative proposals target MRSA infections and could markedly affect healthcare practice. All of the guidance and legislative efforts set the tone for MRSA surveillance and screening practices. As public awareness and the potential for litigation increases, healthcare institutions face ever-increasing pressure to take action to prevent MRSA and laboratory support will become increasingly important.

Multiple Choice Questions

1. What is the genetic target in the commercial MRSA PCR assays?
   A. *mec*A gene
   B. SCC*mec*/*orf*X insertion site
   C. *spa* gene
   D. PVL gene
   E. rpo gene
2. Is the result from the patient an accurate result based on the primers in the MRSA PCR assay?
   A. No, the PCR is false positive
   B. No, the PCR result is false negative
   C. The result is indeterminate
   D. Yes, the isolate is truly an MRSA, albeit a small colony variant (SCV)
   E. Yes, the PCR is always correct compared to culture
3. In light of the issues, what steps would be prudent in order to support infection control active surveillance efforts for this particular case?
   A. Continued monitoring for SCVs and review of concordance between PCR and culture-based methods
   B. Culture of all patients under the age of 21
   C. Discontinue the use of PCR
   D. Treat all patients with vancomycin pre-emptively
   E. Use of blood agar for all surveillance cultures
4. Which variations in *S. aureus* growth can cause discrepancies with PCR results?
   A. All of the growth conditions listed
   B. Anaerobic incubation requirements
   C. Blood requirements
   D. Low density growth
   E. Salt sensitivity

5. Which statement is true regarding MRSA surveillance cultures?
   A. All laboratories are required by clinical laboratory standards to perform PCR
   B. Culture results cannot be expected to agree with PCR as they use different genetic targets to identify MRSA
   C. Healthcare facilities can use active surveillance to support an overall infection prevention program
   D. Passive surveillance is commonly performed with PCR
   E. There are more than eight methods of MRSA PCR commercially available for MRSA, and each has different targets

Answers to Multiple Choice Questions

1. *The correct answer is B.*

The SCC*mec*/*orf*X insertion site is the genetic target in the original commercial MRSA PCR assays. New assays on the market, specifically Cepheid's Xpert MRSA/SA for blood cultures and wounds and the Xpert SA Nasal Complete Assay target the SCC*mec*/*orf*X insertion site, targets within the *mec*A gene and the *spa* gene in *S. aureus*, so results will confirm the presence of either MSSA or MRSA.

2. *The correct answer is D.*

The isolate is truly an MRSA, albeit a small colony variant (SCV), which is still rare but important. Technologists should be aware that hemoloysis and colony morphology for SCVs can differ from typical *S. aureus* colonies.

3. *The correct answer is A.*

Continued monitoring, either ad hoc or as formalized procedural monitoring for SCVs, and review of concordance between PCR and culture-based methods would be prudent.

4. *The correct answer is A.*

All of the growth conditions listed are important caveats, which can cause discrepancies with PCR assays: anaerobic incubation requirements, requirements for blood in agar, low density growth of microbes, and salt sensitivity of some staphylococcal strains.

5. *The correct answer is C.*

Healthcare facilities can use active surveillance to support an overall infection prevention program. While there is debate over the course of action to take, and the need for active surveillance in general, several national and international publications have shown HAIs can be decreased in a cost-effective manner using a variety of active surveillance initiatives.

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Influenza A (H1N1)

36

Benjamin A. Pinsky

Clinical Background

In the summer of 2009 a 12-year-old boy with a history of multiply relapsed acute lymphoblastic leukemia now in his fourth remission on an individualized chemotherapy protocol, presented to his local hospital's emergency room with a two day history of fever to 102.5°C (39.2°C) and upper respiratory symptoms including cough, sore throat, and runny nose. His mother developed similar symptoms approximately one week ago. In addition, he complained of abdominal pain with persistent diarrhea and one episode of emesis. The patient had a history of obstructive lung disease of uncertain etiology for which he used an albuterol inhaler on an "as needed" basis. Since the onset of this acute illness he had been using his inhaler every four hours.

The patient had a central line for the administration of chemotherapy and previously had numerous positive blood cultures with a variety of bacterial organisms. However, current blood cultures were negative and his chest X-ray was unremarkable. Consistent with his presentation, a rapid influenza A antigen test was positive. The patient had not been vaccinated for influenza as neither the seasonal vaccine nor the 2009 A (H1N1) pandemic vaccine was available at the time. He was started on the standard pediatric dose of oseltamivir (Tamiflu) and arrangements were made for the child to be seen by his hematologist early the following morning.

Given the potential significant adverse morbidity and mortality associated with influenza infection in the immunocompromised, including patients being treated for hematologic malignancies, the hematologist admitted the patient to a quaternary medical center for further treatment and supportive care. The hospital stay was uneventful and after completion of the five day course of oseltamivir, the patient's upper respiratory illness resolved. Upon discharge, a direct fluorescent antibody (DFA) test for the presence of influenza A antigen was negative. Further antigen testing was negative two days later, when the patient resumed his chemotherapy regimen. However, nucleic acid testing demonstrated the presence of pandemic, 2009 influenza A (H1N1) RNA.

After three days, the patient again developed fever and upper respiratory symptoms. A nasopharyngeal specimen was positive for influenza A antigen and oseltamivir was restarted.

Question 1: What is your differential diagnosis?

The clinical team considered the possibility that the patient had been reinfected with a different influenza A subtype. However, they were most concerned about persistent infection and the development of oseltamivir resistance.

Reason for Molecular Testing

The goal of molecular testing was to determine the influenza A subtype of the current infection and to evaluate for the presence of a mutation that confers

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Table 36.1 Influenza A subtypes and resistance patterns

Subtype	Oseltamivir	Zanamivir	Adamantanes
Seasonal H1N1	R	S	S
Seasonal H3N2	S	S	R
2009 H1N1	S	S	R

R resistant
S sensitive

oseltamivir resistance. Both of these questions are critical to optimize treatment.

At the time of this case there were two common classes of influenza antivirals; the neuraminidase (NA or N) inhibitors [oseltamivir (Tamiflu) and zanamivir (Relenza)] and the M2 ion channel inhibitors (the adamantanes: amantidine and rimantidine) [1]. In 2009, essentially all of the seasonal H1N1 strains carried the histidine 275 to tyrosine (H275Y) oseltamivir-resistance mutation. These strains were sensitive to both the adamantanes and zanamivir. In contrast, essentially all seasonal H3N2 strains carried the S31N adamantane-resistance mutation and were sensitive to the NA inhibitors. Interestingly, the 2009 A (H1N1) pandemic strain resembled the H3N2 subtype in terms of its resistance profile, and was generally sensitive to the NA inhibitors and resistant to the adamantanes (Table 36.1).

Immunocompromised patients are particularly at risk for the development of resistance to influenza antivirals [2]. Though 2009 A (H1N1) oseltamivir resistance was uncommon at the time of this case (less than 50 reports worldwide), the oseltamivir exposure and clinical course were suggestive of the emergence of a resistant virus. Importantly, the patient's underlying obstructive pulmonary disease contraindicated the use of zanamivir, which requires inhaled dosing and has been associated with a decrease in respiratory function in these patients.

Test Ordered

2009 Influenza A (H1N1) subtyping and H275Y oseltamivir resistance mutation analysis by real time, reverse-transcriptase polymerase chain reaction (rRT-PCR).

Laboratory Test Performed

The laboratory-developed, duplex rRT-PCR assay targeted the 2009 influenza A (H1N1) NA gene. The probes were specific for either the wild-type 2009 A (H1N1) NA sequence or the 823C > T point mutation encoding the H275Y change responsible for oseltamivir resistance. The probes utilized hydrolysis chemistry and were differentially fluorescently labeled on the 5′ end with either FAM or CalFluor560 (HEX-equivalent). On the 3′ ends were black-hole quencher moieties (BHQ). These probes were further modified with a proprietary DNA duplex stabilizing technology (Biosearch Technologies, Novato, CA) that improves specificity and allows single base-pair discrimination.

Numerous rRT-PCR methods, both commercially available and laboratory-developed, are available for the diagnosis of influenza A infections [3]. These assays typically target the highly conserved influenza A matrix (M) gene and are generally ~5–10% more sensitive than viral culture [3, 4]. Because the target is so well conserved, the matrix rRT-PCR tests are able to detect most influenza A subtypes but are unable to distinguish between them.

In order to subtype influenza A, a majority of assays target unique sequences in the influenza A hemagglutinin (HA or H) gene [4]. For example, during the 2009 pandemic in the state of California, county public health laboratories performed individual rRT-PCR reactions to amplify matrix, seasonal H1, and seasonal H3 sequences from respiratory specimens. If positive only for influenza A matrix RNA, the subtype was presumed to be 2009 A (H1N1) and confirmatory rRT-PCR testing targeting the swine-origin H1 was performed at the state public health laboratory.

Another methodology used for the diagnosis of influenza A is traditional RT-PCR, followed by array hybridization, most often to liquid-phase, bead-based arrays. These tests utilize the same nucleic acid targets as the rRT-PCR assays for the identification and subtyping of influenza A, though they may be slightly less sensitive [5]. However, arrays have increased multiplexing capabilities compared to rRT-PCR and therefore allow the simultaneous detection of a large panel of respiratory viral pathogens, most of which have very similar clinical presentations.

The standard molecular approach for Tamiflu resistance testing is pyrosequencing of the NA gene [6, 7]. While sequencing allows the identification of NA mutations other than the 823C > T change, rRT-PCR-based testing may be more sensitive for the detection of specific resistance mutations. At the time this chapter was written, all reported oseltamivir-resistant 2009 A (H1N1) strains carried the H275Y mutation.

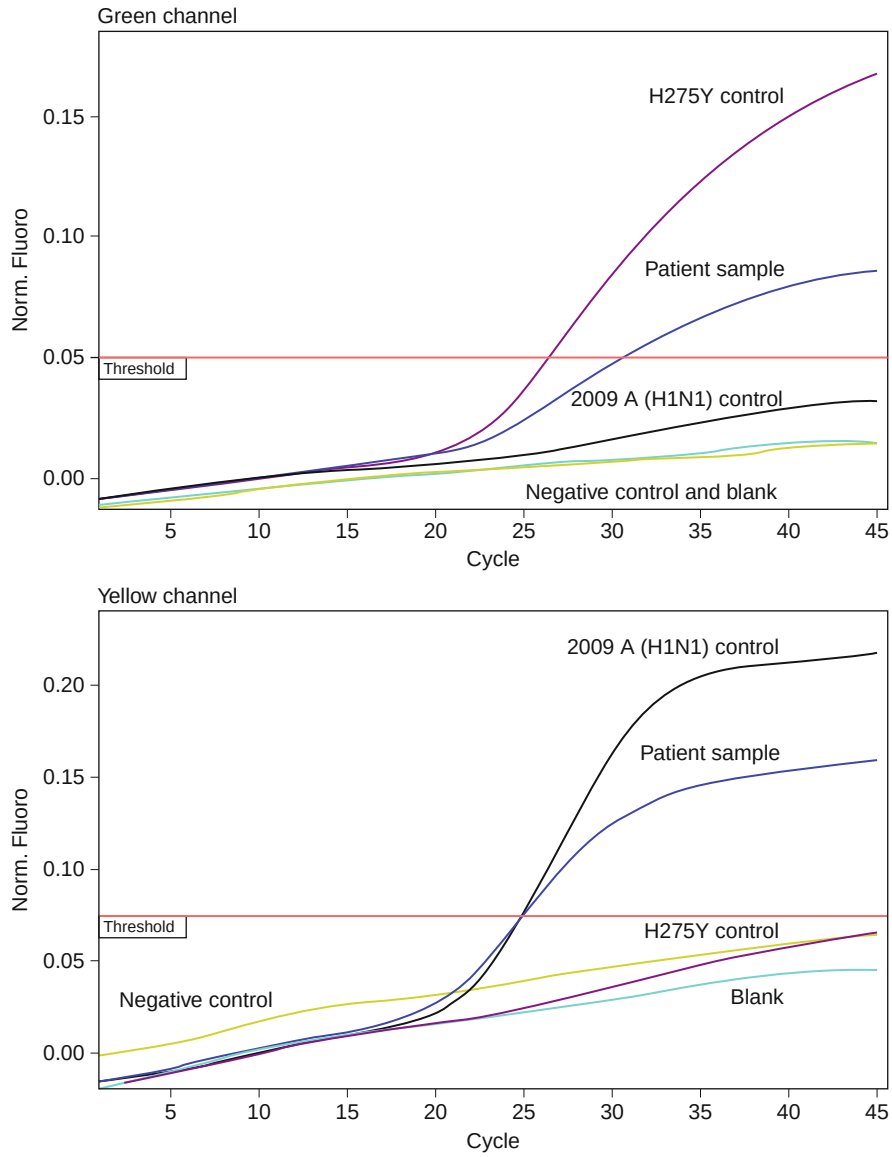

Fig. 36.1 The subtyping of 2009 A (H1N1) with H275Y oseltamivir-resistance mutation testing

Results with Interpretation Guideline

Figure 36.1 displays the results of the 2009 A (H1N1) subtyping and of the H275Y oseltamivir-resistance mutation testing. The criteria for analysis are as follows:

- The blank H2O PCR control must be negative, showing no fluorescent signal above the threshold in both the green and yellow channels.
- The negative influenza A control must be negative, showing no fluorescent signal above the threshold in both the green and yellow channels.
- The positive wild-type 2009 influenza A (H1N1) control must show exponential amplification ONLY in the yellow channel.
- The positive 2009 influenza A (H1N1) H275Y mutant control must show exponential amplification ONLY in the green channel.
- To render an interpretation for patient samples, both the green and yellow channels must be evaluated. Look for the presence or absence of a fluorescent growth curve and the crossing threshold (CT) values to determine the result.

