Chapter 7

Neuromuscular Diseases

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DUCHENNE AND BECKER MUSCULAR DYSTROPHIES

Molecular Basis of Disease

Duchenne and Becker muscular dystrophies (DMD and BMD) are X-linked, allelic, neuromuscular diseases characterized by progressive muscular weakness and degeneration of skeletal muscle. Duchenne muscular dystrophy is the most common X-linked recessive lethal disease, with an incidence of approximately 1 in 3,500 newborns, and approximately one third of cases are the result of new mutations.[1,2] Affected children are usually wheelchair bound by the age of 12 years. As the disease progresses, contractures increasingly develop, leading to asymmetrical spinal deformities. Most patients die at about 20 years of age due to pneumonia related to chronic respiratory insufficiency. The allelic disorder BMD has a milder clinical course and slower disease progression. Becker muscular dystrophy has been estimated to occur approximately one tenth as frequently as DMD, with an incidence of about 1 in 35,000. The majority of BMD patients initially experience difficulties between 5 and 15 years of age, although an onset in the third or fourth decade or even later can occur. By definition the affected patients remain ambulatory until 16 years of age or later, thus allowing clinical distinction from patients with DMD.

The *DMD* gene is the largest human gene isolated, spanning more than 2000 kilobases (kb) of genomic DNA, and is composed of 79 exons that encode a 14 kb transcript, which is translated into a protein named dystrophin.[3,4] Dystrophin is a 427 kilodalton (kDa) cytoskeletal protein consisting of 4 domains: (1) an amino terminus that associates with actin or an actin-like protein, (2) a rod domain consisting of long, flexible rows of 24 alpha helical repeats, (3) a cysteine-rich region, and (4) a unique carboxy terminus.[5] Dystrophin has been shown to be tightly associated with a large oligomeric complex of sarcolemmal glycoproteins through its cysteine-rich domain and carboxy terminus, while the amino-terminal domain interacts with actin or an actin-like protein.

By immunochemistry, dystrophin localizes to the cytoplasmic face of the muscle cell membrane and at postsynaptic membrane specializations in neurons. Dystrophin makes up only 0.002% of total muscle protein but up to 5% of the membrane skeleton. Dystrophin is found in skeletal muscle, smooth muscle, cardiac muscle, and brain. There are slightly different forms of dystrophin messenger RNA (mRNA) in different tissues due to different transcription start sites and alternative splicing. The function of dystrophin is not known for certain, but proposed functions for the protein include important roles for the organization and stabilization of the sarcolemma and in protecting muscle fibers from contraction-induced injury. Patients with DMD have very little or no detectable dystrophin, whereas BMD patients have an altered size and/or quantity of dystrophin.[6] However, the disease etiology may be more complex than a simple loss of dystrophin, because several of the dystrophin-associated proteins that interact with dystrophin also are absent. The dystrophin-associated proteins may be directly involved with the calcium flux in the dystrophic fibers. Thus, the loss of dystrophin may be the first of many steps that ultimately lead to muscular dystrophy.

Utilizing complementary DNA (cDNA) probes derived from the 14 kb mRNA and multiplex polymerase chain reaction (PCR) analysis, approximately 65% of the DMD/BMD cases are due to deletions in the dystrophin gene.[7,8] The deletions are nonrandomly distributed and occur primarily in the center (~80%) and less frequently near the 5′ end (~20%) of the gene. The 200 kb region covering intron 44, exon 45, and intron 45 is the major deletion breakpoint region of the gene. The majority of the larger deletions initiate at the 5′ end of the gene.

There is no apparent correlation between the size or location of the deletion and the severity and progression of the disease. One of the largest deletions (35 exons) identified is in a mild BMD patient. Furthermore, sequences deleted in DMD patients often overlap with

deletions in BMD patients. However, it was proposed that if a deletion disrupts the translational reading frame of the dystrophin mRNA triplet codons, then little or no dystrophin will be synthesized, resulting in the more severe disease, DMD.[9] In the milder disease, BMD, the deletion maintains the translational reading frame, and a partially functional protein is produced. The reading frame hypothesis explains the phenotypic differences observed in about 92% of the DMD/BMD cases. One major exception to the reading frame hypothesis has been the identification of BMD patients with an out-of-frame deletion of exon 3 through exon 7. An alternate splicing mechanism or new cryptic translational start site may account for the production of an altered dystrophin protein and the milder phenotype in these patients. A small number of DMD patients with in-frame deletions have also been identified. The more severe phenotype in these patients may be due to the overall effect of the deletion on the protein conformation or may be the result of mRNA instability. Phenotypic variability has even been observed in several patients who share identical gene deletions. Deletion of exon 45, the most commonly observed DMD deletion, has also been associated with the BMD phenotype. Some genetic variability may be due to other molecules involved in destruction of damaged muscle fibers, in muscle regeneration, or in the cellular response to different hormones.

The large gene size, particularly of the introns, which average 35 kb, may account for part of the high deletion rate; however, in addition to size, other factors must be involved. The observed nonrandom deletion pattern may reflect domain-associated variation in chromosomal stability. For instance, complications related to the maintenance of replication, correct transcription, and proper splicing of such a large gene may play an extremely important role.

