Prospects for the Diagnosis of Malignant Hyperthermia Susceptibility Using Molecular Genetic Approaches

Roy Clifford Levitt, M.D.*

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MALIGNANT hyperthermia susceptibility (MHS) is a clinically heterogeneous, autosomal dominant pharmacogenetic disorder.1 MHS is generally characterized by accelerated metabolism, hyperthermia, and muscle rigidity.2,3 All commonly used potent inhalation anesthetics and depolarizing skeletal muscle relaxants are believed to initiate this syndrome in susceptible individuals.5,4 MHS is widely believed to result from a membrane defect that disrupts calcium metabolism in skeletal muscle. However, the genetic defects in MHS that confer susceptibility to these anesthetic agents and disrupt metabolic regulation remain largely unknown. Efforts to understand the molecular basis of MHS and other Mendelian disorders (those inherited as a single gene) have been greatly aided by recent advances in genetic technology. The clinical application of this technology has provided exciting new opportunities for the diagnosis of hereditary disorders, malignancies, and numerous other diseases5–8 (e.g., autoimmune, infectious, immunodeficiency, and cardiovascular). The purpose of this review is to acquaint the reader with selected molecular genetic techniques that will be useful in the noninvasive presymptomatic and prenatal diagnosis of MHS.

Diagnosis by Direct Molecular Genetic Techniques

The clinical diagnosis of MHS or any Mendelian disorder is easily accomplished once a defective gene or gene product (protein) is identified. Although genes and gene products vary significantly among individuals without producing abnormal phenotypes (nucleotide sequence variation that does not alter the phenotype is referred to as a polymorphism), a true genetic mutation should be unique to affected individuals and should not occur in normal relatives. If a protein is recognized as defective and can be purified, it can be used to isolate and amplify the normal and abnormal genes by propagation in bacterial cells (cloning). The DNA sequence of the normal and abnormal cloned genes can then be examined, providing insight into the structure and function of the defective protein and the pathophysiology of the disorder. Extensive studies on hemoglobinopathies and, more recently, many other heritable disorders have provided considerable information on the nature of mutations that cause such heritable disorders. These and other Mendelian disorders generally arise from either point mutations or gross abnormalities in chromosomal DNA.

Gross abnormalities are characterized as DNA deletions, insertions, or rearrangements within a critical region of a functioning gene. The consequences of these mutations are readily apparent (e.g., the gene is deleted in part or whole, or the gene is interrupted by an insertion or rearrangement). Point mutations are generally confined to the substitution of a DNA base (nucleotide) or a small deletion or insertion of a few base pairs that significantly affects the function of the gene product. Both types of mutations can now readily be demonstrated by molecular techniques. These methods are generally easy to apply to double-stranded DNA isolated from the nuclei of circu-
Fig. 1A: Techniques for demonstrating restriction fragment length polymorphisms (RFLPs). B: Illustration of how restriction fragment length polymorphisms can be associated with an abnormal phenotype. Individuals I-2 and II-3 are normal; I-1, II-1, and II-2 all express an abnormal phenotype. When the chromosomal DNA from these individuals is digested with the restriction endonuclease Msp I (which recognizes the DNA sequence CCGG), a polymorphism within the region recognized by a homologous DNA probe is demonstrated. Two fragments are produced (1.5 and 0.5 kb) from the digestion of chromosome A and one fragment (2.0 kb) from chromosome B. Analysis of the autoradiograph (produced by techniques illustrated in fig. 1A) reveals an association between chromosome B and the abnormal phenotype. If, for example, this abnormal phenotype arises as a result of a mutation that also causes the loss of this Msp I restriction endonuclease site, then this disease can be diagnosed directly by this method (appearance of 2.0-kb fragment on autoradiograph).

lating white blood cells obtained from a blood sample by simple venipuncture.