- Samples in which the fluorescence signal is detected within the first 40 cycles of amplification on the green channel contain 2009 A (H1N1) with the H275Y mutation that confers oseltamivir resistance.
- Samples where there is no value for the CT do NOT contain the mutant 2009 A (H1N1). These samples may have wild-type, oseltamivir-sensitive 2009 A (H1N1) or one of the previous seasonal circulating strains. Go to the yellow channel.
- Samples in which the fluorescence signal is detected within the first 40 cycles of amplification on the yellow channel contain wild-type, oseltamivir-sensitive 2009 A (H1N1).
- If a sample contains a mixture of both sensitive and resistant virus, the sample should be reported as 2009 A (H1N1) with the H275Y oseltamivir-resistance mutation.
- If no CT value is detected on either the green or yellow channels the specimen contains a probable previous seasonal circulating influenza virus. If the sample was positive on the general matrix influenza A rRT-PCR, this result can be reported. If the sample went directly from DFA positive to subtyping and resistance testing, the extracted nucleic acids should be tested on the general Flu A PCR to confirm nucleic acid extraction, the presence of influenza A, and the absence of amplification inhibitors.
- Samples in which the CT value is between 40 and 45 require review by the laboratory director.

Question 2: Is this assay run valid?

Yes. The blank is blank and the negative control is negative in both channels. The H275Y control amplifies only in the green channel and the wild-type control amplifies only in the yellow channel.

Question 3: How would you report this result?

A report for this patient specimen might read:

2009 Influenza A (H1N1) RNA:	*Detected*
H275Y oseltamivir resistance Mutation:	*Detected*

These results are consistent with infection by oseltamivir (Tamiflu)-resistant, 2009 influenza A (H1N1). This genotypic test detects the most common missense mutation (H275Y) associated with oseltamivir resistance. Consultation with the infectious disease service and the use of an alternative therapeutic regimen are recommended

If the sample had contained only the wild-type 2009 A (H1N1) strain, it is important to remind the healthcare provider reading the report that only a single mutation is detected in this test. An example report might read:

2009 Influenza A (H1N1) RNA:	*Detected*
H275Y oseltamivir-resistance mutation:	*Not detected*

These results are consistent with infection by oseltamivir (Tamiflu)-sensitive, 2009 influenza A (H1N1). However, this genotypic test detects only the most common missense mutation (H275Y) associated with oseltamivir resistance and does not rule out the possibility that this virus may have a resistance mutation not detected by this test.

Result Interpretation

Question 4: Does this result explain the patient's clinical course?

Yes. The clinical course is consistent with the development of oseltamivir-resistant influenza A virus following treatment with oseltamivir. There is no evidence for infection with previous seasonal influenza A subtypes and it is very unlikely that the patient was subsequently infected with a second, independent oseltamivir-resistant 2009 A (H1N1) strain.

After receiving this result, the clinical team treated the patient with intravenous zanamivir (at the time an investigational drug) [8]. Other strategies were considered, including an increased dose of oseltamivir and the administration of IVIG (intravenous immunoglobulin), which contains some 2009 A (H1N1) neutralizing antibodies and may provide limited passive immunity.

The patient's symptoms resolved and after two weeks no influenza A RNA was detected in the patient's nasopharyngeal specimens.

Further Testing

For epidemiological purposes, the specimen containing the resistant virus was sent to the state public health laboratory for confirmatory testing. Interestingly, their pyrosequencing approach was unable to detect the resistance mutation.

Question 5: How do you explain this result?

This specimen contained a mixture of wild-type and mutant virus that was below the lower limit of detection for the sequencing assay but above the detection limit for the rRT-PCR test. To resolve this discrepant result, our laboratory used a very sensitive low-copy-number, high resolution melting approach to determine that

indeed, the sample in question contained the mutant virus [9]. In addition, the public health laboratory was able to detect the resistance mutation by sequencing a subsequent sample from this patient that contained predominantly mutant virus by rRT-PCR.

Background and Molecular Pathology

Influenza A is a member of the family *Orthomyxoviridae* [10]. The virus contains a single-stranded, negative-sense, segmented RNA genome, and is subtyped based on its hemaggluttinin and neuraminidase genes. The year 2009 saw the emergence of a novel A (H1N1) subtype derived, in part, from an influenza A virus known to infect swine [11]. This novel A (H1N1) virus spread rapidly through the human population worldwide and represents the first influenza pandemic of the twenty-first century [12].

Influenza infections are transmitted from person-to-person via contact and large particle respiratory droplets [13]. There is a one to four day incubation period and the virus is shed the day before symptoms begin through five to 10 days after illness onset. The signs and symptoms of influenza infection may include fever, myalgia, headache, malaise, nonproductive cough, sore throat, rhinitis, otitis media, nausea, and vomiting. Uncomplicated illness typically resolves in three to seven days, though cough and malaise can persist for more than two weeks. Complications include primary influenza pneumonia, exacerbation of underlying medical conditions, and secondary bacterial pneumonia. The complications are typically highest in those over the age of 65, young children, and patients with underlying disease. Seasonal influenza infections cause significant morbidity and mortality, on average 225,000 hospitalizations and 36,000 deaths per respiratory virus season in the United States. Pandemic influenza has the potential to cause an even greater burden of disease.

Influenza A nucleic acid testing is indicated in patients demonstrating signs and symptoms of respiratory infection in conjunction with clinical and epidemiological risk factors. Influenza A subtyping is indicated to track the local influenza A epidemiology and, as demonstrated in this case, to adjust empiric antiviral therapy (e.g., to discontinue adamantanes if the strain is 2009 H1N1, or to discontinue oseltamivir if the strain is a previous seasonal, circulating H1N1). Furthermore, oseltamivir-resistance testing is indicated in patients who do not show clinical improvement and/or viral clearance after completion of an oseltamivir-containing therapeutic regimen.

Multiple Choice Questions

1. The most common mutation that confers oseltamivir resistance is found in the gene encoding of which influenza A protein?
   A. Hemagglutinin
   B. M2 ion channel
   C. Neuraminidase
   D. Nonstructural protein
   E. Nucleoprotein
2. What is the amino acid change for the most common mutation that confers oseltamivir resistance?
   A. Asn294Ser
   B. Glu198Asp
   C. His275Tyr
   D. Iso222Val
   E. Ser31Asn
3. The use of single-tube rRT-PCR to amplify influenza A virus allows detection of:
   A. Viral complementary RNA
   B. Viral genomic RNA
   C. Viral messenger RNA
   D. A and B
   E. A, B, and C
4. An immunocompromised child presents with a one day history of influenza-like illness. A nasopharyngeal specimen is obtained. What nucleic acid test provides the best chance of identifying the responsible respiratory virus?
   A. Influenza A matrix rRT-PCR
   B. 2009 Influenza A (H1N1) subtyping and H275Y oseltamivir-resistance rRT-PCR
   C. Influenza B rRT-PCR
   D. Respiratory syncytial virus rRT-PCR
   E. RT-PCR/liquid-phase, bead-based respiratory viral array
5. A patient with an upper respiratory, oseltamivir-sensitive, 2009 influenza A (H1N1) infection develops shortness of breath and has a chest X-ray concerning for viral pneumonia. What specimen should you test for the presence of influenza A?
   A. Bronchoalveolar lavage (BAL) fluid
   B. Nasopharyngeal Swab
   C. Plasma
   D. Serum
   E. Throat Swab

Answers to Multiple Choice Questions

1. *The correct answer is C.*
2. *The correct answer is C.*
3. *The correct answer is E.*

Influenza A virus has a single-stranded, negative-sense, RNA genome. The use of single-tube RT-PCR containing both forward and reverse primers allows amplification of genomic RNA (negative-stranded) as well as viral complementary and messenger RNA (both positive-stranded). In contrast, the use of a separate RT reaction with a single primer would allow subsequent amplification of only the negative- or positive-stranded RNA species. For example, an RT reaction with only the forward matrix primer would generate complementary DNA (cDNA) only from the genomic, negative-stranded matrix RNA.

4. *The correct answer is E.*

The signs and symptoms of respiratory viral illnesses are not specific enough to make a definitive diagnosis. Respiratory virus panels often cover influenza A and B, respiratory syncytial virus, metapneumovirus, adenovirus, and parainfluenza 1, 2, and 3. Some tests also detect rhinoviruses and coronaviruses. These panels are particularly important for hospitalized patients where results not only guide patient care but also isolation and infection control.

5. *The correct answer is A.*

Viral pneumonia is a serious complication of influenza A viral infections but may be difficult to distinguish from a secondary bacterial pneumonia. Appropriate diagnosis requires testing a lower respiratory tract specimen, such as bronchoalveolar lavage (BAL) fluid, for viral and bacterial pathogens. Interestingly, 2009 A (H1N1) can present with predominantly lower-tract disease, so testing BAL or endotracheal (ET) aspirate specimens should be considered in patients with severe respiratory illness even in the absence of viral detection in nasopharyngeal swabs [14].

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Bordetella pertussis

37

Alexandra Valsamakis and Christina Newman

Clinical Background

A 37-year-old woman with a history of inherited dilated cardiomyopathy, which had been successfully treated 20 years ago with a heart transplant, developed restrictive cardiomyopathy and accelerated transplant vasculopathy. She was admitted for an inpatient workup for a new heart transplant. She had a six month history of chronic cough associated with volume overload. On admission she complained of rhinorrhea, sinus congestion, and subjective fever. Four days prior to admission she noted the onset of a cough that was different from her chronic complaint. It felt "deeper" than her usual cough, came in paroxysms, and was occasionally productive of sputum. She reported that the cough was so severe, at times, that she vomited. Her immunosuppression regimen consisted of prednisone and cyclosporine. On physical exam she was found to be afebrile, blood pressure was normal, and scattered crackles were heard throughout both lung fields on auscultation. Noteworthy laboratory test results included a white blood cell count of 6,600 cells/mm^3 (normal range, 4,500–11,000 cells/mm^3), with a normal distribution of neutrophils, lymphocytes, and mononuclear cells. Microbiologic testing of expectorated sputum showed an adequately collected specimen with heavy mixed upper respiratory flora on gram stain, and the same organisms by culture after two days. A multiplex PCR test for viruses from a nasopharyngeal swab did not detect respiratory syncytial virus, influenza A virus, influenza B virus, parainfluenza types 1/2/3, human metapneumovirus, rhinoviruses, or adenoviruses. Computed tomography (CT) of the chest demonstrated a new right upper lobe nodular opacity.

Question 1: Based on this information, infection with which organisms should be considered?
Question 2: What additional information in the history would be useful in guiding the differential diagnosis?

Reason for Molecular Testing

This was a case of a young woman on long-term immunosuppression with an acute onset pulmonary process. Sepsis could most likely be excluded due to the absence of consistent symptoms, signs (absence of fever, normal blood pressure), and laboratory results (normal white count). The list of pathogens on the differential diagnosis was very broad; however the clinical data suggested that typical community acquired bacteria (most commonly *Streptococcus pneumoniae*) could be excluded, because the sputum microbiology tests failed to reveal these organisms. The usual viral pathogens could also be excluded as a result of the negative PCR results. In addition, the appearance of the process on imaging was also not suggestive of a viral process (which would appear as an interstitial rather than a nodular infiltrate).

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Given the presentation above, the differential diagnosis would likely focus on atypical bacteria that would not be easily recoverable on routine microbiologic media (so-called "fastidious" organisms). These organisms include *Chlamidophila pneumoniae*, *Mycoplasma pneumoniae, and Legionella pneumoniae*. In this immunocompromised host, *Mycobacterium tuberculosis*, other nontuberculous mycobacteria, *Actinomyces* species, and *Nocardia* species would also be considered given the chest imaging result. The list of potential causative fungi was also long, but *Pneumocystis jerovici* would be worth investigating given her immunosuppressive regimen.

In this case, however, the history of a paroxysmal cough that induced post-tussive emesis was most suggestive of infection with *Bordetella pertussis (B. pertussis)*. The additional history that would be useful is an exposure to anyone known to have pertussis and a recent history of vaccination against pertussis. A tuberculosis exposure history would also be relevant in order to investigate the possibility of latent tuberculosis reactivation, particularly given the apical location of the nodular opacities observed on the CT scan.

When asked about exposures, this patient remembered attending an event, three weeks prior to admission, with a relative who was on antibiotics for pertussis. The patient had been immunized as a child but had not been recently vaccinated against pertussis and, therefore, was at risk of infection due to waning immunity.

Question 3: Which test(s) should be ordered?
Question 4: What specimen should be collected?

