TECHVIEW: GENETICS

Genetic Testing—Present and Future

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The revolution in genetics has led to the determination of the precise genetic basis of common and uncommon hereditary diseases. The first fruits of this revolution are diagnostic—the ability to determine who is and who is not at risk for disease before the onset of symptoms. Such information is becoming essential for proper management of patients and their families. In individuals who inherit mutant genes, simple preventative measures often can reduce morbidity and mortality and allow more thoughtful planning for the future. The benefits of genetic testing are equally important for those family members who are found not to carry the relevant mutation; these individuals are spared unnecessary medical procedures and tremendous anxiety. However, genetic testing is not without its problems.

These problems can be broadly divided into psychosocial or technical in nature. From the societal view, issues related to insurance, employment discrimination, and privacy have garnered much concern and attention (*1*). Additional ethical concerns arise when no effective intervention is available and when prenatal testing is considered for diseases with late onset or minimal effects. The technical challenges associated with genetic testing can be just as formidable and are often overlooked. For example, in many diseases, not all of the genes capable of causing or contributing to pathogenesis are known. Moreover, even when the mutated gene is known, routine genetic testing may fail to identify mutations in 25 to 75% or more of the cases (*2*). As a result of these uncertainties, genetic testing that fails to find a mutation is often inconclusive. Studies have shown that these inconclusive results may be misinterpreted by the patient and physicians and are a source of great anxiety (*2*, *3*). Because of these complex psychosocial and technical issues, it is clear that genetic testing should never be offered to patients without appropriate genetic counseling. In this TechView, we focus on the technical aspects of genetic testing, placing particular emphasis on issues that affect the sensitivity of mutation detection in known disease-causing genes.

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Type of mutation	Typical ease of detection
Nonsense or frameshift mutation in coding sequence	Compliant
Missense mutation in coding sequence	
Intronic mutations affecting splicing	
Interchromosomal rearrangements	
Intrachromosomal rearrangements	
Single exon deleted	
Several contiguous exons deleted	
Entire gene deleted	
3' UTR mutations affecting transcript levels	Refractory
Intronic mutations affecting transcript levels	
Promoter mutations affecting transcript levels	

Types of genetic mutations in hereditary diseases. Mutations are listed by ease of detection from compliant to refractory. Practically, the relative ease of detecting a given mutation type will depend on the specific genetic alterations present and the detection methods used.

Mutation Spectra

There are nearly a thousand different hereditary diseases for which the causative genes are known. In some cases, there are mutation "hotspots," that is, sites within the gene that are mutated in virtually all cases. Examples include sickle cell disease, in which an A to T transversion at codon 6 of the β-globin gene is ever-present, and Huntington's disease, in which virtually all patients have an expanded tract of CAG trinucleotide repeats, creating a long polyglutamine stretch within the huntingtin protein. These mutations can be readily identified with assays designed to detect the specific alterations, such as oligonucleotide-specific hybridizations in the case of sickle cell disease. Oligonucleotide-specific hybridizations are performed with radioactively or fluorescently labeled DNA probes of ~20 bases that are complementary to the mutant sequence. These probes are annealed with polymerase chain reaction (PCR) products derived from a patient's genomic DNA and are prepared using primers from the gene of interest. Under specific conditions, the probes only bind to PCR products that contain an exact match to the mutant-specific oligonucleotide. However, the majority of inherited diseases are caused by diverse mutations scattered along the length of the affected gene(s). In these cases, the precise genetic alteration may be observed in only a single kindred (family).

Just as the distribution of mutations along a gene can be diverse, so too can the nature of these mutations. There are numerous ways in which genes can be mutated (see figure, this page). The ease of detecting these mutations stretches across a continuum between "compliant mutations" and "refractory mutations." Compliant mutations are readily revealed by using one or a small number of routine genetic tests, such as DNA sequencing of PCR products from genomic DNA. Refractory mutations usually are not detected by routine testing. The types of mutations that are refractory will vary depending on the specific tests used but can include deletions encompassing one or more exons, insertions, translocations, or a variety of alterations that affect the expression of the gene at the RNA or protein level. Regardless of whether a particular mutation is compliant or refractory, once a disease-causing mutation is identified in one family member (by any technique), subsequent testing of the other family members for this mutation is relatively straightforward, sensitive, and specific (*4*).