Gross abnormalities in gene structure can be detected by restriction fragment length polymorphism (RFLP) analysis (figs. 1A and 1B) and Southern blotting.10 In RFLP analysis, DNA-modifying enzymes cut double-stranded DNA at recognition sites dictated by the DNA sequence, producing fragments that are then traditionally separated according to size by electrophoresis. Southern blotting is a method of making DNA single-stranded and transferring and binding the sized DNA fragments to a solid support. The polymorphic fragments produced are
generally detected by labeled DNA probes that recognize this gene by hybridization (complementary single strands of DNA recognize each other and produce doublestranded DNA by hydrogen bonding.) Thus, when a restriction endonuclease is used to cleave chromosomal DNA, a unique pattern of fragments can be detected (fig. 1A).

Nearly all gross rearrangements (mutations) can be recognized by RFLPs because the gene structure is altered near a recognition site for one or more restriction enzymes that cut the double-stranded DNA of interest. For example, insertions may produce a larger DNA fragment than normally expected because the added DNA segment lies within the interval (segment of chromosomal DNA) between two restriction enzyme sites. In a similar way, deletions and rearrangements can significantly alter the pattern of restriction fragments produced by the digestion of genomic (pertaining to the entire complement of chromosomes) DNA. Although gross abnormalities are relatively rare in nature, more than 60% of Duchenne and Becker muscular dystrophies,12 as well as certain forms of retinoblastoma,12,13 familial hypercholesterolemia,14 and numerous other disorders,6 are caused by this type of mutation.

A small number of point mutations can be demonstrated directly by virtue of an altered DNA sequence within the recognition site for a restriction enzyme (for detailed description, see fig. 1B). The mutation may add or delete a restriction endonuclease site, producing a RFLP that can be detected as described above. Sickle cell anemia15-18 and hemophilia A19 are examples of diseases in which the mutation can be recognized directly by RFLPs. Presently, only a limited number of restriction endonucleases are known that collectively can recognize only a small portion of the DNA sequence of any particular gene. Therefore, because most point mutations are likely to lie outside these restriction endonuclease recognition sites, they will not be recognized directly by RFLPs.

Allele-specific oligonucleotides (ASOs)20,21 can in theory recognize all known nucleotide polymorphisms and point mutations (fig. 2), regardless of whether they occur within a recognition site for a restriction endonuclease or not. ASOs are synthetic oligonucleotides (usually approximately 20 nucleotides or longer) that under optimal hybridization conditions can be forced to recognize only one allele (gene copy) or homologous DNA sequence, and not one that differs by even a single nucleotide. Oligonucleotides can then be synthesized that recognize all known point mutations within a gene. ASOs are particularly useful for the diagnosis of autosomal dominant and recessive disorders in which a small number of point mutations predominately account for the phenotype (e.g. cystic fibrosis,22 Marfan syndrome,23,24 sickle cell anemia18,21). ASO technology also is being put to use in other important areas of clinical medicine. HLA class II genotyping by ASO analysis has recently been demonstrated to be an efficient technique for improving the selection of unrelated donors for bone marrow transplantation as compared to standard serologic tests.26 Unfortunately, ASO analyses can be used only to detect mutations that have been described. Unrecognized mutations, even very close to a known mutation in the same gene, would not be detected by this technique.

A recently described method amplifies a small fragment of genomic DNA directly. This technique is referred to as the polymerase chain reaction (PCR).27 PCR is a method of selective replication in vitro of a small region of chromosome or DNA of interest (usually 2,000 base pairs or fewer). This method is extremely powerful because, without cloning, it can amplify a small region from a single chromosome. For our purpose in describing PCR, the genomic source of DNA that is amplified will be referred to as a template. PCR, like chromosomal replication in vivo, depends on a number of components: a template, a polymerase that can synthesize the new nucleotide chain, free nucleotides that can be added to the end of the growing nucleotide chain, and various cofactors (Mg++, buffers, etc.). PCR also depends on two oligonucleotide primers (usually 18 nucleotides or longer) that are homologous to the DNA at each end of the region to be synthesized. PCR is a cyclic process with an amplification of the region between the primers by 2n (where n equals the number