B. pertussis is a fastidious gram-negative coccobacillus. Successful recovery in culture requires growth on specific medium (such as Regan-Lowe medium). Culture for this organism must, therefore, be specifically requested. Growth is usually detected by three to four days of incubation but can take longer. Consequently, cultures are usually held for at least seven days prior to resulting as "no growth detected". Staining of specimens with monoclonal antibodies against *B. pertussis* was used in the past as a rapid detection method but has now largely been replaced by molecular tests due to suboptimal sensitivity. Molecular tests for detection of *B. pertussis* DNA have rapidly become the methods of choice for detection, due to their sensitivity and relatively rapid time-to-result.

B. pertussis has a tropism for ciliated respiratory epithelial cells of the posterior nasopharynx and lower respiratory tract. Thus, optimal specimens for organism detection are posterior nasopharyngeal swabs or aspirates. Dacron or rayon swabs are recommended because they can be used for culture or PCR-based assays. Alginate swabs can be used for culture, but these swabs inhibit PCR and are not recommended for PCR-based tests [1].

Test Ordered

The tests that were ordered were PCR (after specimen collection with a rayon nasopharyngeal swab) and culture (after specimen collection with an alginate swab) for *B. pertussis*.

Question 5: How is nucleic acid extraction modified to allow efficient recovery of B. pertussis *DNA by PCR?*
Question 6: What are the appropriate quality controls in a PCR-based test for B. pertussis*?*

Laboratory Test Performed

B. pertussis DNA was assayed using a real-time PCR-based laboratory-developed assay. The sample was incubated with proteinase K at 56° for 15 min to facilitate bacterial cell lysis. DNA was extracted using an automated instrument that captures and elutes nucleic acid from magnetic beads. Real-time PCR was performed using commercially available primers and FAM-labeled probe in a SmartCycler instrument (Cepheid) to amplify and detect the repetitive element IS*481*. A proprietary, commercially obtained internal control was amplified with commercially available primers and detected with Texas Red-labeled probe to allow discrimination between true-negative and false-negative results due to sample-specific real-time PCR failure. Internal control reagents (target and primers/probe) were added to all clinical samples and controls. Other quality control samples were a positive control consisting of a control swab inoculated with a *B. pertussis* reference strain and a negative control (phosphate buffered saline). Both controls were extracted with other samples. Culture was performed by inoculating solid Regan-Lowe medium with the alginate swab sample.

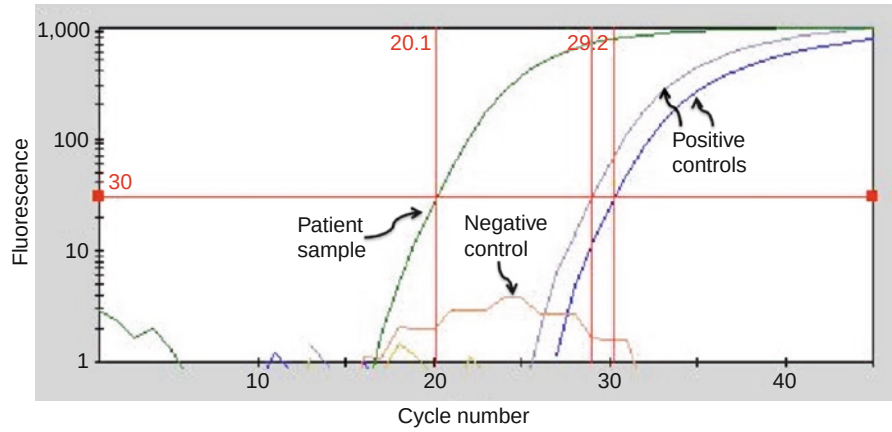

Fig. 37.1 Real-time PCR data for the patient sample, two replicates of positive control, and the negative control

Results with Interpretation Guideline

Figure 37.1 demonstrates the results of the *B. pertussis* real-time PCR test. The threshold discriminating background from true fluorescence was set at a fluorescence intensity midway through logarithmic amplification. Fluorescence above this background was detected after 29, 30, and 20 PCR cycles for the positive controls and the patient sample, respectively. The acceptable range for the positive control specimen was defined as fluorescence detectable between 28 and 31 PCR cycles; this range was determined by testing ten replicates on three different days. Fluorescence signal from the negative controls remained below the background threshold throughout all cycles ($N=45$). The Texas Red fluorescence plot associated with the internal amplification control demonstrated signal above the background threshold after 28 PCR cycles in all reactions (data not shown). No organism was detected by culture after 10 days.

Result Interpretation

The detection of fluorescence in positive control reactions at a cycle within the predefined range demonstrates that the real-time PCR test was performing with expected efficiency. Fluorescent signal from the patient sample approximately ten cycles prior to the positive control demonstrates IS*481* amplification and detection, suggesting the presence of *B. pertussis*. The pattern of signals from the negative control reaction (no fluorescence in IS*481* reactions, fluorescence in internal control reactions) suggests that the signal observed in the patient sample is not due to contamination. If the positive IS*481* control reaction had demonstrated no signal, or fluorescence at a cycle number greater than the upper acceptable limit (>32 PCR cycles for this particular control) and the patient sample demonstrated no signal, the test would have been repeated due to unexpectedly low PCR efficiency that might have produced a false-negative patient result. If the negative control demonstrated fluorescence signal in the IS*481* reaction, the assay would have been repeated due to the potential for a false-positive patient result caused by contamination. Testing would also have been repeated if the patient sample failed to demonstrate fluorescence signal in both the IS*481* and internal control reactions (and all external controls performed as expected).

*Question 7: Does amplification of IS*481 *prove that* B. pertussis *is the cause of disease?*

The final interpretation of real-time PCR for the detection of *B. pertussis* is dependent upon the amplification target. Assays that target IS*481* are highly sensitive because this sequence is present in high copy number (80–100) in the *B. pertussis* genome. However, similar sequences are found in another species, *Bordetella holmesii*. Studies to link this organism to a pertussis-like syndrome have identified the organism in some populations but not others and it has been implicated as a cause of sepsis [2, 3]. Discrimination between *B. pertussis* and *B. holmesii* is not particularly necessary from a treatment perspective because these organisms are susceptible to the same antibiotics. Nonetheless, when providing a result, it is important to

acknowledge assay cross-reactivity with an interpretive comment such as: "A positive result does not assure that *B. pertussis* DNA is present because this assay will also detect *Bordetella holmesii*, an uncommon human pathogen that can be treated with the same antibiotics as *B. pertussis*. Positive PCR results should be correlated with clinical findings."

The pertussis toxin promoter is another common PCR and real-time PCR target. This sequence is present as a single copy in *B. pertussis* only. Therefore, it is specific, but relatively insensitive compared to IS*481*.

Question 8: In this patient, how does one interpret conflicting PCR and culture test results?

The sensitivity and time-to-result of nucleic acid amplification methods make them highly desirable for *B. pertussis* detection. *B. pertussis* detection rates are three to fourfold greater by PCR than by culture [4]. However, there is considerable concern for the potential of false-positive results with amplification tests. Hence, the US Centers for Disease Control (CDC) suggest that culture and nucleic acid testing both be performed due to the specificity of culture methods. Interpretive problems arise when the results of molecular and culture tests differ, most commonly when nucleic acid amplification is positive and culture is negative, as occurred here. This case underscores the importance of confining molecular testing to patients with symptoms highly suggestive of *B. pertussis* infection such as multiple coughing paroxysms, post-tussive "whoops," or post-tussive emesis. The existence of an exposure, as was reported here, also supports the accuracy of the real-time PCR test result.

Further Testing

Further infectious disease testing was not indicated for this patient.

Background and Molecular Pathology

Despite the availability of a vaccine since the mid 1940s, pertussis continues to be a problem today, with the most recent (2008) estimated incidence of 4.18 cases/100,000 in the USA [5]. Following an incubation period of seven to 10 days, the classic disease in unvaccinated individuals occurs in three phases: (1) the "catarrhal phase," consisting of mild upper respiratory symptoms and lasting approximately two weeks, (2) the "paroxysmal phase" starting in the second week of symptoms, typified by episodes of uncontrollable coughing often followed by deep inspiration (the inspiratory "whoop" of whooping cough), and lasting two to eight weeks, and finally, and (3) the "convalescent phase" when coughing decreases in severity and frequency over several weeks. Complications of classic disease include pneumonia (due either to *B. pertussis* or to secondary infection) and central nervous system disorders such as seizures and encephalopathy, thought to result from the severe hypoxia induced by coughing. The force of coughing during the paroxysmal phase commonly produces side effects such as fractured ribs, hernias, and rectal prolapse.

Pertussis in young infants can produce severe morbidity and high mortality rates, particularly in neonates. In the USA, 90% of pertussis deaths occur in infants < four months of age [5, 6]. The presenting symptom is usually apnea because coughing can be difficult to discern in this population. Pronounced lymphocytosis is common, and the degree correlates with disease severity. Sequelae of infection include pneumonia, hypoxia-induced seizures secondary to apnea, and an often-fatal pulmonary hypertension syndrome.

Question 9: Why did this patient develop pertussis, despite having likely been immunized as a child?

Prior infection and immunization do not confer lifelong immunity to pertussis. Immunity induced by immunization and natural infection wanes almost completely after approximately 10 years [7, 8]. Since this individual was likely immunized as a child, she had become susceptible to infection. Infections such as this have become common. The most recent US data demonstrate that ~50% of pertussis cases occur in the highly vaccinated population above 10 years of age [5]. Although infection can range from asymptomatic to classic pertussis, these individuals usually present with a history of weeks of chronic cough during which they have been infectious.

Diagnosis by culture or molecular methods is typically difficult in adults and adolescents. Organism loads are lower in vaccinated adults and adolescents compared to infants with pertussis [9]. In addition, the organism burden is typically low late in infection, when most individuals seek medical care. Serology

can be useful in this setting. In the case presented above however, the onset of suggestive symptoms occurred during hospitalization when the patient was being extensively evaluated. As a consequence, testing was likely performed earlier in the course of the disease than would normally occur. Despite this early testing, organism loads were probably low, as suggested by the positive PCR but negative culture results.

In the developed world, immunized adolescents and adults are thought to be the reservoir of infection due to a combination of factors that delay treatment, including delayed presentation for medical care, difficulty in ascertaining the diagnosis by culture or molecular tests, and care provider failure to consider pertussis as an etiology of disease.

B. pertussis is susceptible to orally dosed macrolide antibiotics (inhibitors of bacterial protein synthesis). Erythromycin is commonly used. Azithromycin and clarithromycin are options to avoid the gastrointestinal side effects of erythromycin. Resistance to macrolides is rare. Treatment is usually instituted to eradicate the organism and abort transmission rather than to alleviate symptoms because it is not thought to have much of an effect after onset of the paroxysmal stage. Household contacts of a confirmed case should receive prophylactic antibiotics because *B. pertussis* is highly infectious.

Pertussis can be prevented through vaccination. The current vaccines are subunit vaccines consisting of purified bacterial components that produce fewer side effects than the original cellular (unpurified) vaccines. They are available only in combination with diphtheria and tetanus vaccines. The vaccines licensed for use in children and adolescents/adults have different formulations, in that the latter contain lower concentrations of *B. pertussis* antigen. For children, a four-dose vaccination series is recommended at two, four, six, and 15–18 months of age [10]. A single booster dose is advocated for adolescents (at 11–18 years) and adults (at 19–64 years) [11, 12]. To prevent the devastating effects of neonatal pertussis, a booster prior to pregnancy or in the immediate postpartum period is recommended. A booster is also recommended for adults anticipating contact with an infant <12 months of age [12, 13].

Diagnostic testing, particularly nucleic acid amplification assays, should only be performed when the pretest probability of infection is high. Although asymptomatic infection can occur, no public health entity has recommended surveillance testing by any method. Nucleic acid tests should also not be used as "test of cure" after antibiotics because *B. pertussis* DNA is detectable after viable organisms have been eradicated by treatment. In one study of neonates with pertussis, all cultures were negative after one week of erythromycin therapy whereas 56% of subjects had DNA detectable by PCR [14].

A positive *B. pertussis* test result (molecular or culture) has public health implications. Pertussis is a reportable illness in the USA and public health authorities must be notified of a positive result by law in many jurisdictions. Laboratories performing such testing should put appropriate notification mechanisms in place. Pertussis is highly contagious via droplets and infection control strategies (isolation in single bedrooms and caregiver/visitor use of disposable gowns, masks, and gloves) should be implemented for inpatients with proven pertussis. The laboratory should also have mechanisms for alerting hospital infection control practitioners of such patients.