Partial gene duplications have been identified in 5% to 8% of patients. Unlike the deletion distribution, approximately 80% of the duplications are located at the 5′ end of the gene and only 20% in the central gene region. Out-of-frame duplications occur in DMD patients and in-frame duplications in BMD patients, thus suggesting that the reading-frame genotype-phenotype hypothesis also holds true for duplications.

Small mutations (point mutations and small deletions and duplications) in the dystrophin gene also have been identified in DMD patients.[10] The majority of these mutations have been unique to individual patients and have resulted in a truncated dystrophin protein lacking part or all of the C-terminus. The truncated proteins are presumably unstable, and little or no dystrophin is produced. Therefore, these types of mutations provide little information on structural/functional relationships in the dystrophin protein. The identification of DMD mutations that do not cause protein truncation may provide us with further insight into the function of dystrophin, as well as defining the essential regions and conformations necessary for dystrophin stability. A DMD missense mutation was found in the actin-binding domain.[11] The patient was shown to have correctly localized dystrophin, thus indicating that an intact actin-binding domain is essential for function. The distribution of small mutations is fairly random throughout the gene sequence. However, whereas less than 5% of the gene deletions are found upstream of exon 55, more than 40% of the small mutations are located in this same region of the gene.[12]

Clinical Utility of Testing and Available Testing

The most important impact of DNA testing for DMD/BMD has been on presymptomatic diagnosis and the correct diagnosis of patients with DMD/BMD-like symptoms, as well as for carrier detection and prenatal diagnosis. The western immunoblot is the most sensitive test for the diagnosis of DMD or BMD, but requires a muscle biopsy from the patient. Proteins from the muscle biopsy specimen are separated by electrophoresis, transferred to a membrane, and incubated with antibodies against specific dystrophin domains. Dystrophin from unaffected patients is 427 kDa in size. The complete absence of dystrophin is very specific for the diagnosis of DMD, whereas the presence of an altered molecular weight form or reduced amount of dystrophin, or both, is consistent with BMD. It must be emphasized, however, that the clinical diagnosis and phenotype predictions should never be made exclusively on the basis of dystrophin western blot analysis. Patients with markedly reduced dystrophin levels may have the milder BMD phenotype. Furthermore, western blot analysis is generally not sensitive enough to detect female carriers.

Southern blot analysis using a full-length dystrophin cDNA clone as a probe detects 65% of deletions in affected DMD/BMD patients, as well as gene duplications. The cDNA probes detect the site of the mutation, so meiotic recombination events are irrelevant. Therefore, the chance of diagnostic error is greatly reduced. Diagnostic strategies are based on initially identifying deletions in the affected patient.[13] The deletions are simply detected by examination of Southern blots for the presence or absence of each exon containing genomic restriction fragments, which hybridize to the cDNA probe. However, Southern blot analysis requires the use of a radioisotope, is labor intensive, and is time-consuming.

Before performing Southern blot analysis with the *DMD* cDNA probes, most laboratories perform an initial deletion screen using multiplex PCR[14] to amplify specific deletion-prone exons within the *DMD* gene. Deletion of specific exons from a patient's sample results in absence of the corresponding ethidium bromide–stained amplification products by gel electrophoresis analysis (Figure 7-1). Mul-

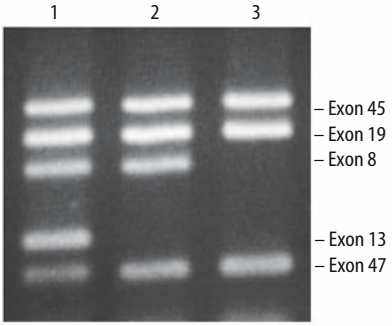

Figure 7-1. Multiplex DNA amplification of DMD exons 8, 13, 19, 45, and 47. Lane 1: normal control; lane 2: DMD patient with exon 13 deletion; lane 3: DMD patient with exon 8 and 13 deletions.

tiplex PCR, using primer sets for about 20 different exons, detects approximately 98% of the deletions in the *DMD* gene. In contrast to Southern blot analysis, which may require several cDNA hybridizations and take several weeks to complete, multiplex PCR testing can be completed in one day. This makes the technique ideal for prenatal diagnosis, when time is critical.

The identification of a deletion in a DMD patient not only confirms the diagnosis but also allows accurate carrier testing for other family members. Carrier status is determined by gene dosage analysis, which is used to assess whether a female at risk of being a carrier exhibits no reduction or 50% reduction in band intensity in those bands that are deleted for the affected male relative. A 50% reduction (single-copy intensity) for the deleted band or bands on the autoradiograph indicates a deletion on one of her X chromosomes and confirms her carrier status. Either Southern blot or quantitative PCR can be used for gene dosage analysis. Dosage determinations permit direct carrier analysis and eliminate the inherent problems of Southern blot analysis without gene dosage (recombinations, noninformative meioses, unavailability of family members, and spontaneous mutations). This is important since unlike affected males, carrier females are generally asymptomatic, and creatine phosphokinase (CPK) is elevated in only approximately two thirds of known carriers.

In 35% of families with undefined mutations, linkage analysis using microsatellite markers must be used for carrier detection and prenatal diagnosis. Microsatellite markers, which correspond to short tandem repeats (di-, tri-, or tetranucleotides) and tend to be highly polymorphic in repeat number, have been found in several locations in the *DMD* gene and have significantly improved linkage analysis for DMD/BMD.[15] The method relies on the coinheritance of the disease gene with those DNA microsatellite sequence variations known to be located within the disease gene. Thus, even when the responsible gene mutation remains unknown, the linkage technique allows the mutation to be traced through an affected family for prediction of carrier or disease risk.