Fig. 2. The use of allele-specific oligonucleotide analysis. Individuals I-1, II-1, and II-2 are affected with a Mendelian disorder (solid symbols). In this example, a C-to-A nucleotide substitution in the DNA sequence of one chromosome causes the disorder. Only those individuals affected with this disorder will demonstrate this abnormal gene by allele-specific oligonucleotide analysis.
of cycles in the process). During the first cycle, the double-stranded template is denatured (separating the strands) by heating. Oligonucleotide primers are annealed to each complementary template strand by cooling. (There is a large excess of primers so they are much more likely to anneal to the template than to the complementary strand.) These oligonucleotide primers are then extended by the polymerase (adding nucleotides in succession) to synthesize replicas of each strand of the original template. During the second cycle, the products of the first cycle and the original template are denatured, annealed to primers, and replicated by the polymerase. If we start with one double-stranded template molecule, after the first cycle of PCR we would have two; after the second cycle we would have four; after the third cycle we would have eight; and so on (for a detailed description, see fig. 3).

This method obviates the need to cleave large amounts of genomic DNA with a restriction endonuclease, separate the fragments by electrophoresis, and transfer them from the gel to a solid support to detect a mutation by RFLP analysis (fig. 1A). The small fragment of genomic DNA containing the mutation that alters the recognition site for a restriction endonuclease can be amplified to high concentration by PCR. The RFLP can then be demon-

FIG. 3. Amplification of DNA by polymerase chain reaction. The components shown are combined for the polymerase chain reaction. The double-stranded template DNA is denatured (i.e., the strands are separated) using heat, and then the components are cooled, allowing each primer to anneal to a template strand. The polymerase (thermostable) then extends the primers by adding nucleotides complementary to those on the template strand. An excess of primer ensures that the template strands do not reanneal with each other. Primer extension produces another template in each case. When this process is repeated (heat denaturation, primer annealing, primer extension) an amplification of 2^n results (where n = the number of cycles). During a typical polymerase chain reaction amplification, approximately 2 h is required to complete 30 cycles.
strated directly in this PCR fragment by restriction endonuclease digestion\(^{20}\) (fig. 1B). The entire process from blood collection to the description of a mutation would take hours with PCR, rather than days. Alternatively, this PCR fragment can be bound directly on a solid support and hybridized with an ASO to demonstrate a mutation that cannot be detected by RFLP analysis.

Further refinements in these methods use a labeled ASO as one of the PCR primers (see fig. 4); the labeled ASO amplifies only the mutant allele of interest and can be detected directly by autoradiography without time-consuming DNA hybridizations with an ASO.\(^{8}\) For example, in figure 4, if primer A recognizes a specific mutant sequence, then the PCR product would be expected only when this allele was present. This is true because we can force the oligonucleotide primer to recognize only the mutant sequence and not the normal allele. The prenatal diagnosis is now routinely carried out for numerous genetic disorders.\(^{6,8}\) For example, the sensitivity and specificity of the prenatal and presymptomatic molecular genetic diagnosis of sickle cell anemia is essentially 100%. The specificity of direct molecular genetic techniques remains at 100% in heterogeneous disorders (similar phenotypes produced by different mechanisms or genetic defects); however, the sensitivity is inversely related to the number of unidentified mutations. These methods are limited by the inability to detect unknown genetic defects within the same gene or in genes elsewhere. However, it is likely that ASO with PCR will be the direct method of choice for diagnosing the defects that produce MHS.

**Prospects for the Diagnosis of Malignant Hyperthermia Susceptibility by Direct Methods**

Similar to most other genetic disorders, MHS is recognized only by an abnormal phenotype. The MHS phenotype is currently described as a fulminating malignant hyperthermic reaction to triggering anesthetics *in vivo* or an abnormal caffiene–halothane contracture test *in vitro*.\(^{27,28}\) Little is understood at present as to how the MHS phenotype arises. A number of membrane proteins might alter calcium regulation after exposure to triggering anesthetics, if they were defective. Therefore, as in most genetic disorders, it will be necessary to localize and identify the causative gene(s) for MHS before identifying the defective protein responsible in each case. Studying how the abnormal and normal genes differ will ultimately provide an understanding of the altered proteins function and of the pathophysiology involved.