Multiple Choice Questions

1. For which of the following uses is PCR for *B. pertussis* detection indicated?
   A. To assess the risk of infection of nursing home visitors who were unknowingly exposed at the beginning of an outbreak
   B. To decide whether to stop antibiotics in a two-month-old boy hospitalized for pertussis
   C. To decide whether to stop antibiotics in an 85-year-old woman hospitalized for pertussis
   D. To diagnose the cause of two weeks of chronic cough and post-tussive emesis in a 30-year-old woman
   E. To diagnose the cause of sleep apnea in a 42-year-old man
2. Culture in addition to PCR has been recommended by public health authorities because:
   A. Antibiotic resistance is common among *B. pertussis* isolates and should be documented
   B. False-positive PCR results can be more readily detected
   C. New variants of *B. pertussis* can be identified
   D. Only *B. pertussis* isolates with certain carbohydrate utilization profiles are pathogenic
   E. PCR is not as sensitive as prolonged culture

3. Which of the following specimens is optimal for detection of *B. pertussis* by PCR?
   A. Calcium alginate swab of anterior nares
   B. Calcium alginate swab of posterior nasopharynx
   C. Dacron swab of the anterior nares
   D. Rayon swab of the posterior nasopharynx
   E. Rayon swab of the throat
4. Compared to pertussis toxin promoter, the target sequence IS*481* is:
   A. Equally sensitive and specific
   B. Less sensitive and specific
   C. Less sensitive, but more specific
   D. More sensitive and specific
   E. More sensitive, but less specific
5. *B. pertussis* DNA is detected by a pertussis toxin promoter-based PCR assay in a 19-year-old girl with a one week history of paroxysmal cough that is pronounced at night. She should be treated with:
   A. Ampicillin
   B. Azithromycin
   C. Aztreonam
   D. Ceftriaxone
   E. Metronidazole

Answers to Multiple Choice Questions

1. *The correct answer is D.*

A chronic cough severe enough to induce post-tussive emesis are symptoms suggestive of pertussis and this individual was likely susceptible to infection because she was probably vaccinated >10 years previously. Individuals who are exposed to *B. pertussis* are given prophylactic antibiotics. Treatment is administered for standardized periods to ensure eradication of viable organism; *B. pertussis* DNA can be detected for weeks after successful treatment. Pertussis-induced sleep apneas are commonly observed in neonates and young infants, not adults.

2. *The correct answer is B.*

The potential for false-positive PCR results is a public health concern. The Centers for Disease Control has recommended testing for *B. pertussis* by culture and PCR as a way to monitor the accuracy of positive PCR results. It should be noted however that nucleic acid amplification technologies are more sensitive than culture, therefore the finding of false-negative culture/true-positive PCR is likely to occur. Resistance to commonly used antibiotics is rare.

3. *The correct answer is D.*

B. pertussis is tropic for ciliated epithelial cells of the posterior nasopharynx and lower respiratory tract. Dacron or Rayon swabs are suitable for PCR; alginate can inhibit Taq polymerases.

4. *The correct answer is E.*

IS*481* is a repetitive element found in *B. pertussis* and *B. holmesii*. The pertussis toxin promoter is present as a single copy only in the genome of *B. pertussis*. Assays that detect IS*481* are therefore more sensitive but less specific than those that detect the pertussis toxin promoter.

5. *The correct answer is B.*

Macrolide antibiotics such as azithromycin are active against *Bordetellae*. Treatment is useful for preventing secondary infections later in disease (during paroxysmal stage). Late treatment is generally not thought to ameliorate cough symptoms.

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Human Metapneumovirus

38

Sophie S. Arbefeville and Aaron D. Bossler

Clinical Background

The patient was a 10–month-old Caucasian male who was brought to the clinic by his mother. She reported that for the past five days her son had a fever of 103°F, difficulty breathing, and cough. She treated the fever with ibuprofen and acetaminophen and the patient improved, but worsened again after three days. The mother took her son to a local physician who prescribed albuterol nebulizer for home usage, but this did not improve his symptoms. The day prior to the current presentation the patient had decreased activity, increased "lethargy", decreased appetite, difficulty nursing, laborious breathing, and decreased urine output. He also vomited and had two loose stools. At this point the mother decided to bring her son to the clinic for further care. While in clinic, his temperature was recorded at 38.6°C and oxygen saturation was 91%. His five-year-old sister was reported to have had similar symptoms, but not as severe. The patient's past medical history included no prior hospitalizations, and no asthma or albuterol use. His immunizations were up to date, including recent receiving of the novel H1N1 influenza A vaccination.

On physical examination blood pressure was 136/90, pulse 158, tympanic temperature 37.8°C (100°F), number of respirations 58, and oxygen saturation by pulse oximetry 96%. His height, weight, and head circumference were normal for age. He appeared lethargic, sleeping in mother's arms, and could be slightly aroused on exam. Head, ears, eyes, nose, and throat examinations were normal except for copious amounts of clear rhinorrhea. No erythema or exudates were observed. Heart rate and rhythm were regular with normal S1 and S2, without gallops, clicks, or murmurs. Lungs had coarse breath sounds with expiratory crackles throughout. Bowel sounds were present throughout and the abdomen was nontender, nondistended, and without organomegaly. Extremities demonstrated capillary refill in two to three seconds, were warm and without rashes, ecchymosis, or petechiae.

The assessment was of a 10–month-old male patient with mild to moderate respiratory distress, likely secondary to a viral respiratory infection versus pneumonia and mild dehydration.

Question 1: Several viruses can cause respiratory infection requiring hospitalization. Which is the most common?

Reason for Molecular Testing

The US Department of Health and Human Services estimates the annual frequency of the common cold to be more than one billion, making viral respiratory tract infections the most common seen in children and adults. Usually the infections are mild, restricted to the upper respiratory tract and self-limited. In the elderly, immunocompromised patients and particularly in the

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very young, these infections can lead to more severe illness involving the lower respiratory tract and are responsible for infections such as bronchitis, bronchiolitis, and pneumonia. In infants and young children, viral acute respiratory tract infection is a leading cause of hospitalization and death with infection by respiratory syncytial virus (RSV) being the most common virus leading to hospitalization [1–3]. Rapid identification of the infectious agent is important for the proper management and treatment of the infected patient and for implementation of appropriate hospital epidemiologic measures to reduce nosocomial transmission to at-risk patients. Rhinovirus, influenza A and B, RSV, and adenovirus are the most common causes of viral respiratory infection.

Question 2: There are several methods that can be used to detect respiratory virus infections. Which is/are the least sensitive?

Respiratory virus detection can be performed using several different techniques, including rapid antigen detection enzyme immunoassays, fluorescent antibody detection of viral antigens in patient's specimens, culturing for viral growth using various cell culturing methods, and molecular-based methods. Membrane-based enzyme immunoassays (EIAs), optical immunoassays (OIAs), and immunochromatographic lateral flow (ICLF) assays provide quick turn around time in 15–30 minutes and can be performed during a clinic visit. However, they are hampered by poor sensitivity requiring 10^5–10^6 viral particles to give a positive result compared to culture methods, which require approximately 10 viral particles, or compared to molecular assays, which demonstrate detection of as low as two to five copies of viral genomic nucleic acid [4–7]. Direct fluorescent antibody (DFA) detection of viral antigens on centrifuged cellular material (cytospun) made directly from patient specimens (nasopharyngeal swab, aspirate, or wash) demonstrates greater detection rates than the rapid antigen assays and provides results in a relatively short time frame of three to four hours. However, compared with PCR-based methods the detection rate with antigen detection methods is still lower [8]. Conventional cell culturing methods are sensitive and have been considered the gold standard for the detection of a wide range of viruses, but are laborious and require time for growth of the virus. Even though culture methods have been optimized by combining multiple cell lines and use of shell vial spin amplification cultures, they still require one to two days for results and are not as sensitive as molecular-based methods of detection.

Molecular detection of respiratory viruses offers several advantages. Most importantly, it has greater sensitivity for infection than viral culture and the lower limit of detection has been shown to be around two to five copies of the viral target [6, 9, 10]. In addition, molecular assays can be performed more rapidly than culture, taking up to eight hours depending on the method of detection. This, combined with the greater sensitivity, helps to decrease the use of antibiotics. Molecular methods are also better at detecting multiple infections, providing a wide range of viruses that can be detected at one time [11].

Test Ordered

Molecular testing for respiratory virus detection was ordered.

Laboratory Test Performed

The patient's nasal swab specimen was processed using a laboratory-developed multiplexed reverse transcription (RT) real-time polymerase chain reaction (PCR) assay for the detection of influenza A and B, novel influenza A (H1N1 subtype), respiratory syncytial virus (RSV), human metapneumovirus (hMPV), parainfluenza virus (PIV) types 1, 2, and 3, adenovirus, and rhinovirus, along with an internal control for extraction and amplification of human β-2-microglobulin.

Results with Interpretation Guideline

The RT real-time PCR results can be visualized in a two-dimensional plot; the product of the PCR amplification is represented by the fluorescence intensity (y-axis) versus the number of PCR cycles (x-axis). The crossing threshold (C_t), or cycle threshold value reflects the number of rounds of PCR replications at which the fluorescence generated within a reaction crosses the fluorescence threshold, a fluorescent signal significantly above the background fluorescence. The C_t value assigned to a particular reaction reflects the

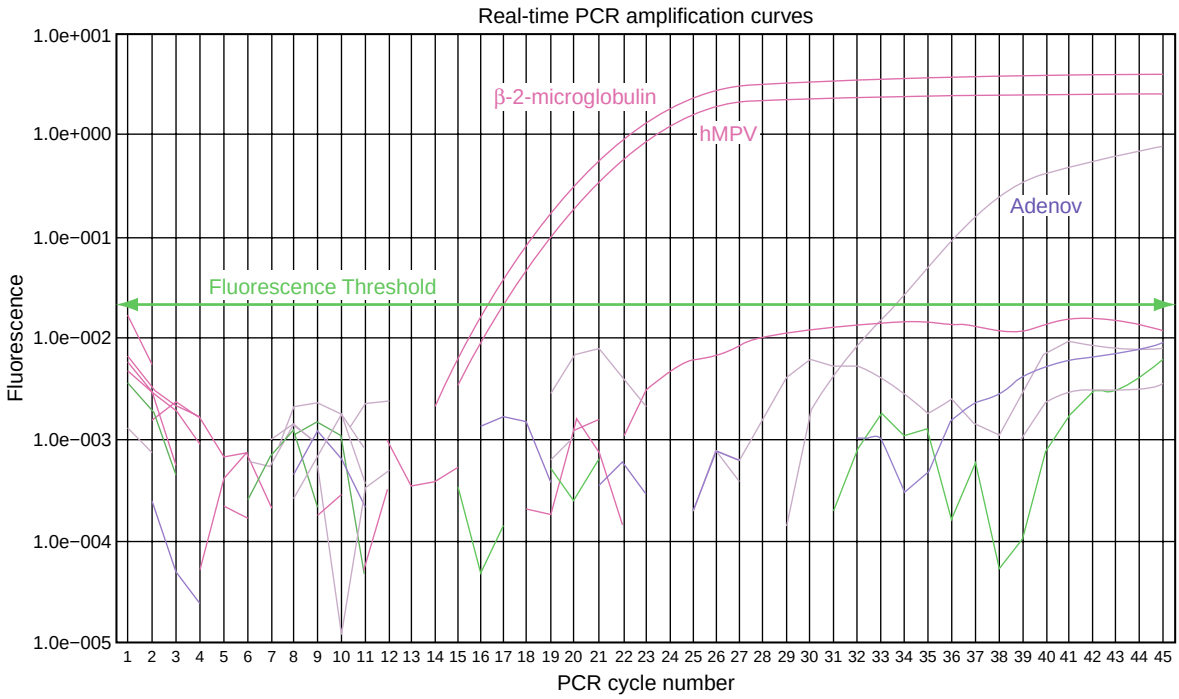

Fig. 38.1 Real-time PCR amplification curves for hMPV and adenovirus plotted against PCR cycle number (x-axis) and fluorescence (y-axis). The *green bar* represents the fluorescence threshold

point during the reaction at which a sufficient number of amplicons have been generated during the early exponential phase. Thus, there is an inverse relationship between the viral load and C_t such that the lower the C_t the higher the viral load. In general, the presence of an amplification curve demonstrates the presence of the viral genomic sequence, and the crossing threshold value represents a semiquantitative measure of the amount of viral nucleic acid present in the specimen. False positives are occasionally detected at C_t's of 40 or greater and should be viewed with caution. Repeat testing is recommended along with close clinical correlation.

RT real-time PCR amplification curves were present for hMPV (crossing threshold or $C_t = 17$) and adenovirus ($C_t = 34$) (Fig. 38.1). Amplification of the β-2-microglobulin internal control was also observed. Amplification was negative for all of the other viruses represented by the angled lines below the threshold.

Question 3: What is your interpretation of these results in the context of this patient?

Result Interpretation

Viral nucleic acid detection is an effective tool to diagnose respiratory virus infection. PCR-based methods along with RT and the advent of real-time PCR in which the product of amplification is simultaneously detected as the reaction progresses are sensitive means for detection. In this case the patient demonstrated a low CT for hMPV and higher CT for adenovirus. We speculated that the adenovirus infection may have preceded the infection with hMPV, was now resolving, and that the timing and combination of these two infections contributed to the severity of respiratory distress for this infant.

Further Testing

Positive results for hMPV and adenovirus indicated that no further testing was required for a pathogen source.