Interpretation of Test Results

The analysis of gene mutations and protein determinations has greatly improved diagnosis, carrier detection, and prenatal counseling. The multiplex PCR deletion test will confirm the clinical diagnosis in approximately 65% of affected DMD and BMD patients. A major advantage of the Southern blot analysis is the detection of an additional 5% to 8% of patients with duplications. The standard multiplex PCR test detects deletions but not duplications. However, if there is any question of the diagnosis after negative results by deletion and duplication molecular testing, western blot analysis of the dystrophin protein should be performed on a muscle biopsy specimen.

When gene dosage testing indicates that the mother does not have the deletion present in the affected child, she still has an uncertain risk of carrier status, due to the possibility of germline mosaicism.[16] Cases of germline mosaicism in DMD have been reported, in which a deletion is transmitted to more than one offspring by a mother who shows no evidence of the mutation in her somatic cells. Cases of germline mosaicism have important counseling implications. First and most obvious is the need to perform carrier studies on all female siblings of affected males, regardless of the outcome of testing for the mother. Furthermore, a negative deletion result in a mother does not rule out a recurrence risk for future pregnancies, and prenatal diagnosis still should be offered. The exact recurrence risk in germline female carriers is unknown because the risk is related to the size of the mutant clone in the mosaic mother. However, in these cases the recurrence risk for subsequent pregnancies is significantly increased relative to what had initially been perceived as a new mutation with a low recurrence risk. It has been estimated that mothers of apparently sporadic DMD cases have an approximate 15% recurrence risk in future pregnancies.

Linkage analysis can provide valuable information but is limited by the possibility of recombination between the polymorphic marker and the unknown mutation, the presence of sporadic mutations, and unavailability of family members. The intragenic recombination rate over the entire length of the DMD gene is estimated to be as high as 12%. The high recombinational error rate can be partially overcome by using microsatellite markers throughout the gene. Linkage analysis results often are extremely limited for extended family members of isolated cases of DMD/BMD, due to the possibility of the occurrence of a new mutation. Linkage analysis indicates only whether the female at risk inherited the same X chromosome as the affected male, not whether she is a carrier of a defective gene. Furthermore, since the gene mutation remains unidentified, a correct clinical diagnosis is essential. This is extremely important with patients presenting with the milder BMD, since this phenotype can overlap with other neuromuscular disorders.

Laboratory Issues

Today, there are many laboratories offering dystrophin-deletion testing. Most laboratories use multiplex PCR, and amplify 16 to 23 deletion-prone *DMD* exons. Since the deletion hotspot is from exon 44 to exon 53, more exons are amplified in this region. Although the 5′ deletions are generally larger, it is important for laboratories to amplify about every third or fourth exon at the 5′ end of the gene; otherwise, smaller deletions are missed. The multiplex strategies are a deletion test and will not detect duplications. Although Southern blot analysis is both costly and labor-intensive, duplications will be detected. Unlike the deletion distribution, duplications are found in about 5% to 8% of the patients and are located primarily at the 5′ end of the gene. The identification of duplications is important both for the confirmation of the diagnosis and for accurate carrier studies for other family members. When laboratories encounter challenging nondeletion types of cases, Southern blot analysis should be performed. Proficiency testing for both *DMD* deletion testing and carrier testing is offered through the College of American Pathologists (CAP), and proficiency specimens are sent to participants twice per year.

MYOTONIC DYSTROPHY

Molecular Basis of Disease

Myotonic dystrophy (DM) is the most common inherited form of muscular dystrophy affecting adults, having an incidence of approximately 1 in 8,000 individuals. DM is an autosomal dominant, multisystem disorder characterized by progressive muscle weakness and myotonia. The diagnosis can be problematic because of the wide range and severity of symptoms. Often, affected individuals have children before they are diagnosed. A severe congenital form of DM results in mental retardation, respiratory distress, hypotonia, and in many cases death due to respiratory complications shortly after birth. The congenital form is seen in the offspring of women who are themselves mildly affected.[17] The clinical genetic phenomenon of anticipation occurs in DM. Anticipation denotes progressively earlier appearance of a disease in successive generations, generally with increasing severity.

The DM mutation has been characterized as an unstable trinucleotide repeat present on chromosome 19q13.3.[18–20] A polymorphic CTG repeat ranges in size from 5 to approximately 30 repeats in the normal population. Mildly affected patients have 50 to 80 repeats, whereas more severely affected individuals have more than 1,000 repeats. The number of repeats varies between affected sibs and increases through generations in parallel with an increasing severity of the disease. Expansion of the CTG repeat between generations accounts for the clinical genetic anticipation phenomenon.