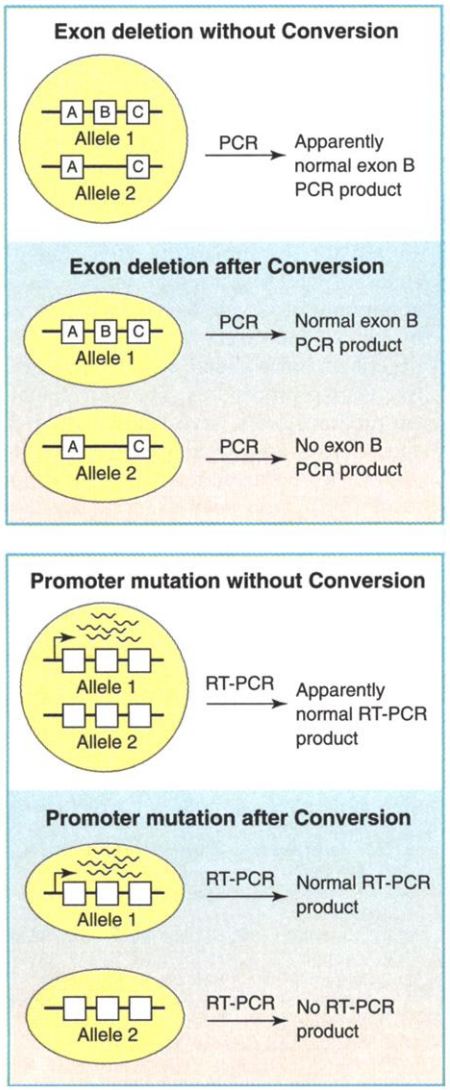
Direct Detection of Compliant Mutations

Direct methods, such as DNA sequencing, document the existence of a genetic variant while revealing its exact nature. In contrast, indirect methods document that a sequence variation is present, but they require a subsequent direct method to elucidate the nature of the variant. This disadvantage of indirect methods is counterbalanced by their relative simplicity and lower cost.

Currently, there are only two direct methods: DNA sequencing and microarray analysis. DNA sequencing is the current gold standard, against which all other methods are judged. DNA sequencing technologies have improved dramatically in the past few years, and the recent introduction of capillary electrophoresis has considerably simplified several aspects of the process and made certain aspects of the testing procedures automatable (*5*). Nevertheless, DNA sequencing does not reliably detect all mutations even when they are predicted to be readily detectable (compliant). For example,

in cases where the mutation affects only one allele (copy) of the gene, the peak heights corresponding to wild-type and mutant alleles are not always present at the predicted ratio of 1:1 (*6*, *7*). Distinguishing these mutations from other normal variations in the baseline can be difficult. Therefore, most genetic testing laboratories sequence both strands of DNA to achieve maximum sensitivities and to provide independent confirmation of any putative mutations identified.

Microarrays contain thousands of specific oligonucleotides bound to glass slides through one of two methods. One method involves spotting technologies, in which presynthesized oligonucleotides are printed onto slides with the aid of a robot. The second method involves synthesis of oligonucleotides in situ on the glass slides, using a photolithographic process similar to that used by microchip manufacturers. In either case, the oligonucleotides bound to the slide include those that are complementary to all possible base substitutions and a subset of small deletions and insertions (*8*). Fluorescently labeled PCR fragments from the genes to be tested are then hybridized to the microarrays. Though the up-front costs for producing microarrays for specific genes are high, economies of scale could eventually reduce such costs for relatively common hereditary diseases. There are still significant problems with microarray technologies, however, which have limited their application. For example, detection of homozygous mutations (both copies of the affected gene carry the same mutation) with microarrays is much easier than detecting heterozygous mutations (the two copies of the affected gene carry different mutations, or one copy is defective and the other normal) (*8*). Unfortunately, heterozygous mutations are more the norm than the exception. Microarrays also cannot be used to detect insertions of more than a single nucleotide without substantially increasing the number of oligonucleotides that must be immobilized.



Examples of refractory mutations detected through Conversion. (top) Analysis of diploid DNA templates by genomic PCR reveals only the normal PCR product of exon B, even though exon B is missing from one allele. The deletion is obvious from PCR analysis of hybrids containing the defective allele, in which no PCR product is obtained. **(bottom)** Mutations affecting the expression of the gene are revealed through analysis of the RT-PCR products of the haploid hybrids, whereas analyses of diploid patient cells are uninformative or more difficult to interpret.