Background and Molecular Pathology

Human metapneumovirus (hMPV), identified in 2001 by van den Hoogen *et al*. in Dutch children has been found in upper respiratory specimens of adults and children worldwide [12]. It is a member of the *Paramyxoviridae* family with RSV and Parainfluenza. hMPV is subclassified in the *Pneumovirinae* subfamily and belongs to the *Metapneumovirus* genus. It is an enveloped, single-stranded, nonsegmented, negative-sense RNA virus. Two serotypes have been identified dividing the hMPV into two groups, A and B, and further genetic studies have divided each group into two genetic sublineages (A1, A2, B1, B2) [13]. Infection usually occurs in the winter and spring months in the temperate regions [14]. Infection rates in children have been reported at 6–7% in China [15], Singapore [16], and the USA [17]. It is now recognized that hMPV is one of several viral pathogens that can cause respiratory symptoms in the absence of other pathogens and is, moreover, the second leading cause of acute bronchiolitis in hospitalized children [18]. Cell culture of the virus is fastidious requiring an incubation time of two to three weeks before cytopathic effect is seen. Relatively few cell lines, like LLC-MK2, efficiently support hMPV replication [19] and require the addition of trypsin to the culture medium to allow processing of the HMPVF protein into its mature form, a prerequisite for virus infectivity [20].

Adenovirus is another common cause of viral respiratory tract infection, particularly in young children. This is a nonenveloped, icosahedral virus with a double-stranded DNA genome. Adenovirus infection is one of the more common causes of febrile illness in pediatrics and may present with gastrointestinal symptoms such as vomiting or diarrhea in infants. Occasionally acute respiratory distress may occur.

Coinfection with multiple respiratory viruses has been documented in several studies and raised the suggestion that coinfection might increase the severity of disease, particularly for hMPV [15, 21–23]. The incidence of dual respiratory viral infection varies from 10% to 30% in hospitalized infants [18]. Gláucia Paranhos-Baccalà et al. reported increased dual infection (34.1%) in their pediatric intensive care unit population compared to their pediatric short-term unit population (15.2%).

Viral nucleic acid detection is an effective tool to diagnose respiratory virus infection. PCR-based methods along with RT have been most commonly described. RT is the synthesis of a DNA complement (cDNA) from the viral RNA target strand using the enzyme reverse transcriptase. It is necessary because most respiratory viruses have RNA genomes, which are susceptible to RNase degradation during collection and processing making RNA less stable to use than DNA. The resulting cDNA is amplified using PCR amplification. Real-time PCR, in which the product of amplification is detected at the same time as the reaction progresses, has several advantages. It allows for multiplexing capabilities or the detection of multiple viruses in a single well because of the different fluorophores that can be used. Also, the detection is performed during amplification so that amplicons are contained in a closed system and the risk of laboratory contamination is reduced. PCR-based target amplification methods are most commonly used; however, other molecular detection methods have been described [24].

Commercially available PCR-based systems for detection and identification of respiratory viruses that have been FDA cleared include the ProFlu+, ProParaFlu+, and Pro hMPV + assays from Gen-Probe, Inc. (San Diego, CA), the xTAG Respiratory Viral Panel from Luminex Corp. (Austin, TX), and the Verigene Respiratory Virus Nucleic Acid Test from NanoSphere, Inc. (Northbrook, IL). All of these assays are approved for use on nasopharyngeal swabs and all of them use internal controls for amplification. Both the ProFlu + and Verigene Respiratory Virus Nucleic Acid Test detect influenza A and B and RSV whereas the xTAG Respiratory Viral Panel detects these along with parainfluenza virus (PIV) types 1, 2, and 3, adenovirus, hMPV, and rhinovirus. The ProParaFlu + assay detects all three PIV types and the Pro hMPV + assay detects hMPV. All three manufacturers reported that their influenza assays are capable of detecting the 2009 novel influenza A H1N1.

The three Gen-Probe assays use RT real-time PCR with hydrolysis-type fluorescent probe design for detection. This type of method is simple to use, combining the amplification and detection in one platform, thus maintaining a closed system and decreasing the risk of PCR contamination. The Verigene® Respiratory Virus Nucleic Acid Test from Nanosphere, Inc. uses

a unique, single-use microfluidics cartridge in which the products of the multiplex RT-PCR are captured to a microarray format. Detection is performed using gold nanoparticle probes which are illuminated with a fixed wavelength light source and detected with a single image sensor in the dedicated detection instrument, the Verigene® System. The assay includes two internal controls: a process control for sample isolation and an inhibition control for amplification. It has been demonstrated to be highly reproducible with 100% detection of moderate levels of virus. Varying limits of detection have been reported ranging from 0.05 to 50.0 TCID50 (50% tissue culture infectivity dose)/mL, depending on the viral strain. It is highly specific with no cross-reactivity with over 38 viruses and bacterial genera (data derived from the clinical trial information in the manufacturer's product insert).

The xTAG™ Respiratory Viral Panel (RVP) assay is also based on multiplexed RT-PCR but uses fluorescently color-coded microsphere (bead) hybridization for subsequent detection and identification of 12 respiratory viruses and subtypes. The detection is accomplished by labeling the RT-PCR amplification products with biotin containing deoxynucleotide triphosphates (dNTPs) through a target-specific primer extension. This also incorporates a proprietary tag sequence for hybridization to the virus-specific probe on the color-coded bead. After hybridization, phycoerythrin conjugated to streptavidin is bound to the biotin-labeled primer extension products and the fluorescent signal is read on the Luminex xMAP™ instrument. The instrument takes two readings: one for the identification of the color codedbead and the other for the detection of the phycoerythrin signal attached to the primer extension product. The assay includes a separate lambda phage amplification control and an MS-2 bacteriophage internal control for extraction and amplification. Sensitivities range from 78.3% to 100% depending on the virus. The assay does not adequately detect adenovirus species C, or serotypes 7a and 41, and the primers for detection of rhinovirus cross-react with enterovirus. Detection rates for influenza have been shown to be 5–10% more sensitive than those of DFA and culture [25]. Specificity is also high, ranging from 91.3% to 100%.

In addition to the FDA-cleared assays, many laboratories have developed their own multiplex respiratory viral molecular assays using commercial analyte-specific regents (ASR) or have designed their own laboratory-developed tests (LDTs), according to their needs and capacities [8]. Examples of LDTs using ASRs include the FimArray respiratory pathogen panel (Idaho Technology, Inc., Salt Lake City, UT), the Infinity respiratory viral panel (AutoGenomics, Inc., Carlsbad, CA), and the ResPlex II (Qiagen, Inc., Valencia, CA). These tests all require independent validation and verification by each laboratory to unequivocally demonstrate their intended use.

Multiple Choice Questions

1. Hospitalization is common for infants and very young children presenting with wheezing or rapid, labored breathing and a low-grade fever. What is the leading cause of infection for hospitalization of these patients?
   A. Adenovirus
   B. Herpes virus
   C. Influenza virus
   D. Respiratory syncytial virus
   E. *Streptococcus pneumoniae*
2. Which of the following techniques is the most sensitive for detection of common respiratory viruses?
   A. Conventional culture of a nasal swab specimen
   B. Direct fluorescent antibody testing of a sputum specimen
   C. Membrane-based enzyme immunoassays performed on a nasal swab specimen
   D. Real-time reverse transcription polymerase chain reaction (RT-PCR) performed on a nasal swab specimen
   E. Viral culture testing of a bronchoalveolar lavage specimen
3. Which answer is incorrect regarding hMPV infection?
   A. Coinfection with other respiratory viruses is commonly reported
   B. It has been identified worldwide
   C. It is a member of the *Paramyxoviridae* family and is subclassified in the *Pneumovirinae* subfamily
   D. Patients present with symptoms of the common cold including cough, coryza, wheezing, and fever
   E. There is seasonal presentation of infection particularly in the summer months in temperate climates

4. Reverse transcription real-time PCR reactions include which of the following steps?
   A. Creation of cDNA
   B. Hybridization to a fluorescently labeled bead
   C. Nucleic acid extraction
   D. RNA amplification
   E. Signal amplification generated by a biotin-labeled probe
5. No amplification of the internal control in a PCR-based assay for respiratory virus detection most likely indicates which of the following?
   A. If no viruses were detected then the reaction should be repeated
   B. Multiple viruses are present in the patient's specimen
   C. No virus is present in the patient's specimen
   D. No results can be reported if one or more of the viral amplifications are positive
   E. The specimen was sufficient and should be reported as negative

Answers to Multiple Choice Questions

1. *The correct answer is D.*

Respiratory syncytial virus is the leading cause of hospitalization of infants and very young children presenting with wheezing or rapid, labored breathing or apnea and fever.

2. *The correct answer is D.*

Real-time reverse transcription polymerase chain reaction (RT-PCR) performed on a nasal swab specimen is the most sensitive method, because molecular methods have been demonstrated to be more sensitive than any other method of detection for respiratory viruses.

3. *The correct answer is E.*

In temperate regions, presentation is typically in the winter and spring months. All of the other answers are correct.

4. *The correct answer is A.*

The RNA is not amplified but is copied into a DNA template that is then amplified and detected. Nucleic acid extraction of the patient's specimen is performed prior to testing. Hybridization to a fluorescently labeled bead is performed with the xTAG™ Respiratory Viral Panel (RVP) assay.

5. *The correct answer is A.*

If no viruses were detected then the reaction should be repeated. Internal controls are important for determining when the reaction is suboptimal, which could be due to the presence of an inhibitor, poor performance of the reagents, reaction conditions, or suboptimal specimen.

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BK Virus

39

Benjamin A. Pinsky

Clinical Background

The patient was a 45-year-old male with end-stage renal disease secondary to autosomal dominant polycystic kidney disease who received an HLA-matched renal allograft from a living, related donor: his younger brother. During the pretransplant workup, a nonsense mutation (c.4609 G > T, p.Glu1537X) in exon 15 of the polycystin-1 gene, *PKD1*, was identified in the patient, whereas targeted *PKD1* sequence analysis of the donor was negative for mutation [1].

Following an unremarkable transplant surgery, the patient received maintenance immunosuppressive therapy with prednisone, tacrolimus, and mycophenolate mofetil, and was monitored at least weekly for laboratory markers of kidney dysfunction. Three months post-transplant, the patient had a mildly increased serum creatinine and a decreased estimated glomerular filtration rate.

Question 1: What is your differential diagnosis?

The differential diagnosis of late acute renal allograft dysfunction can be divided into three categories: prerenal, postrenal, and intrinsic-renal causes. Prerenal causes of late acute dysfunction include volume depletion and renal artery stenosis, whereas urinary tract obstruction and lymphocele are causes of postrenal dysfunction. Intrinsic renal causes include immunosuppressant toxicity, acute rejection, recurrence of primary disease, and infection.

Question 2: How would you narrow the differential?

Serum creatinine levels remained mildly elevated following a cautious trial of fluid replacement therapy, ruling out volume depletion. Ultrasound examination found no evidence of renal artery stenosis, urinary tract obstruction, or lymphocele, suggesting that the allograft dysfunction was not due to one of these common pre- or postrenal causes. While the patient did have liver cysts due to his underlying genetic disease, there was no mass effect and the cysts did not appear to compromise the transplanted organ. Serum levels of tacrolimus and mycophenolate mofetil were both in the therapeutic range, consistent with adequate immunosuppression and inconsistent with tacrolimus nephrotoxicity. Urinalysis revealed pyuria, hematuria, and cellular casts, findings compatible with interstitial nephritis. This can be seen in infection, acute rejection, and drug-mediated nephrotoxicity.

Reason for Molecular Testing

The findings described are suggestive of an intrinsic renal cause for the patient's renal dysfunction. Molecular testing in this clinical scenario may help to distinguish a common infection affecting kidney transplant patients, polyomavirus-associated nephropathy (PVAN), from acute rejection. This distinction is critical because the treatments for these diagnoses are diametrically opposed. Whereas PVAN requires decreased immunosuppression, antirejection therapy calls for intensification of the immunosuppressant regimen. An improper diagnosis can result in graft loss.

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Test Ordered

Quantitative PCR for BK virus detection on a urine specimen.

Question 3: Is this an appropriately ordered test?

Yes. Approximately 1–10% of renal transplant patients will develop PVAN [2]. BK virus accounts for >95% of cases [2]. JC virus, a related polyomavirus, has also been found in patients with PVAN, but is more commonly associated with progressive multifocal leukoencephalopathy (PML), a rare demyelinating disease of the central nervous system [3].

The quantitative detection of BK virus DNA in the urine is recommended for noninvasive PVAN screening. Furthermore, a persistent urine level of $>10^7$ copies/mL for more than three weeks has been proposed as a threshold value for the diagnosis of presumptive PVAN. It is suggested that renal transplant recipients be screened for PVAN at least every three months during the first two years post-transplantation and annually thereafter until year five. Screening is also recommended when allograft dysfunction occurs and when surveillance biopsies are performed [4].