The CTG repeat is located within the 3′ untranslated region of the *DMPK* gene that encodes a protein kinase, named myotonin protein kinase. Since protein kinases are involved in signal transduction pathways in all cells in the body, a defective protein kinase may explain how a single gene defect could result in the diverse symptoms characteristic of DM. However, since the repeat is not in the protein coding region of *DMPK*, the molecular mechanism by which the mutation exerts its dominant expression is difficult to explain. A study using quantitative reverse transcription–PCR (RT-PCR) and a radioimmunoassay demonstrated that decreased levels of mRNA and protein expression are associated with the adult form of DM,[21] suggesting that the dominant nature of DM is likely the result of a dosage-dependent mechanism. However, in another report, expression studies on samples from congenital DM cases demonstrated marked increases in the steady-state levels of the *DMPK* mRNA.[22] In contrast to a reduction of myotonin kinase, the authors proposed that the effect of the mutation would be a nonregulated hyperphosphorylation of kinase substrate by high levels of myotonin kinase.

Another possible disease mechanism is that the repeat expansion affects the expression of other genes in the region. Since the CTG expansion is located in the 3′-untranslated region of the gene, downstream gene expression, like that of DM locus-associated homeodomain protein (DMAHP) may be downregulated. Finally, other studies have suggested a novel type of pathogenic mechanism in which the *DMPK* mRNA with long CUG repeats, and not the protein, results in the pathology. Novel RNA-binding proteins that specifically bind to CUG repeats may be depleted by excessive CUG repeats, or abnormalities in these proteins may disrupt the metabolism of *DMPK* mRNA and other transcripts. Further studies are necessary to define precisely the mechanism by which the repeat expansions cause the DM disease symptoms.

Clinical Utility of Testing and Available Testing

The majority of clinically significant mutations can be identified by Southern blot analysis; however, PCR is essential for small CTG expansions (<100 repeats). Although the PCR test is less expensive and faster than Southern blot analysis, longer repeats often are not reliably amplified. Southern blot analysis can be performed using *Hind* III restriction digestion and the probe pMDY1,[19] which spans the repeat area (Figure 7-2). The probe pMDYI detects a *Hind* III polymorphism with normal alleles of 9.5 and 8.5 kb, the frequencies of which are approximately 0.60 and 0.40, respectively. The polymorphism is due to a 1 kb insertion telomeric to the CTG repeat, and is almost in complete linkage disequilibrium with the CTG repeat mutation in most populations.[20] The mutation is found on the larger 9.5 kb allele, suggesting that there were a limited number

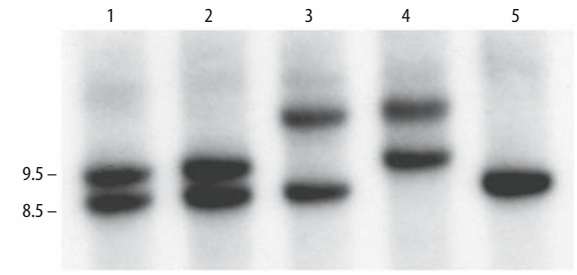

Figure 7-2. Autoradiogram of a Southern blot for myotonic dystrophy. Normal alleles are 8.5 and 9.5 kb. Lanes 1, 2, and 5: unaffected individuals; lanes 3 and 4: expanded and normal alleles in DM patients with repeat expansions.

of ancestral mutations that occurred on a chromosome having the 1 kb insertion. Alternatively, the larger allele may be predisposed to DM mutations. Typical increases in the range of 1 kb to 4 kb are observed on Southern blots in the DM population. Many of the larger expansions are detected as smears, indicating somatic cell heterogeneity of the expanded alleles. Molecular testing is extremely helpful in identifying individuals who are asymptomatic or exhibit equivocal symptoms, such as cataracts. These smaller expansions are often detected using alternate digestions, which reduce the normal size fragment, such *BamH* I, or by PCR amplification across the repeat region. De novo mutations have not been described in DM, which is consistent with the linkage disequilibrium data. To account for the maintenance of the mutation in the population, it was proposed that in DM families there is a high incidence of minimally expanded alleles, which produce few symptoms and are stably transmitted over several generations.[23] Therefore, for counseling purposes, it becomes important to identify in which side of the family the mutation is segregating.

Several cases of reverse mutations have been reported in DM, whereby there is a spontaneous correction of a deleterious expansion mutation upon transmission to an unaffected offspring. The mechanism for the DM reverse mutations remains unknown. A gene conversion mechanism, whereby the normal parental allele replaces the expanded allele, may best account for the reversion events in DM. The reversions may provide an explanation of the nonpenetrance observed in some DM families.

Interpretation of Test Results

The discovery of an expanded repeat sequence in DM has greatly improved our ability to confirm the diagnosis in symptomatic patients, to detect DM carriers who are asymptomatic or who show few of the classical signs of the disease, and for prenatal testing when a parent has been diagnosed with the disorder. Although closely linked restriction fragment length polymorphism (RFLP) markers were available for several years, linkage analysis is not as accurate as direct DNA testing for the mutation, especially when there is an uncertain diagnosis or when key family members are unavailable for testing. Also, linkage test results provide no information regarding the severity of the disease. Molecular testing has largely replaced muscle biopsy, muscle enzyme study, and electromyography as the first diagnostic procedure.