Nearly a decade ago, it was hypothesized that mutational analyses could be carried out with a comprehensive array of very short oligonucleotides (*9*, *10*). The advantage of this strategy is that a single, universal microarray containing every possible oligonucleotide of a defined length could be used to analyze any gene. Though it has not yet been possible to implement this approach in a general fashion, advances in hybridization and microarray fabrication technologies may make this strategy viable in the future.

Indirect Methods

A number of clever indirect methods for detecting mutations have been developed (*11*). Some of these methods—for example, single-strand conformation polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE)—exploit the differential electrophoretic migration of nucleic acids that vary by as little as a single base. Denaturing high-performance liquid chromatography (DHPLC) is a related technique that detects a variation in structure between mutant and wild-type molecules but uses HPLC instead of gel electrophoresis for separation and is thereby highly automatable (*12*). Certain slab gel electrophoresis–based methods can likewise be automated through the implementation of capillary electrophoresis (*13*). In capillary electrophoresis, DNA molecules are driven to migrate through a viscous polymer by a high electric field and are separated on the basis of charge and size. Though the electrophoretic principle is identical to that used in slab gel electrophoresis, the separations are done in individual glass capillaries rather than gel slabs, facilitating loading of the samples and other aspects of automated sample handling.

Another group of powerful, indirect methods use chemical or enzymatic cleavage of the DNA, which exploits the bulges or bends in the DNA duplex that are created by mismatched mutations (*14*). It is also possible to take advantage of the alterations in protein structure resulting from certain genetic alterations. For example, nonsense mutations, frameshifts, and skipped exons can often be revealed by Western blot analysis of endogenous proteins or through analysis of proteins synthesized in vitro from PCR products (*15*).

Labs with experience in the above techniques often report sensitivities for detecting compliant mutations that are competitive with those achieved with DNA sequencing (*11*). In addition to their high throughput and relatively low cost, some indirect methods can be multiplexed, thereby allowing the evaluation of several exons simultaneously. Likewise, microarray technologies are ideally suited to such simultaneous analysis of multiple exons, whereas DNA sequencing is not.

Methods for Detecting Refractory Mutations

Many mutations are difficult or impossible to detect with the techniques described above. For example, if the genomic region examined is deleted from the mutant allele, the PCR product that is obtained from genomic DNA will be exclusively derived from the wild-type allele, leading to the false conclusion that this region of the gene was "wild type." Other mutations can affect the expression or processing of mRNA from the affected allele, through mutations of promoter sequences, 5′ or 3′ untranslated regions, or introns. These regions often encompass genomic segments 10 to 1000 times as large as the coding regions of the gene, and it is not practical to examine such a large number of nucleotides even with the indirect methods noted above.

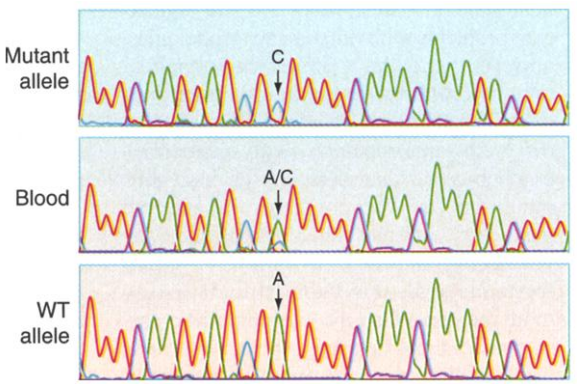
How can these refractory mutations be detected? Intronic mutations that affect splicing can be revealed through the analysis of RNA, and some investigators have recommended that both RNA and DNA be analyzed routinely to maximize sensitivity (*16*). Polymorphisms (benign sequence

variants that are present in a fraction of normal individuals) within an mRNA transcript, when present, can be used to assess relative levels of expression of the two alleles of the gene (*17*). Deletions of one or a few exons can theoretically be detected by quantitative hybridizations, quantitative PCR, or Southern blotting. Large deletions should be detectable with fluorescence in situ hybridization methods. The combined application of several of these techniques could improve the sensitivity of genetic tests, but so far such combinations have not been widely implemented for a variety of practical reasons.