Laboratory Test Performed

A laboratory-developed BK virus quantitative PCR assay was performed. Viral nucleic acid was extracted from urine using an automated, silica-coated, magnetic bead-based system. Prior to extraction, an internal control was added to the sample in order to confirm nucleic acid extraction and to control for amplification failures due to PCR inhibition. The internal control contains sequences from the Green Fluorescent Protein (GFP), a gene first isolated from the Jellyfish, *Aequorea victoria*, flanked by primer binding sites identical to the BK virus target sequences. BK virus DNA was detected and quantitated by targeting the viral VP1 gene. The VP1-specific primers amplify both the target DNA and the internal control. However, differentially fluorescently labeled hydrolysis probes complementary to VP1 (fluorescein-labeled) and GFP (CalFluor560-labeled) allow detection of both BK DNA and the internal control in the same reaction. These reactions were performed on a real-time PCR instrument. Quantitation of the amplified products was accomplished using a standard curve generated with a plasmid containing the entire BK virus genome [5].

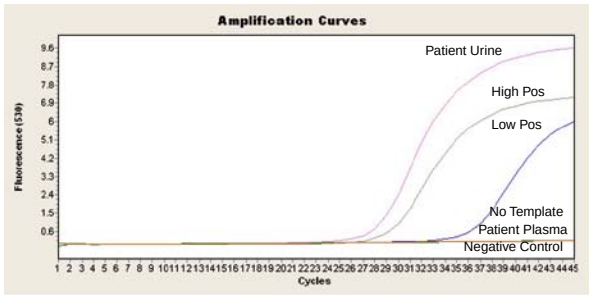
Numerous PCR methods, both commercial and laboratory-developed, are available for the detection and quantitation of BK virus [6]. These assays typically target conserved regions of the VP1 or T antigen genes and are generally significantly more sensitive and specific for BK viruria than urinary cytology [7, 8].

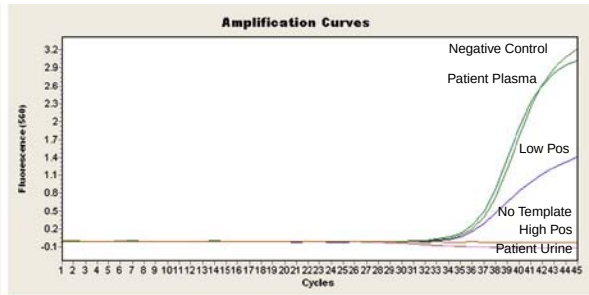
An important caveat to the evaluation of quantitative BK virus testing is that an international quantitative calibration standard does not yet exist. Therefore, values obtained from different assays should be interpreted with caution, especially if trends in viral load are being monitored. For example, the choice of material used to make the standard curve (using a plasmid versus mixed positive patient urine) can result in a one-log difference in quantitation [9]. Essentially any assay variable (including primer-probe target, probe type, and extraction method) could have a similar effect. Other viruses commonly monitored in the transplant setting, Epstein-Barr Virus (EBV) and Cytomegalovirus (CMV), also currently lack quantitative calibration standards, and their testing is subject to the same cautions [10, 11].

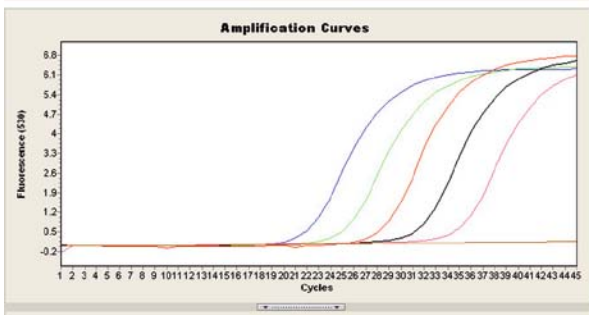
Results with Interpretation Guideline

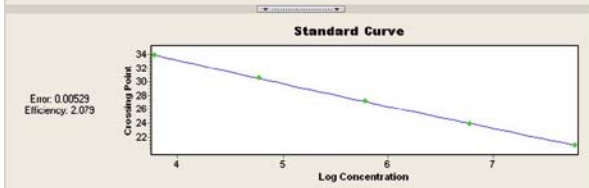
Figure 39.1 displays the results of the BK virus quantitative PCR. The criteria for analysis are as follows:

- The no template control must be negative, showing no fluorescent signal above the threshold in both the BK and IC (internal control) channels, 530 and 560 nm, respectively.
- The negative BK control must be negative, showing no fluorescent signal above the threshold in the 530 nm channel.
- The negative BK control must show a fluorescent signal in the IC 560 nm channel with a crossing threshold within three standard deviations (SD) of the mean of previous runs.
- The high positive and low positive control values should be within 2 SD of the mean of previous runs.
- It is necessary to look at the data for a sample's BK result (Channel 530) and its corresponding IC result (Channel 560) to render an interpretation. Look for the presence or absence of a fluorescent amplification curve and the cycle threshold (CT) value to









Results	CT (530 nm)	BKV Copies/mL	CT (560 nm)
No Template Control	ND	ND	ND
Negative Control	ND	ND	34.95
Low Positive	35.09	2.83×10^3	34.32
High Positive	28.02	3.25×10^5	ND
Patient Urine	26.91	7.11×10^5	ND
Patient Plasma	ND	ND	35.42

ND = Not Detected

Quality Control

	Mean	Acceptable Range
Low Positive	2.26×10^3	$5.59 \times 10^2 – 3.96 \times 10^3$
High Positive	2.94×10^5	$2.07 \times 10^5 – 3.80 \times 10^5$
Negative Control	34.88	33.53 – 36.22

Fig. 39.1 Quantitative BK virus testing in urine and plasma

determine whether the result on the patient's urine sample is positive or negative.

- Using the values assigned to the standard curve, the instrument's software automatically assigns a numeric value to positive results for patient samples. This value represents the number of copies of BK DNA per mL of sample.
- If the specimen is negative for BK but the IC is not amplified (which would be evidenced by lack of a CT or a CT value ≥ 3 SD from the mean), the sample needs to be re-extracted and reanalyzed on the next run. If the IC remains negative after re-extraction, the specimen may be inadequate or may contain inhibitors. A new sample may need to be collected and resubmitted, if clinically indicated.
- If the specimen is positive for BK but the IC is not amplified (No CT or CT value ≥ 3 SD from the mean), the reaction is acceptable and the BK quantitation can be reported. This typically occurs at high BK viral loads due to competition for primers, since the BK target and the IC share the same primer-binding sequences.

Question 4: Is this assay run valid?

Yes. The no template control is blank in both the 530 and 560 nm channels. The negative control is negative in the BK channel but IC amplification is adequate. Both the high positive and low positive control values are within 2 SD of the mean for this control lot.

Question 5: How would you report this result?

A report for this patient specimen might read:

BK virus DNA	*Detected*
Result	*711,000 copies/mL*
Result log10	*5.85*
Method	*Polymerase Chain Reaction (PCR) nucleic acid amplification*
Linear Range	*1,000–60,000,000 copies/mL (3.00–7.78 log10)*
Disclaimer	*This test was developed and its performance characteristics determined by the performing laboratory. It has not been cleared or approved by the US Food and Drug Administration. Such approval is not required for tests validated by the performing laboratory*

Result Interpretation

Question 6: Does this result explain the patient's clinical course?

Not necessarily. Although the patient has laboratory evidence of renal dysfunction and BK viruria, this BK level in urine does not meet the recommended criterion for presumptive PVAN. If BK were not detectable in urine, BK nephropathy could be ruled out and acute rejection or drug-mediated toxicity would have been the more likely diagnoses. However, the moderately high BK viruria in this clinical scenario requires further testing.

Further Testing

Quantitative PCR for BK virus detection was performed on plasma collected concurrently with the urine sample (Fig. 39.1).

Question 7: How do you explain this result?

BK virus DNA was not detected in the plasma of this patient. BK viruria in the absence of BK viremia suggests reactivation and replication of BK virus in the renourinary tract without renal parenchymal involvement. Therefore, the patient was unlikely to have PVAN and a renal biopsy was required for further characterization of the patient's graft dysfunction.

Renal biopsy on this patient showed lymphocytic inflammatory infiltrates and active tubulitis consistent with acute rejection, without findings of tacrolimus toxicity, viral inclusions, immunohistochemical detection of polyomavirus antigen, or *in situ* hybridization for BK DNA. Intensification of the immunosuppressant regimen resulted in normalization of the patient's renal function and a subsequent renal biopsy showed resolution of the inflammatory infiltrate. The patient continued to be closely monitored for persistent BK viruria and despite antiviral therapy, several months later developed BK viremia with associated graft dysfunction and histopathologic findings of PVAN. This case demonstrates the delicate balance that must be maintained by the immune system to achieve successful transplantation.

Background and Molecular Pathology

BK virus was originally identified from the urine of a renal transplant patient (initials B.K.) with ureteric obstruction [12]. BK virus belongs to the family *polyomaviridae*, which includes JC virus and the simian virus, SV40 [13]. It is a nonenveloped, double-stranded DNA virus with a ~5 kb genome. The genome has an early region encoding the small and large T antigens, a late region encoding the viral capsid proteins VP (viral protein) 1, VP2, and VP3, and a noncoding regulatory region. The VP1 gene displays considerable genetic heterogeneity and this genetic variation has led to recognition of several viral genotypes; I, II, III, and IV. Though this genetic heterogeneity may affect accurate quantitation, BK genotyping is not yet thought to have clinical utility [9].

Primary infection with BK virus is prevalent in childhood and more than 90% of adults are seropositive [14]. Primary infection may be asymptomatic, but can also be associated with upper respiratory symptoms and transient viruria. After primary infection, the virus remains latent in the urogenital tract. Asymptomatic reactivation and intermittent shedding of virus in the urine occurs spontaneously in both immunocompetent and immunosuppressed patients. However, BK virus-associated diseases are more common in the immunosuppressed, particularly renal transplant patients, and include hemorrhagic cystitis, ureteral stenosis, and polyomavirus-associated nephropathy, as discussed in this case.

Quantitative PCR assays have emerged as the "gold standard" for polyomavirus detection and are a part of the noninvasive diagnostic approach to this pathogen in renal transplant patients [2]. In addition, these assays are used to monitor the response to therapy, which primarily involves a reduction in immunosuppression, though agents with antipolyomavirus activity, such as leflunamide and cidofovir, are also often part of the therapeutic regimen [2]. A diagnosis of presumptive PVAN can be made in biopsy-negative cases with persistently elevated viruria ($>10^7$ viral copies/mL) or viremia ($>10^4$ viral copies/mL) for more than three weeks [4]. It is important to note that these threshold values are the recommendations of an expert panel and may need to be re-evaluated in the context of the quantitative assay being used.

BK viruria typically precedes viremia by one to three months [2, 6]. Therefore, the monitoring of BK DNA levels in the urine of asymptomatic renal transplant patients allows early detection of those at risk of developing PVAN. BK viremia is more likely to be associated with active BK virus infection and is thought to better correlate with nephropathy [15].

Multiple Choice Questions

1. BK virus is in the same virus family as:
   A. Adenovirus
   B. Herpes simplex virus
   C. Human papilloma virus
   D. Parvovirus B19
   E. Simian virus 40
2. Polyomaviruses are associated with:
   A. Merkel cell carcinoma
   B. Polyomavirus-associated nephropathy (PVAN)
   C. Progressive multifocal leukoencephalopathy (PML)
   D. B and C
   E. A, B, and C
3. PVAN most commonly occurs in the context of:
   A. Diabetes
   B. Hematopoietic stem cell transplant
   C. HIV
   D. Renal transplant
   E. Small bowel transplant
4. The best way to ensure the portability of quantitative nucleic acid amplification testing results is to:
   A. Require all quantitative testing be sent to a central reference lab
   B. Require exactly the same assays be used in every lab
   C. Require more stringent proficiency testing criteria
   D. Require more stringent quality control criteria
   E. Require the use of international quantitative calibration standards
5. A patient travels 8 hours by bus every two weeks to be seen by his transplant physician at an academic, quaternary medical center. In the intervening weeks, he is seen by his local primary care physician. Because of persistent BK viruria, BK virus quantitative PCR on plasma is monitored at each visit. This testing is performed at two different laboratories using different assays. Measures of renal dysfunction and therapeutic drug monitoring remain unchanged. The BK results are as follows:

Week	Laboratory X (copies/mL)	Laboratory Z (copies/mL)
1	25,500	–
2	–	1,500
3	31,000	–
4	–	1,100
5	22,300	–

What is the best interpretation for these results?
   A. The patient's viremia is fluctuating. The tests offered by the laboratories are equivalent
   B. The patient's viremia is fluctuating. The test offered at Laboratory X has increased analytical sensitivity compared to Laboratory Z
   C. The patient's viremia is stable. The test provided at Laboratory Z has an approximately one-log negative bias compared to Laboratory X
   D. The patient's viremia is stable. The test offered at Laboratory X has decreased analytical specificity compared to Laboratory Z
   E. The patient's viremia is stable. The tests offered by the laboratories are equivalent

Answers to Multiple Choice Questions

1. *The correct answer is E.*

BK virus is in the family *polyomaviridae* which includes Simian virus 40 (SV40) and JC virus, as well as the newly described WU (Washington University), KI (Karolinska Institute), and Merkel Cell polyomaviruses [16]. Interestingly, SV40 can transform primary human cells and the p53 tumor suppressor was initially identified as a cellular protein associated with the SV40 large T antigen. Though this virus has made significant contributions to our understanding of cellular transformation and tumorigenesis in model systems, it has not yet been convincingly shown to be associated with human cancers in epidemiologic studies [17].