The triplet repeat size does correlate with muscular disability and is inversely related with the age of onset of the disease. However, there is a significant overlap of repeat size in patients with differing severity. When unrelated affected individuals with small to moderate differences in repeat sizes are compared, accurate prediction of the severity of the disease in each case is generally difficult. However, when a child has a significant increase in allele size compared to the parent, the child will almost certainly become symptomatic at an earlier age of onset and will have more severe disease. Lastly, as a result of the somatic heterogeneity observed in DM, genotype/phenotype associations derived from leukocytes may not be as accurate as the measurement of the repeat size in the affected tissue (muscle, heart, others). CTG expansions may be 2- to 13-fold greater in the DNA isolated from skeletal muscle than in the DNA from leukocytes.[24]

Laboratory Issues

There are many molecular pathology laboratories offering DNA testing for DM. The majority of laboratories are using a combination of PCR and Southern blot testing. The PCR allows accurate quantitation of the number of CTG repeats for normal alleles and those with small expansions. For the large CTG expansions, Southern blot analysis is required. All patients with DM have a CTG expansion in the 3′ region of *DMPK*, and no other types of mutations have been identified in the gene. However, the gene for the rarer dominantly inherited myotonic dystrophy type 2 (DM2), which usually results in a more proximal myotonic myopathy and some of the multisystem manifestations of myotonic dystrophy, has been identified.[25] Myotonic dystrophy type 2 is caused by a very large CCTG expansion in intron 1 of the zinc finger protein 9 gene (*ZNF9*). Gene testing for DM2 should be considered in patients with a DM-like phenotype who have a negative test for the *DMPK* CTG expansion. Proficiency testing for DM is available through the CAP, and proficiency specimens are sent to participants twice per year.

SPINAL MUSCULAR ATROPHY

Molecular Basis of Disease

The autosomal recessive disorder proximal spinal muscular atrophy (SMA) is a severe neuromuscular disease characterized by degeneration of alpha motor neurons in the spinal cord, which results in progressive proximal muscle

denervation and atrophy resulting in the symptoms of weakness and paralysis. Spinal muscular atrophy is the second-most common fatal autosomal recessive disorder after cystic fibrosis, with an estimated prevalence of 1 in 10,000 live births.[26] Childhood SMA is subdivided into three clinical groups on the basis of age of onset and clinical course. Type I SMA (Werdnig-Hoffmann disease) is characterized by severe, generalized muscle weakness and hypotonia at birth or within the first three months after birth. Death from respiratory failure usually occurs within the first two years of life. Children affected with Type II SMA are able to sit, although they cannot stand or walk unaided, and survive beyond four years of age. Type III SMA (Kugelberg-Welander syndrome) is a milder form, with onset during infancy or youth, and patients may walk unaided.

The survival motor neuron (*SMN1*) gene has 9 exons and is the primary SMA-causing gene.[27] Two almost identical *SMN* genes are present on 5q13: the telomeric, or *SMN1*, gene that is the SMA-determining gene, and the centromeric, or *SMN2*, gene. The *SMN1* gene exon 7 is deleted in approximately 94% of affected patients, while small, more subtle mutations have been identified in the majority of the remaining affected patients. Deletions of other genes in the SMA region most likely mark the extent of the deletion and may modify the severity of the disease. Although mutations of the *SMN1* gene are observed in the majority of patients, no phenotype-genotype correlation was observed, because *SMN1* exon 7 is absent in the majority of patients independent of the type of SMA. This is because routine diagnostic methods do not distinguish between a deletion of *SMN1* and a conversion event whereby *SMN1* is replaced by a copy of *SMN2*. Several studies have shown that the *SMN2* copy number influences the severity of the disease.[28–30] The number of *SMN2* gene copies varies from 0 to 3 copies in the normal population, with approximately 10% of unaffected individuals having no gene copies of *SMN2*. However, milder patients with type II or III SMA on average have more gene copies of *SMN2* than do type I SMA patients. It has been proposed that the extra *SMN2* gene copies in the more mildly affected patients arise through gene conversions, whereby the *SMN2* gene is copied either partially or totally into the telomeric *SMN1* locus.

Five base pair differences exist between *SMN1* and *SMN2* transcripts, and none of these differences change amino acids. Since virtually all individuals affected with SMA have at least one *SMN2* gene copy, the obvious question that arises is, Why do individuals with *SMN1* mutations have an SMA phenotype? The *SMN1* gene produces predominately a full-length transcript, whereas the *SMN2* copy produces predominately an alternate, exon-7-deleted product. The inclusion of exon 7 in *SMN1* transcripts and exclusion of this exon in *SMN2* transcripts is caused by a single nucleotide difference at +6 in *SMN* exon 7. Although the C-to-T change in *SMN2* exon 7 does not change an amino acid, it does disrupt an exonic splicing enhancer (ESE), which results in the majority of transcripts lacking exon 7. Furthermore, the importance of the exon 7 region was suggested by Talbot et al.[31] by demonstration that a highly conserved tyrosine-glycine (Y-G) dodecapeptide motif is encoded by this exon region and is crucial for the oligomerization and function of the SMN protein. Therefore, SMA arises because the *SMN2* gene cannot completely compensate for the lack of SMN1 protein function when the *SMN1* gene is mutated. However, the small amounts of full-length transcript generated by *SMN2* are able to prevent in utero lethality due to a complete lack of SMN1 protein, and produce a milder type II or III phenotype when the copy number of *SMN2* genes and transcripts is increased.