The first genetic locus (MHS-1) shown to cause MHS was localized on human chromosome 19q13.1 by two laboratories simultaneously. McCarthy et al.\(^{29}\) established linkage by chromosomal homology with a similar disorder in pigs. The linear order of genes on the pig and human chromosomes suggested conservation of chromosomal structure and a possible location for the homologous unmapped gene in the human. MacLennan *et al.*\(^{30}\) also mapped MHS-1 to chromosome 19q13.1 along with the ryanodine receptor gene (RYR-1) or calcium release channel in skeletal muscle, and, on the basis of linkage, suggested that the MHS phenotype arises from a defect in RYR-1. Central core disease is commonly considered to be invariably associated with MHS. The recent mapping of central core disease to the same subchromosomal region as MHS-1 confirms this chromosomal localization for at least one form of MHS and raises the possibility that central core disease may arise from a mutation in the same gene.\(^{31,32}\)

A systematic search for the genetic alterations in RYR-1 from both porcine and human MHS individuals has disclosed a single genetic mutation.\(^{35-37}\) A replacement of cytosine 1843 in the complementary DNA (cDNA, a stable copy of the mRNA) of normal pigs with thymidine in MHS pigs, was the only nucleotide substitution found that resulted in an amino acid substitution.\(^{35-37}\) The resulting substitution of a cysteine for arginine 615 was cor-
related with, and likely to be causative of, MH in more than 450 swine of six breeds.\textsuperscript{55} Therefore, in all strains of pigs that were examined, it appears that a single mutation in RYR-1 may be causative of MHS.\textsuperscript{55} The analysis of approximately 70 human families believed to be predisposed to MH has revealed the corresponding substitution in one family, making this potential cause of MHS rare.\textsuperscript{54}† Clearly, in contrast to the pig, a single genetic mutation does not appear to explain MHS in the human. However, this rare ryanodine receptor mutation can be directly demonstrated by a RFLP resulting from the loss of a Rsa I restriction site within a MHS RYR-1 PCR fragment.\textsuperscript{54}

The likelihood of genetic heterogeneity (similar phenotypes produced by different mechanisms) in MHS makes the practical application of direct molecular detection somewhat more complicated. Multiple genetic etiologies have been proposed in MHS\textsuperscript{1} because of the highly variable clinical expression.\textsuperscript{1-3} Genetic heterogeneity is further supported by an association between MHS and numerous other neuromuscular disorders,\textsuperscript{56} including Duchenne muscular dystrophy, myotonic dystrophy, myotonia congenita, nonspecific myopathies, and the King-Denborough syndrome. The King-Denborough syndrome is characterized by MHS, myopathy, and severe dysmorphic features.\textsuperscript{57} The sporadic occurrence of this disorder suggests that it may arise from a new mutation. Furthermore, the involvement of multiple organ systems suggests either a gross abnormality in the DNA with an effect on multiple genes or a mutation in a single gene with pleiotropic (multiple) effects. Gross chromosomal abnormalities have aided the localization and isolation of mutant genes in a number of disorders. High-resolution cytogenetic studies may prove useful as a diagnostic test in the King-Denborough disorder, if a gross chromosomal abnormality is at fault. The association between Duchenne dystrophy (which maps to the X chromosome) and myotonic dystrophy (which maps to 19q13.2) with MHS is not explained by the genetic mapping data (linkage data) described above. The genetic locus for myotonia congenita has also been excluded from the MHS-1 region of chromosome 19.\textsuperscript{58,59} Hence, the mapping data support the notion that there may be a variety of genetic defects that can cause MHS. Recent linkage studies in my laboratory\textsuperscript{60} confirm that at least one additional, perhaps common, genetic locus (MHS-2) can produce the MHS phenotype. Others also are finding MHS families that do not map to 19q13.1.\textsuperscript{41}‡ MHS-2 has not yet been mapped but has been excluded from the 19q13.1 linkage group surrounding MHS-1.