2. *The correct answer is E.*

The development of Merkel cell carcinoma, a neuroendocrine tumor of the skin, is associated with the Merkel cell polyomavirus. JC virus is associated with progressive multifocal leukoencephalopathy (PML) and, of course, BK virus is associated with PVAN.

3. *The correct answer is D.*

Approximately 1–10% of renal transplant patients will develop PVAN. The disease occurs almost exclusively in transplanted kidneys and only rarely in the native kidneys of transplant recipients, or in patients with hematopoietic stem cell transplants, malignancy, or HIV. BK virus is associated with hemorrhagic cystitis in hematopoietic stem cell transplant patients.

4. *The correct answer is E.*

Traceable and commutable international quantitative calibration standards are critical for standardization and result portability. For example, international standards are available for HIV, HBV, and HCV

nucleic acid amplification tests. The other potential answers do not directly address this fundamental issue.

5. *The correct answer is C.*

These laboratories likely use different BK assays and the lack of an international standard results in systematic bias (values consistently too high or too low). Bias in quantitative assays can be identified using Bland-Altman plots [18]. In these graphs, the differences between the values obtained with the two methods are plotted against their average.

Given that the patient is clinically stable, it is unlikely that his viral load is fluctuating so consistently and dramatically on alternating weeks, eliminating answers A and B. Answer E is illogical.

For answer D it is worthwhile to review the definitions of sensitivity and specificity. Diagnostic sensitivity [true positives/(true positives + false negatives)] and diagnostic specificity [true negatives/(true negatives + false positives)] are statistical measures of a binary (positive or negative) classification test. Analytical sensitivity refers to the smallest amount of substance that can accurately be measured by an assay (lower limit of detection and lower limit of quantitation). Analytical specificity is defined as the ability of an assay to measure one particular organism or substance. If, for example, Laboratory X's test unintentionally amplified JC in addition to BK, one might see the results described above. However, this would require reactivation of both JC and BK viruses and is less likely than answer C.

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Respiratory Viruses

40

Donna M. Wolk

Clinical Background

A six-year-old child of Asian descent presented to the Bone Marrow Transplant Out-Patient Clinic with a two day history of a febrile upper respiratory tract infection. The child had recent contact with several extended family members during the winter holiday break. At the time of allogeneic hematopoietic stem cell transplantation (HSCT) three months prior to presentation, the patient was seropositive for cytomegalovirus (CMV) and seronegative for human immunodeficiency virus (HIV), hepatitis B, and hepatitis C.

During her wait in the clinic, her condition rapidly deteriorated with worsening cough, dyspnea, and tachycardia. She was admitted to the pediatric intensive care unit where, upon examination, the child had obvious evidence of respiratory distress with severe tachypnea (>50 breaths per minute) and intercostal retraction. Pulse oximetry revealed hypoxia with an oxygen saturation of 0.92. Chest auscultation demonstrated audible diffuse high-pitched wheezing with fine inspiratory crackles and bilobar rales. A chest radiograph indicated obliterative bronchiolitis with hyperinflation of both lung fields and diffuse extensive pulmonary infiltrates. Laboratory studies revealed a hematocrit of 24%, a white blood cell (WBC) count of 2.3×10^6/mL (leukopenia), lymphopenia (1.1×10^6/mL), and a normal platelet count. Other history and the family history were unremarkable.

Histopathologic and cytologic examination of a bronchoalveolar lavage (BAL) revealed no abnormalities. Gram's stain was reported as "few polymorphonuclear cells and few mixed flora"; no yeast was identified. Bacterial, fungal, and mycobacterial cultures were pending.

Question 1: What are the major patient risk factors that made this patient or her respiratory samples a good candidate for molecular testing?

Reason for Molecular Testing

The patient was an immunocompromised child with acute respiratory distress and known exposure to other children and adults during the winter respiratory virus season. Rapid and sensitive molecular detection of viral infections is essential in select populations such as transplant patients, children, the elderly, the chronically ill, and the immunocompromised [1]. In these patients, clinical goals are aimed at reduction of morbidity, mortality, and at directing specific therapy if possible [2–5]. Whereas respiratory infections in the immunocompetent patient may be nothing more than inconvenient, the same infections in critically ill or immunocompromised individuals can be life-threatening and warrant antiviral therapy or removal of immunosuppressant therapy. Therefore, rapid and sensitive detection methods have important clinical ramifications.

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Molecular multiplex methods are emerging as useful tools to support the aims of rapid and sensitive detection of most respiratory viruses. Several key clinical issues highlight the importance of molecular testing for respiratory viruses. First, viruses can cause similar symptoms, but may have very different clinical implications [6]. Second, in otherwise healthy patients, a rapid viral diagnosis is valuable because it can potentially shorten hospitalization or decrease the need for antimicrobial therapy [2]. Third, in the transplant population, rapid testing is more critical because viral infections can interfere with the success of transplants and with therapeutic interventions or can predispose patients to other infections [3, 5]. Additionally, because viral shedding is prolonged in transplant patients, they are at increased risk for development of antiviral resistance [3, 5]. Finally, rapid viral testing is important for infection prevention in hospitals.

Question 2: List the most appropriate specimen and laboratory tests, which would be logically requested to assess this patient for the presence of a respiratory virus infection.

Test Ordered

A nasopharyngeal (NP) swab was collected and submitted for rapid respiratory syncytial virus (RSV) antigen testing and influenza antigen testing. A bronchoscopy was performed and a BAL specimen was submitted to the anatomic pathology laboratory for histopathologic and cytologic examination. In addition, the BAL was collected and transported to the microbiology laboratories on wet ice and submitted for respiratory virus molecular multiplex panel testing (respiratory multiplex). Testing was also requested for bacterial, fungal, and mycobacterial culture. A third shift employee inadvertently placed the unpreserved BAL specimen in the −20°C freezer overnight prior to testing.

Laboratory Test Performed

Historical virological methods have limitations in sensitivity, specificity, speed, cost, or sample type and do not fully support rapid testing goals for confirmation of respiratory viruses. While new single-plex molecular methods are rapid and accurate for viral detection, their collective use is cost-prohibitive and impractical when applied to multiple viral pathogens. For this reason, a respiratory multiplex test was performed.

With simultaneous differentiation of 12–17 common viruses within five to eight hours, the respiratory multiplex assays are currently among the most rapid methods for confirmation of respiratory viruses. Over the past several years, these assays have displaced a combined standard of practice consisting of routine viral culture, rapid viral antigen testing, and testing with single-plex PCR. With their advent came the awareness that viral coinfections were previously underestimated. Reports of increased numbers of coinfections are relevant, as infections may require diagnosis of all involved pathogens for effective treatment [7]. Multiple viruses can be detected in over 10% of samples.

There are several Luminex-based respiratory multiplex assays commercially available. However, to date only one is FDA-cleared: the Luminex xTAG™ RVP assay, distributed by Abbott Molecular and Luminex Molecular Diagnostics (Toronto, Canada).

Note: Since the writing of this chapter, another multiplex system was FDA-approved, the Idaho Technology Film Array Respiratory Panel.

xTAG RVP

The Luminex xTAG™ RVP assay is accurate, sensitive, and currently FDA-cleared for nasopharyngeal swab samples, for detection of RSV, influenza A and B, parainfluenza 1, 2, and 3, metapneumovirus, adenovirus, and rhinovirus. The assay uses multiplex PCR and fluid microsphere-based array detection on the Luminex x-MAP system (flow cytometer based). In one study, a total of 360 frozen respiratory specimens, consisting of 70 culture-positive nasal and nasopharyngeal washes, were tested and results compared to those obtained with a combined reference standard of cell culture and *Taq*Man RT-PCR assays. The reported sensitivity of this version of the assay for individual viruses ranged from a low of 63.3% for RSV to a high of 100% for influenza B [8]. In another study the RVP test detected 180 of 183 true positives, for a sensitivity of 98.5% [9]. Similarly, in another comparison, the RVP assay compared to in-house molecular methods as the sensitivity was determined to be 91.2% with a specificity of 99.7% [10].

ResPlex II

The ResPlex technology is based on target-enriched multiplex-PCR (Tem-PCR™), which includes a template enrichment step that is designed to increase assay specificity by using very small amounts of gene-specific primers to reduce primer–dimer formation and nonspecific background [11]. This assay uses a "target-enriched amplification", which has been previously described [8, 11, 12], based on the ResPlex assay (Genaco Biomedical Products, Inc, Huntsville AL), but which was modified by Qiagen to include an improved internal control based on the human genome and an inhibitor control, designed to maximize sensitivity to PCR inhibitors.

The ResPlex II assay version 2.0 is currently available for research use only (RUO), and detects influenza A and B (FLU A, FLU B), parainfluenza virus 1–4 (PIV-1, PIV-2, PIV-3, PIV-4), respiratory syncytial virus A and B (RSV A and B), human metapneumovirus (hMPV), rhinovirus (RHV), and coxsackie virus/echovirus (CVEV). In addition, the assay incorporates an external positive control to monitor PCR inhibition, as well as a control primer set, which is based on amplification of the human iduronate-2-sulfatase (*IDS*) gene, located on the X chromosome. The *IDS* primer set will amplify DNA from human cells and ensures that sample collection was appropriate to deliver some human cells in the patient specimen.

After template enrichment, the ability to amplify gene targets is increased by use of a pair of high concentration proprietary super-primers with high affinity for *Taq* polymerase, that maximize the efficiency of target amplification. RNA extracts are subjected to reverse transcription (RT)-PCR, followed by the initial PCR. The advantages of the Resplex II include the assay's ability to rapidly detect multiple viruses in less than one eight hour work shift. This was the assay used for testing our patient's sample. The assay was verified and validated using CLIA '88 requirements for laboratory-developed tests (LDTs).

Results with Interpretation Guideline

Rapid RSV Antigen

Upon hospital admission, the rapid RSV antigen test was performed on the NP swab. Typically, rapid antigen assays are comprised of a sample preparation step followed by addition of the sample to a membrane test well. Wash fluid is then added, followed by the addition of alkaline phosphatase-conjugated monoclonal antibodies to RSV proteins. This mix is incubated at room temperature, after which the membrane well is washed, substrate reagents are added, and the test result is read after five minutes. If the patient result demonstrates a visible purple line or, for some tests, a purple triangle, then it can be concluded that RSV antigen is present in the patient sample. In each test batch, a positive control is included, and should become purple, indicating correct test performance.

ResPlex Assay

Respiratory multiplex assays rely on multiplex RT-PCR and PCR to amplify various genetic regions of common respiratory viruses. Once genetic targets are amplified, they are hybridized to solid-phase probes located on beads, which are tagged with fluorescent molecules to identify each individual target. After amplicon binds to the beads, they are passed through a Luminex system, a flow cytometer which can identify the bead and the bound amplicon by its corresponding fluorescence. Mean Fluorescence Intensity Units (MFI) are the results generated by the Luminex software and are used to determine whether the test result is positive or negative. A cutoff of 250 MFI was used in this example to differentiate between positive and negative samples.

Several controls are used in this assay. 1) For the external positive control, which is based on amplification of the *IDS* gene, no MFI cut-off is established for the ResPlex assay. However, it is prudent for laboratories to identify a human cell cutoff to ensure appropriate specimen collection. In our laboratory, an IDS cutoff was established to ensure cellular quality of the specimen. 2) The assay's internal controls (IC) is an additional positive control to monitor PCR inhibition. 3) Because of the cost of performing quality controls on all viruses with each assay, it is typical to test two to four known viruses per batch and to rotate positive viruses to assess test performance with all viruses in a short period of time. 4) For users of these assays, it is recommended that at least 20% of all samples tested are controls called "no template controls"