Recent evidence supports a role for SMN in small nuclear ribonuclearprotein (snRNP) biogenesis and function.[32] The SMN protein is required for pre-mRNA splicing. Immunofluorescence studies using a monoclonal antibody to the SMN protein have revealed that the SMN protein is localized to novel nuclear structures called "gems," which display similarity to and possibly interact with coiled bodies, which are thought to play a role in the processing and metabolism of small nuclear RNAs. SnRNPs and possibly other splicing components require regeneration from inactivated to activated functional forms. The function of SMN is in the reassembly and regeneration of these splicing components. Mutant SMN, such as that present in SMA patients, lacks the splicing-regeneration activity of wild-type SMN. SMA may be the result of a genetic defect in spliceosomal snRNP biogenesis in motor neurons. Consequently, the motor neurons of SMA patients are impaired in their capacity to produce specific mRNAs and as a result become deficient in proteins that are necessary for the growth and function of these cells.

Clinical Utility of Testing and Available Testing

The molecular diagnosis of SMA consists of the detection of the absence of exon 7 of the *SMN1* gene (Figure 7-3). Although this is a highly repetitive region and there is the almost identical centromeric *SMN2* copy of the *SMN1* gene, there is an exonic base pair difference that allows distinction of PCR products of *SMN1* from those of *SMN2* using restriction-site generating PCR (RG-PCR) followed by restriction enzyme digestion. The absence of detectable *SMN1* exon 7 in SMA patients is being utilized as a powerful diagnostic test for SMA, with a sensitivity of approximately 94%. Limitations of this diagnostic test are the inability to detect nondeletion mutations of *SMN1* and the inability to determine carrier status for SMA.

Since SMA is one of the most common lethal genetic disorders, with a carrier frequency of 1 in 40 to 60, carrier

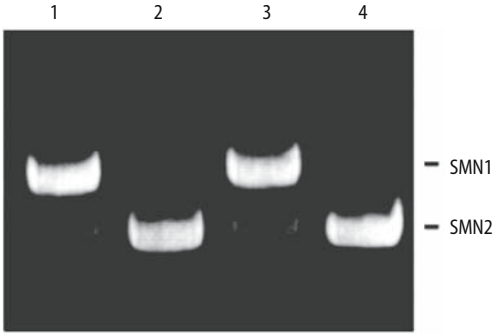

Figure 7-3. Restriction enzyme digestion of RG-PCR products distinguishes *SMN1* from *SMN2* exon 7 PCR products. Lanes 1 and 3: normal controls with *SMN1* present and *SMN2* deletion; lanes 2 and 4: SMA patients with *SMN1* deletion and *SMN2* present.

testing is useful to many families. Carrier detection for the heterozygous state is technically challenging because the SMA region is characterized by the presence of many repeated elements. The *SMN* gene is present in two almost identical copies, *SMN1* and *SMN2*. However, the *SMN2* gene copy number fluctuates: approximately 5% of normal individuals lack the *SMN2* copy, whereas many of the more mildly affected SMA patients have three or more copies of *SMN2*. Thus, a straightforward dosage assay using the *SMN2* gene as the internal control would not be reliable, and the copy number of *SMN2* affects the efficiency of amplification of the *SMN1* exon 7 region. Quantitative PCR assays, using alternative internal controls, are used for the identification of SMA carriers and determine the number of copies of *SMN1* and *SMN2* genes.[28,33]

Interpretation of Test Results

The presence of *SMN1* exon 7 does not entirely exclude a diagnosis of SMA. Although the absence of both copies of the *SMN1* gene is a very reliable and sensitive assay for the majority of SMA patients, about 6% of affected patients have other types of mutations in the *SMN1* gene that will not be detected by PCR deletion testing.[34] Most of these patients will be compound heterozygotes, with one *SMN1* allele deleted and the other allele with a point mutation or other small insertion or deletion. If the clinical suspicion remains high after a negative deletion test, then dosage carrier testing to determine whether there is a single copy of *SMN1* should be considered. A dosage testing result of two copies of the *SMN1* gene greatly reduces the likelihood of SMA.

The carrier test has two limitations. The first is the presence of de novo mutational events in the *SMN1* gene. The de novo mutation rate for this gene has been observed to be approximately 2%, which is high when compared to most autosomal recessive disorders.[35] The second limitation of the carrier test is the finding of two *SMN1* genes on a single chromosome. The allele frequency of the 2-copy *SMN1* chromosome is approximately 2% in the general population. The finding of two *SMN1* genes on a single chromosome has serious genetic counseling implications, because a carrier individual with two *SMN1* genes on one chromosome would have the same dosage result as a noncarrier with two *SMN1* gene copies on each chromosome 5. Approximately 5% of parents of a single affected SMA child have two *SMN1* gene copies by dosage analysis. Thus, although, the finding of normal dosage significantly reduces the risk of being a carrier, there is still a recurrence risk of future affected offspring for individuals with two *SMN1* gene copies. Thus, risk assessment calculations using Bayesian analysis are essential for the proper genetic counseling of SMA families (see chapter 5).

Laboratory Issues

There are many molecular pathology laboratories offering testing for the homozygous deletion of *SMN1*. The majority of laboratories utilize RG-PCR of exon 7 and PCR of exon 8 in two separate reactions, with restriction enzyme digestion to differentiate *SMN1* from *SMN2* PCR products, and report results for both *SMN1* exons 7 and 8. Some laboratories only test for exon 7 deletion. Carrier testing is being offered by a fewer number of laboratories. Proficiency testing for the homozygous *SMN1* deletion is offered by the CAP. These proficiency specimens are sent to participants twice per year. External proficiency testing is not available for dosage carrier testing.