Genetic heterogeneity is a common theme in human genetic disease. Elliptocytosis is a hereditary disorder characterized by an abnormal shape of erythrocytes and occasionally mild-to-moderate hemolytic anemia. This phenotype is apparently produced by genetic defects in one of three separate genes.\textsuperscript{45-47} Mutations in the α-spectrin gene on chromosome 1q22-25,\textsuperscript{45,44} the β-spectrin gene on 14q32,\textsuperscript{46} and the gene for protein 4.1 on 1p36.2-p34\textsuperscript{45} all have been shown to produce a similar red cell appearance or phenotype. Similarly, hereditary spherocytosis has been shown to arise either from genetic mutations in the α- or β-spectrin genes on chromosomes 1q22-q25 and 14q32 respectively.\textsuperscript{46,47} Osteogenesis imperfecta is a heritable disorder characterized by brittle bones. Osteogenesis imperfecta type III is characterized by the autosomal dominant inheritance of progressive deformation of bones, dentinogenesis, hearing loss, and very short stature. Osteogenesis imperfecta type III is commonly caused by various point mutations in either the COL1A1 (collagen 1A1) gene on chromosome 17 or the COL1A2 gene on chromosome 7.\textsuperscript{48} Separate mutations in these genes also can produce the similar osteogenesis imperfecta type IV phenotype.\textsuperscript{48} Ehlers-Danlos syndrome is another heterogeneous group of heritable disorders of connective tissue.\textsuperscript{49,50} Ehlers-Danlos syndrome type VII is characterized by extreme joint hypermobility and dislocations, skin hyperextensibility, easy bruising, and abnormal scarring.\textsuperscript{50} This disorder appears to be caused by a deficiency in processing of the N-propeptide of type I procollagen.\textsuperscript{49} Genetic mutations in three separate genes (COL1A1, COL1A2, and procollagen N-proteinase) have been associated with this phenotype.\textsuperscript{49}

Only speculation can be offered as to how a genetic defect(s) in each of a number of genes could produce the MHS phenotype by altering the regulation of myoplasmic calcium. However, both the clinical characterization and genetic mapping data clearly support genetic heterogeneity in this disorder. It is likely, therefore, as in numerous other genetic disorders, that a number of common mutations in different genes produce the MHS phenotype.

The rapid noninvasive presymptomatic diagnosis of MHS can be carried out, like any routine preoperative screening test, using the direct molecular methods discussed above, once the genetic mutations that cause this disorder have been characterized. The more genetic defects there are that can produce the MHS phenotype, the more difficult and time-consuming it will be to identify each of them. For these reasons, indirect molecular genetic techniques are likely to be required to identify the abnormal gene in many cases and to follow its inheritance to establish the clinical diagnosis of MHS.
Diagnosis by Indirect Molecular Genetic Techniques

Chromosomes are organized in a linear fashion. Therefore, the genetic locus (physical location) of the defective gene that produces MHS will be inherited with closely surrounding genetic markers. Polymorphisms in and around a gene (which have no known effect on the individuals phenotype) occur an average of about once in every 500 nucleotides. At present, the indirect diagnosis of a heritable disorder is dependent on the use of these polymorphisms as genetic markers. These polymorphisms usually are recognized by the molecular techniques described above (RFLP, ASO, and PCR analyses). Genetic markers are used to demarcate the interval of chromosome surrounding a mutation, which allows the clinician to follow its inheritance (figs. 5 and 6). Therefore, knowledge of the exact mutation is unnecessary for diagnosis of the disorder. As an example, in figure 5, markers 1–4 would be useful to evaluate the inheritance of the putative MHS locus. Markers 2 and 3 are in close proximity to the MHS locus and are least likely to be separated by homologous recombination of this gene. Hence, the inheritance of markers 2 and 3 would indicate that, most likely, the subchromosomal segment between them, including the MHS locus, has also been inherited. Figure 6 illustrates how polymorphic DNA markers can be associated with a phenotype and used as a noninvasive method of diagnosis (linkage analysis). Individuals I-1, II-2, II-3, II-6, and III-1 are MHS, and individuals I-2, II-1, II-3, and II-6 are normal. These data can be used to establish a close association between markers 2A and 3A and the MHS phenotype in this family and to provide a likelihood estimate with confidence limits as to whether individuals III-2 and III-3 inherited the mutant gene. In this example, individual III-2 is likely to be normal, and III-3 is likely to be MHS. The same analysis can be used for other individuals at risk within this family. Recombination occurs in individual II-4 between markers 3 and 4 and in III-1 between markers 1 and 2, illustrating that these markers would be less useful to predict the inheritance of MHS. This approach is limited because the data from this type of indirect analysis cannot be generalized to other families. In addition, to demonstrate an association between the MHS phenotype and a particular set of chromosomal markers, the family must be of sufficient size, and the phenotype must be known for a sufficient number of family members. The sensitivity of indirect techniques is directly dependent on the frequency of recombination exhibited between a disorder and surrounding DNA markers. The closer that markers can be found to the disorder (the lower the rate of recombination), the higher the sensitivity of the test.