Sample	Well	Accession	RSVA	RSVB	OC43	BocV	229E	PIV2	PIV1	INFB	PIV3	PIV4	NL63	RHV	CVEV	INFA	ADVB	ADVE	HMPV	HKU1	IDS	IC	Comments
1	A1	Patient 1 (Case patient)	12	1403	35	30	39	25	22	26	359	17	16	18	28	7	15	15	2153	27	629	2191	RSVB, PIV-3, HMPV
2	A2	Patient 2	8	16	13	25	13	20	25	19	23	20	22	28	21	15	25	13	15	18	758	2378	NEG
3	A3	Patient 3	35	18	18	9	26	10	28	24	33	13	13	38	43	3764	14	14	16	25	112	817	INFA
4	A4	Patient 4	12	16	35	30	39	17	22	26	22	17	16	18	17	7	15	15	36	27	629	2191	NEG
5	A5	Patient 5	24	4	24	23	61	15	22	15	43	20	53	657	2090	24	6	19	32	32	479	2201	RHV,CVEV
6	A6	Patient 6	1	30	20	27	27	18	10	17	27	12	26	86	46	19	28	19	6	10	450	2176	NEG
7	A7	Patient 7	4600	21	24	39	20	17	19	14	30	23	82	83	21	353	21	2	23	36	500	716	RSVA, INFA
8	A8	Patient 8	7	18	14	14	4	11	20	7	26	26	40	23	18	1790	19	9	13	16	215	2461	INFA
9	A9	Patient 9	20	14	9	25	5	6	31	5	22	11	48	36	27	12	2153	27	16	16	747	1086	ADVB
10	A10	Positive IDS Control	0	25	13	25	16	24	13	14	14	22	16	35	16	13	21	12	10	22	1431	85	POS IDS-PASS
11	A11	Positive Control (CVEV-OC43)	22	24	606	24	64	18	17	13	43	28	36	12	1890	19	11	24	21	6	1291	640	POS QC-PASS
12	A12	Negative Control (No template)	17	26	23	17	10	15	8	6	30	9	3	19	15	21	19	23	21	8	22	2890	NEG QC - PASS
13	B1	Negative Control (No template)	2	22	23	28	5	2	9	19	19	28	19	27	7	13	26	9	22	22	28	2813	NEG QC - PASS
14	B2	WIPE TEST -1	8	16	13	25	13	20	25	19	23	20	22	28	21	16	25	13	15	18	758	2378	NEGATIVE-PASS
15	B3	WIPE TEST-2	35	18	18	9	26	10	28	24	33	13	13	38	43	9	14	14	16	25	112	817	NEGATIVE-PASS
16	B4	WIPE TEST -3	22	39	15	18	25	23	15	119	24	24	32	67	10	26	35	25	21	28	148	914	PASS, question background
17	B5	Negative Control (No template)		23	17	15	14	16	22	0	31	6	34	49	13	16	12	29	14	13	17	2786	NEGATIVE-PASS

Black cells indicate a positive reaction; grey cells indicate negative results that are higher than the typical baseline
RSV = respiratory syncytial virus; OC43 = coronavirus; BocV = bocavirus; 229E = coronavirus 229E; PIV = parainfluenza virus; NL63 = coronavirus NL63; RHV = rhinovirus; INF = influenza, CVEV = coxsackie/enterovirus; ADV = adenovirus; HMPV = human metapneumovirus; HKU1 = coronavirus HKU1; IDS = control for presence of human cells and appropriate specimen collection; IC = internal control .

Fig. 40.1 Grid with mean fluorescent units (MFI) from the ResPlex II assay. The grid depicts patient samples, positive and negative (no template) controls, and results of a monthly wipe test to survey the laboratory for presence of possible amplicon contamination

(NTC), also known as Negative Controls, to help ensure that there is no amplicon contamination in patient specimens. No substantial signal should occur in the NTC wells.

For our patient, the respiratory multiplex testing result of the BAL was reported the next day (Fig. 40.1).

Question 3: Is this assay run valid? Could you accept and report the patient results?

The assay is valid. *IDS* controls exhibit strong signals above the background fluorescent reading and are indicative of good sample collection techniques. (Exceptions can be noted for strong positive virus samples, because virus target amplification will compete with both the *IDS* and IC controls.) Three NTC controls constitute more than 20% of all samples and the NTCs are all negative, indicating a clean, contaminant-free test batch. The positive controls are positive for the two viruses placed into the run. No problems were noted with any controls.

Question 4: How would you interpret the result grid and identify the viruses for the patient?

Positive results are indicated by a black box in this example. The patient is positive for RSVB, PIV-3, and hMPV. Three viruses in one sample are rare, but have been documented. As opposed to cell culture, in which multiple viruses are rarely observed, the advent of molecular respiratory multiplex testing has demonstrated that multiple infections are relatively common.

Result Interpretation

Question 5: How would you explain and reconcile the discrepancy between the rapid antigen test and the respiratory multiplex assay?

Like any laboratory method, rapid antigen tests can produce false-negative results. Molecular tests are known to produce positive results when antigen tests

are negative. Likewise, because of the lability of RSV, molecular tests can produce a false-negative result if RSV is present in low density and the transport of the specimen is suboptimal.

Question 6: How would you justify or prove that these viruses were not the result of laboratory contamination?

All three NTCs (negative controls) were clearly negative, delivering MFI well below the MFI cutoff of 250. Positive sample wells are evenly distributed among the viruses, with no focus on one particular virus, which may indicate contamination or a true increase in prevalence. The assay was performed in winter when the respiratory virus prevalence is high, consistent with assay results.

Further Testing

Other lower respiratory tract infections could exist and given the patient's transplant status, the lower respiratory sample, the BAL, may need to be tested for cytomegalovirus, herpes simplex virus, and, in some cases, strains of adenovirus that are not included in the respiratory multiplex tests [6].

Background and Molecular Pathology

Lower respiratory infections are the most common cause of mortality and severe disease among immunosuppressed patients and the most common cause of death among human stem cell transplant (HSCT) patients [13]. Pulmonary infiltrates caused by infections must be distinguished from noninfectious causes. The ability to rapidly diagnose or rule out infection is critical in order to initiate proper therapy (antibacterial, antiviral, antifungal, or other), minimize lung damage, and limit the time spent on potentially toxic empiric therapeutic regimens.

Rapid initiation of therapy for influenza A and RSV have been documented to reduce severity of disease [14]. For human metapneumovirus, colonization has been reported, however, as have cases of severe pneumonia, respiratory failure, transplant rejection, and death [4, 15]. Important considerations for users of all multiplex assays include challenges related to nucleic acid extraction, amplicon control, and multiplex assay validation.

RSV

RSV undoubtedly is the most important respiratory viral pathogen in pediatric transplant patients and the largest cause of pediatric hospitalizations and emergency room visits for lower respiratory infections (LRI) and acute bronchiolitis in children younger than five years [6, 16, 17]. Rapid antigen tests are commonly used to cohort patients based on their RSV status during the peak of the annual RSV season. However, rapid antigen tests can produce false-negative results and molecular RSV testing is more accurate. Negative results should be confirmed with culture or a molecular RSV assay.

HPMV

Since 2001, hMPV has been established as a significant respiratory pathogen in children and adults [18, 19]. It has been reported to be the second most common cause of bronchiolitis in infants, and causes infections such as acute otitis media in older children and mild upper respiratory infection (URI) or asymptomatic carriage in healthy adults. Severe illness has been described in the elderly, adults with underlying conditions, and the immunocompromised [18, 19].

Parainfluenza Virus

Human PIV-1, PIV-2, and PIV-3, are important causes of respiratory infection in all age-groups. In both children and adults, PIV causes URI, such as croup, as well as LRI in infants, young children, the elderly, those with chronic comorbidities, and the immunocompromised [20]. These pathogens are second only to RSV as a cause of hospitalizations for LRI in children [20]. The clinical significance and epidemiology of human PIV-4 are not well understood and this virus is not detectable by all currently used assays. PIV-1 and 3 are the most common and the best studied.

Nucleic Acid Extraction

Working with enveloped RNA viruses is a challenge for all clinical laboratories. Enveloped viruses are more sensitive to degradation during transport than

those without envelopes. Therefore, preanalytical systems must be optimized and monitored. Moreover, RNA viruses are more susceptible to damage caused by the freeze–thaw process. The enveloped RNA respiratory viruses include influenza, parainfluenza, respiratory syncytial virus, and human metapneumovirus. The addition of DNA viruses, such as adenovirus and bocavirus, which are included in some multiplex panels, necessitates the need for use of extraction methods that combine DNA and RNA extraction.

Amplicon Control

One important facet of workflow and assay performance for the Luminex-based assays is the strict requirement for adherence to amplicon control and unidirectional workflow. All assays have the potential to produce high levels of amplification, and no formal amplicon control, such as treatment with uracil-*N*-glycosylase, exists for any of the respiratory panel assays, because the assays do not support incorporation of uracil into the PCR amplicon. Therefore, for these assays, extreme caution must be used when performing manipulation of the multiplex amplicon during the bead hybridization steps. For users of these assays, it is recommended that at least 20% of all samples tested are no template controls (NTCs) to help ensure that there is no amplicon contamination in patient specimens. This practice adds costs and must be considered when assessing the cost benefit of the multiplex assays. Stringent amplicon control practices should be used with these and similar assays.

Continuous Assay Validation

Though several published reports describe respiratory multiplex test performance, many describe a relatively limited number of specimens, considering the wide genetic variety of known respiratory viruses. Therefore, despite FDA-clearance or publications, users of these assays must maintain constant vigilance to emerging strains of viruses and other issues that could impact test results. Genetic variation issues are complicated by the fact that users do not have access to proprietary genetic sequences used by commercial suppliers. Laboratory directors will need to keep close contact with physicians and public health authorities in their region to maintain awareness of genetic variability emerging in local virus strains and submit these strains to the commercial vendors for assessment. In some cases, it may be necessary to perform backup cultures or a single-plex molecular test if the clinical evidence strongly suggests a viral etiology but no virus has been detected.

Despite the limitations, when compared to combined reference standard methods, the respiratory multiplex methods are rapid and accurate for detection of respiratory viruses. These methods generally provide equivalent or improved performance over culture, fluorescent antibody, and rapid antigen methods. Laboratory directors need to balance the speed and user-friendly approach of testing multiple viruses with assay performance for labile enveloped viruses, and continue to assess performance of multiplex methods with other methods and in the context of clinical symptoms.

Multiple Choice Questions

1. From the list below, select the most likely cause(s) of the patient's respiratory distress?
   A. Adenovirus
   B. Herpes simplex virus
   C. HMPV, parainfluenza virus 3
   D. RSV, HMPV, parainfluenza virus 3
   E. RSV only
2. Are all three viruses identified in our patient involved in the respiratory disease?
   A. It is impossible to determine whether all three viruses were involved in the infection, because some of these viruses are commonly found in people without disease
   B. No, only RSV causes respiratory disease that is severe enough to cause this patient's respiratory distress
   C. No, these combined viruses are unlikely to cause disease, because human cells can only be infected with one virus at a time
   D. Yes, all these three common respiratory pathogens could be involved in causing the respiratory distress in a patient with these risk factors
   E. Yes, the patient is predisposed to mixed infections because of her Asian descent

3. What is (are) the most likely event(s) that led to the patient's infection with multiple viruses?
   A. Ethnic descent in a transplant patient
   B. Immunosuppression and exposure to multiple family members
   C. Immunosuppression and seropositivity to CMV
   D. The patient's age
   E. The patient's gender
4. In light of these results, what would you tell a physician who calls to question your laboratory's results for this patient with three viruses identified?
   A. Only RSV is pathogenic enough to cause disease of this severity
   B. The results are reliable
   C. The results are unreliable
   D. The results are valid; however it may be prudent to resubmit a second specimen for testing
   E. The laboratory has experienced contamination; a new specimen should be collected and sent to a reference laboratory for testing
5. Given that the specimen was inadvertently frozen, what could you do for the patient to avoid a recollection of the BAL?
   A. Heat the frozen specimen to 95°C to remove nucleases
   B. Nothing, there is no way to avoid recollection
   C. Re-extract the specimen stored at −20°C
   D. There is no need for a recollection of the specimen
   E. Use the cytology specimen

Answers to Multiple Choice Questions

1. *The correct answer is D.*

The sample is positive for RSV, hMPV, and parainfluenza virus 3. These three viruses are typically associated with severe respiratory disease, especially RSV.

2. *The correct answer is D.*

This patient was a child with multiple risk factors during the winter respiratory virus season. It is very plausible that all three common respiratory pathogens would be involved in the disease process and that each contributed to the respiratory distress observed in this patient.

3. *The correct answer is B.*

The child's immunosuppression and exposure to multiple family members during the peak of winter respiratory season are likely factors that would contribute to respiratory virus infection. The child's age is also a factor, as pediatric viral infections are relatively common. Ethnic decent and gender would not typically contribute to elevated risk factors for respiratory disease.

4. *The correct answer is B.*

The results appear to be reliable. All quality control, the positive and negative controls, the NTCs, and the wipe tests were negative. Whereas one wipe test had a slightly elevated fluorescent value (119 for Influenza B), it was not above the 250 MFI required for the test to be interpreted as positive. Furthermore, there was no evidence of a pattern of contamination within the result plate – no other patients were positive for Influenza B, nor was there any other patient positive or had low level signal for any of the three viruses present in our patient. Of note, prudent laboratory practice would warrant continued monitoring and laboratory cleaning to ensure no amplicon contamination is present in the laboratory.

5. *The correct answer is D.*

After review of test results and discussion with the patient's physician about the case, it was determined that there was no need for a recollection of the specimen. Although storage at 2–8°C would be optimal, storage for a short period of time at −20°C is a marginally acceptable practice for storage prior to RT-PCR and PCR for respiratory viruses. Long-term storage at −20°C is not recommended because enzymes can remain active and degrade nucleic acids, particularly RNA, in the specimen. In this case, storage did not appear to be severely detrimental to test results and the laboratory consultation, explaining the limitations to the physician was sufficient, in light of the viruses identified. Because this may not always be the case, close communication between the laboratory consultant and the clinician is warranted. It is not surprising to find that a rapid antigen test for RSV is negative while the RT-PCR is positive; this can occur due to increased analytical sensitivity of the RT-PCR. Despite the fact that the sample was inadvertently frozen, the specimen is acceptable and apparently had good yield of nucleic acid as evidenced by the high *IDS* control and the fact that three viruses were identified. Long-term specimen storage would need to occur at −80°C.

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