MITOCHONDRIAL ENCEPHALOMYOPATHIES

Molecular Basis of Disease

Mitochondria are semiautonomous replicating cellular organelles with their own genetic material. Each mitochondrion contains multiple copies of the mitochondrial DNA genome (mtDNA), with replication, transcription, and translation machineries separate from the cellular machineries for these functions. Human mtDNA encodes 13 polypeptides of the respiratory chain subunits, 28 ribosomal RNAs (rRNAs), and 22 transfer RNAs (tRNAs) in a circular double-stranded genome of approximately 16.5 kb.

Unlike nuclear DNA, in which each cell contains pairs of chromosomes, one of maternal and the other of paternal origin, mtDNA is inherited exclusively from the mother. This type of transmission is called maternal inheritance. Although both sexes are equally affected by mitochondrial diseases, inheritance of the disorder is from the mother. Mitochondrial mutations are often present in only some of the mtDNA molecules of a cell (heteroplasmy). Heteroplasmy occurs because mitochondria segregate randomly into daughter cells during mitosis, which results in cells containing both mutant and wild-type mtDNA. Thus, the

proportion of heteroplasmic mutation may vary widely between different tissues or even between different cells of the same tissue. The proportion of mutant to wild-type mtDNA plays a role in determining the clinical variability and severity often observed in the mitochondrial disorders. However, the phenoytpe-genotype correlation in the mitochondrial disorders is complex and is influenced by age, the type and extent of respiratory chain disruption caused by the mutation, and the tissue-specific threshold for the pathogenic effect.

The term "mitochondrial encephalomyopathies" is used to describe mitochondrial disorders in which both muscle and the central nervous system (CNS) are affected. These disorders are multisystemic, with diverse clinical features due to defects in the mitochondrial function. This chapter is restricted to those disorders in which the mutation event involves mtDNA, in contrast to the many nuclear genetic disorders that result in mitochondrial pathology. This occurs when the nuclear encoded protein functions in the mitochondria. This chapter discusses Kearns-Sayre syndrome (KSS), mitochondrial encephalomyopathy with lactic acidosis and strokelike episodes (MELAS), and myoclonic epilepsy with ragged-red fibers (MERRF).

Kearns-Sayre Syndrome

The common features of KSS include progressive external ophthalmoplegia (PEO), pigmentary degeneration of the retina, and defects of cardiac conduction.[36] The typical affected patient presents before the age of 20 years with PEO and pitosis. This is followed by the pigmentary retinal degeneration and cardiac conduction block. Other features of the disorder may include ataxia, deafness, dementia, and diabetes mellitus. The most common type of mutation found in KSS is a deletion of mtDNA (Figure 7-4), and almost of all these deletions occur sporadically.[37] Approximately one third of KSS cases are due to a common 4977 bp deletion, which is associated with direct repeats at the deletion junction. The severity of KSS depends on the extent of heteroplasmy and the tissue distribution of structurally altered mtDNA. An extreme KSS phenotype occurs when the frequency of deleted mtDNA in muscle cells is greater than 85%. In contrast, when lower levels of heteroplasmy for the deletion are present, then PEO may be the only symptom.

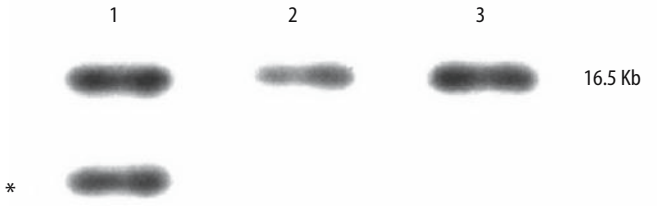

Figure 7-4. Autoradiogram of a Southern blot of muscle mitochondrial DNA. Lane 1: Kearns-Sayre patient with heteroplasmic deletion; lanes 2 and 3: unaffected individuals. The normal allele is 16.5 kb and the deleted allele fragment is indicated by the asterisk.

Mitochondrial Encephalomyopathy with Lactic Acidosis and Strokelike Episodes

MELAS patients are usually normal at birth but develop stunted growth, intermittent vomiting, seizures, and recurrent cerebral insults resembling strokes during the first years of life.[38] An episodic course follows, with recurrent strokelike episodes, only partial recovery, and eventual deterioration, with death from respiratory failure often before 20 years of age. Milder adult-onset cases have been reported. Approximately 80% of all MELAS cases are the result of an A3243G point mutation in the mtDNA gene encoding $tRNA^{Leu}$.[39] The point mutation alters the normal structural conformation of the tRNA, thereby impairing protein synthesis. The A3243G mutation occurs in the heteroplasmic state, with variation among different tissue types. When the mutation is present in greater than 90% of the mtDNA of the muscle tissue, there is increased likelihood of recurrent strokes and classic MELAS manifestations. However, when the heteroplasmy of the mutation is less than 90%, later onset and more moderate symptoms may occur.