This physical association can also be used in research to identify a previously unknown location for a defective

![Polymorphic DNA marker diagram](image_url)
gene, by demonstrating that the phenotype produced by the gene is consistently inherited along with a particular set of DNA markers (they cosegregate at meiosis because they are located in close proximity on the same chromosome and are not readily separated by homologous recombination). These mapping studies are laborious and require data from sufficient numbers of affected families to generate likelihood estimates for the inheritance (linkage) of the disease phenotype with a set of DNA markers.

In addition, the results of these mapping studies are critically dependent on the validity of the phenotype. However, once these linkage relationships are firmly established, they provide a sensitive, noninvasive technique for the presymptomatic diagnosis of MHS. The surrounding markers can be used to identify the chromosome (haplotype) and region on the chromosome that carries the defective gene in a particular family. Ultimately, the map locations generated in this fashion may also suggest gene candidates that may harbor the genetic mutation(s) that cause MHS. Studying the DNA sequence of these candidate genes may ultimately disclose a mutation, which can be used as a direct, highly accurate, and specific method of diagnosis in the future.

Prospects for the Diagnosis of Malignant Hyperthermia Susceptibility by Indirect Methods

The clinical diagnosis of MHS can now be attempted by indirect molecular methods. However, a diagnosis can be offered only to those individuals whose family members clearly demonstrate an association between the MHS phenotype and DNA markers closely spaced around MHS-1 on 19q13.1. The closer the DNA markers are located to the MHS-1 locus, the less likely they will be separated from the chromosomal interval carrying the disease gene by homologous recombination. Therefore, the precision of the diagnosis will depend on the reliability of the phenotype established in the other family members and the proximity of the DNA markers used that flank the disease gene. Furthermore, the lack of an association between DNA markers on 19q13.1 and the MHS phenotype cannot exclude the diagnosis because the disorder may be produced by a mutation elsewhere in the genome. Mapping MHS-2 (and potentially other MHS loci) will eventually provide indirect molecular methods of clinical diagnosis in individuals whose disorder does not originate from a mutation at the MHS-1 locus.

Summary

MHS is a heterogeneous pharmacogenetic disorder in the human that is likely to be caused by one of a variety of genetic defects, in one of a number of genes. Direct molecular methods will provide a rapid, efficient, non-invasive, and low-cost screening test once the causative genetic mutations have been identified. However, until this objective is met, indirect molecular genetic methods can be used to demonstrate the inheritance of an abnormal gene in certain family members at risk. This requires localizing the gene that produces the abnormal phenotype to a subchromosomal segment by linkage analysis and showing the coinheritance of MHS and DNA markers in a number of family members. Indirect molecular genetic methods are likely to be particularly useful in the diagnostic evaluation of children too small to be biopsied in families where others have been biopsied or their phenotypes are known. It appears likely that molecular genetic methods will not eliminate the usefulness of the muscle biopsy and caffeine–halothane contracture test in the near future. Rather, these diagnostic tests will complement one another and significantly improve our understanding of the complexity of this disorder.

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References


46. Goodman SR, Shiffer KA, Casoric LA, Eyster ME: Identification of the molecular defect in the erythrocyte membrane skeleton

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