As described recently by our group, an approach called Conversion (see figure, previous page) can considerably simplify the detection of refractory mutations (*18*). Patient cells are fused with a specially designed rodent cell line, creating hybrids that stably retain a subset of the human chromosomes. About one quarter of the derived hybrids contain a single copy of any human chromosome of interest. The diploid nature of the human genome (two copies of each gene) is thereby "converted" to a haploid state in which mutations are easier to detect because they are not accompanied by the normal sequence of the wild-type allele. Two examples of refractory mutations that can be detected through Conversion are shown in the figure on the previous page. In the first example, deletion of a single exon is obvious from genomic PCR analysis of hybrids: no product is observed from the hybrid containing the mutant allele. In the second, a mutation affecting the expression of one allele is simply revealed through the analysis of reverse transcriptase–PCR (RT-PCR) products. Compliant mutations are also revealed more clearly through the analysis of converted samples (see figure, previous page), because the signal-to-noise ratios are dramatically increased (from a maximum of 50:50 with conventional techniques to nearly 100:0 after Conversion). It is important to note that Conversion is not a substitute for the detection methods described above, but rather is an adjunct that provides improved nucleic acid templates that can maximize the sensitivity of conventional methods (see figure, this page). Disadvantages of the Conversion approach include the increased time and expense associated with the hybrid generation and screening process.

Cross Talk Between Basic Science and Genetic Testing

DNA sequencing and related methods can reveal the presence of sequence variations but cannot determine the functional consequences of such variations. Although mutations predicted to produce truncated proteins (such as nonsense mutations and frameshifts) can generally be assumed to be causal, other mutations are more difficult to interpret. In particular, missense mutations cannot be assumed to be causal without additional information. Extensive databases for many genes and diseases have been compiled, and these can be extremely useful for interpreting the significance of missense mutations and other sequence variants (*11*).



Base substitution mutations can be problematic. The intensities of the signals representing the mutant and normal sequences are not always equivalent in sequencing reactions derived from genomic DNA templates. In the example shown, the sequence of the PCR product in the sample derived from a patient's white blood cells exhibited a relatively small mutant "C" (blue) peak in comparison to the normal "A" (green) peak. Separation of the two alleles through Conversion reveals that one allele was wild type (WT), with a homogenous "A" (green) peak while the other allele was mutant, with a homogenous "C" (blue peak).

Equally important are studies of the function of the encoded protein. Common missense mutations can be tested for their effects on such functions and incorporated into the databases. Genetic counselors should be able to use all available information, through Bayesian analysis, to predict risk in individual families (*19*).

The contribution of basic research to genetic testing is not a one-way street. In many hereditary diseases, conventional genetic testing reveals mutations in only a subset of kindreds. This always raises the question of whether the kindreds without mutations have mutations in other genes. As genetic testing improves, with detection of virtually all mutations, these questions will be definitively answered. Future gene-hunting efforts can thereby be directed to the kindreds that truly lack mutations in known genes, opening the door to discovery of new genes that may further elucidate pathogenesis. A recent example of this principle was established through the examination of families affected by Li-Fraumeni syndrome (characterized by a marked susceptibility to cancer), who did not have mutations in the tumor suppressor gene *p53* despite extensive investigation. The discovery that some of these families had mutations in *Chk2*, a checkpoint-regulating gene with protein kinase activity, provided compelling insights into the regulation and function of p53 (*20*).

Prospectus

Genetic testing in the future will no doubt involve a combination of methods designed to fit the mutation spectrum of specific patients and genes. For example, simple hybridization methods can identify over 90% of the mutations in cystic fibrosis patients within certain ethnic groups, and more sophisticated methods can be reserved for the remaining 10%. For diseases in which refractory mutations are commonly observed, Conversion followed by any of the direct or indirect methods described above should lead to very sensitive testing procedures. The best diagnostic medical tests have sensitivities and specificities approaching 100%, and it is not overly optimistic to expect that genetic testing will meet such exacting standards in the future.

References and Notes

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21. The authors are grateful to the members of their laboratory for critical reading of the manuscript. Under a licensing agreement between the Johns Hopkins University and GMP Companies, the authors are entitled to a share of royalty received by the University on sales of products involving Conversion. The University and the authors also own GMP Companies stock, which is subject to certain restrictions under University policy. The terms of this arrangement are being managed by the Johns Hopkins University in accordance with its conflict of interest policies.