Myoclonic Epilepsy with Ragged-Red Fibers

MERRF is a rare maternally inherited disorder in which the full expression includes muscle weakness, myoclonus, generalized seizures, ataxia, and deafness.[40] The hallmark morphologic change seen in the muscle biopsy is the ragged-red fibers. The term "ragged-red fibers" refers to large clumps of abnormal mitochondria that accumulate beneath the sarcolemma and are stained red with the Gomori trichome stain. The majority of MERRF cases are the result of a point mutation (A8344G) in the $tRNA^{Lys}$ gene. The MERRF mutation, like the MELAS $tRNA^{Leu}$ mutation, diminishes overall mitochondrial protein synthesis. Similar to the other mitochondrial disorders, a more classic MERRF phenotype is observed when the mutation is present at higher levels in the muscle and nerve.

Clinical Utility of Testing and Available Testing

The symptoms of the mitochondrial disorders are often nonspecific and are common to many other neuromuscular diseases. As a result, the mtDNA diseases are often considered only after many other diagnoses have been excluded. However, genetic testing for mitochondrial diseases is becoming increasingly available. Southern blot analysis or long-range PCR is used to detect the deletions observed in KSS. Total DNA used for this testing should be obtained from a muscle biopsy. Use of a tissue with rapidly dividing cells, such as blood, will often lead to false-negative results since the heteroplasmy of deleted mtDNA

is shifted toward wild-type mtDNA. PCR-RFLP testing is used for the common tRNA point mutations found in MERRF and MELAS. Patients with MERRF have high levels of the mutant mtDNA in the blood, and therefore blood is an appropriate specimen for testing. In contrast, patients with the MELAS mutation often have low levels of the mutation in the blood, leading to false-negative results when blood is used for testing.

Interpretation of Test Results

If clinical suspicion is strong and the blood results are negative, then mtDNA from a muscle biopsy should be tested. Since the disorders are highly clinically heterogeneous and there is considerable phenotypic overlap, the classic MERRF and MELAS point mutations and the KSS deletions frequently are ordered together and tested as a mitochondrial test panel. The A8344G MERRF mutation is associated with other phenotypes, including Leigh syndrome, myoclonus or myopathy with truncal lipomas, and proximal myopathy. The A3243G MELAS mutation can result in milder phenotypes, including sensorineural deafness with diabetes. Furthermore, these mutations sometimes are found in asymptomatic relatives of the index case.

Laboratory Issues

If the common mtDNA mutations are not detected, more extensive testing for rarer mutations may be useful for diagnosis. Sequencing of the mtDNA is available from several specialty laboratories, which may identify a new mutation. However, since the mtDNA is very polymorphic, new mutations must be carefully verified.

KENNEDY DISEASE

Molecular Basis of Disease

Spinal and bulbar muscular atrophy (SBMA), or Kennedy disease, is a rare X-linked, slowly progressive, adult-onset motor neuropathy.[41] The age of onset is usually between 30 and 50 years and is characterized by muscle cramps, proximal and bulbar weakness, and fasciculation. Endocrine abnormalities, including gynecomastia and testicular atrophy, are common. The disease is caused by a CAG trinucleotide repeat expansion in the coding region of the androgen receptor gene (*AR*).[42,43] The CAG repeat found within the first exon is polymorphic in normal populations, and ranges in length from 10 to 36 repeats. Patients with SBMA have a CAG repeat expansion that does not overlap with the normal population and ranges from 40 to 62 repeats. Similarly to other trinucleotide repeat disorders, the CAG repeat length correlates with disease severity and age of onset. However, considerable variability in age of onset is seen among family members with similar CAG repeat lengths, suggesting that factors other than the size of the repeat modulate the onset and severity of the disease. While *AR* repeats in the unaffected range are stably transmitted, expanded repeats in SBMA patients are transmitted less stably and tend to increase in size by paternal transmission.

The pathogenic mechanism of SBMA expansion involves a gain of a toxic function of the protein product. The mutant allele is both transcribed and translated, arguing against a loss of function mechanism. Individuals with mutations producing a loss of AR protein function have testicular feminization and do not have the motor neuropathy seen in SBMA patients. The exact mechanism by which the expanded polyglutamine tract in the AR protein produces the neuropathy of SBMA is uncertain.

Clinical Utility of Testing and Available Testing

Despite distinct clinical features, SBMA is often misdiagnosed due to the heterogeneity of manifestations and lack of full expression in some family members. Many of the cases are sporadic, and some patients have only mild signs of motor neuron disease and mild gynecomastia. Amytrophic lateral sclerosis, type III SMA, hereditary motor and sensory neuropathy, limb-girdle muscular dystrophy, and facioscapulohumeral muscular dystrophy are included in the differential diagnosis for some patients affected with SBMA.[43] The PCR amplification of the repeat sequence within the first exon of the *AR* gene provides an accurate confirmation of the diagnosis of SBMA, distinct from other neuromuscular disorders. In addition, carrier females and young asymptomatic males may be identified by molecular testing for a repeat expansion with implications for genetic counseling and potential early treatment.

Interpretation of Test Results

The definitive diagnosis of SBMA is by the analysis of the CAG repeat in the *AR* gene. All patients with SBMA have a CAG repeat expansion, and no other related mutations in the *AR* gene have been identified. Some studies have described a correlation between repeat size and age of onset, but interfamilial and intrafamilial variation is large for any given repeat length.

Laboratory Issues

Several molecular pathology laboratories offer DNA testing for SBMA. The PCR test is accurate and inexpensive to perform, and requires only a blood sample. PCR with electrophoretic analysis of the PCR products allows for accurate assessment of the repeat size. External proficiency testing is not available for SBMA.

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