Post-natal molecular diagnosis of inherited diseases

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ABSTRACT

Molecular diagnostics is being revolutionized by the completion of the human genome project and by the development of highly advanced technologies for DNA testing. The coupling of polymerase chain reaction with a panel of tools for gene mutation identification has led to the development of methods for detecting known mutations or screening for unknown sequence alterations. Assays based on nucleic acid hybridization with short oligonucleotide probes or on the use of DNA modifying enzymes have been developed for single nucleotide polymorphism genotyping in inherited diseases caused by a small number of mutations, and protocols based mainly on heteroduplexes analyses have been employed for gene scanning in diseases associated with a high number of different and private DNA variations. Here we describe the most important methods that are commonly utilized in molecular diagnostic laboratories.

INTRODUCTION

Molecular diagnostics is a discipline that combines laboratory medicine with the knowledge and technology of molecular genetics. Its aim is to provide a sensitive alternative to protein-based current methodologies by developing DNA/RNA-based analytical methods for monitoring human pathologies. This is accomplished by the identification of the disease-causing mutations which may be both known or unknown.

Completion of the human genome project have generated within a few years vast information regarding molecular alterations either causing inherited disorders or predisposing to diseases (1). Many projects in current human genetics aim to dissect complex traits by making use of DNA markers, mainly by single nucleotide polymorphisms (SNPs), which are currently used for whole scanning of genomes to gain first indication of interesting regions that contribute to the traits under investigation or in more focused candidate gene association studies. Once a SNP or a group o SNPs has been identified as a disease marker, it can be used for diagnostic application. In this case specific criteria of the DNA variations typing methods are required, such as simple protocols, which can be easily applied for medium throughput applications, short operational time reducing labour costs, rapid analysis, which may be crucial to save the life of a diseased (e.g., infected) patient and reduced costs (2).

With the advent of polymerase chain reaction (PCR), the battery of diagnostic tools for gene mutation screening was significantly enriched and DNA amplification was coupled to a rich repertoire of methodologies for detecting known mutations or screening for unknown sequence alterations inside the human-associated disease loci (3, 4).

In clinical diagnostics, methods for SNP genotyping are applied in the diagnosis of a number of inherited diseases caused by a relatively small number of mutant alleles. In other situations, when the disease is associated with a high number of different and private mutations spread all over the gene or the mutant alleles are so rare that each family carries its own mutation, the routine clinical diagnostics must be based on mutation scanning over a complete gene (5). Consequently, in the last years a number of rapid, robust, cost-effective and efficient methods with the capacity to detect known and unknown sequence variations on a medium-large scale have been developed (1). Here we describe only the methods that are frequently utilized in molecular diagnostic laboratories.

DETECTION OF KNOWN MUTATIONS

Genotyping methods for the identification of known DNA variations are based on nucleic acid hybridization with short oligonucleotide probes or on the use of DNA modifying enzymes.

Allele-specific mutation detection of amplified DNA based on hybridization of PCR products to allele-specific oligonucleotide probes (ASO) can be applied in two formats. The first is the dot-blot approach, whereby PCR products are immobilized on a membrane and hybridized to labelled ASO probes. Because of its simplicity it became one of the most widely adopted methods in molecular diagnostics, using either radioactive or nonradioactive probes. The dot-blot format is most useful when large numbers of samples are being screened for a small number of mutant alleles (6). The second approach is the reverse dot-blot, whereby ASO probes are immobilized on a membrane and hybridized to labelled PCR products. It can be considered the founding principle behind genotyping microarrays. The reverse dot-blot is a widely used tool for routine screening of numerous mutant alleles in several disease-associated genes (7). Automated

platforms for preparing the reverse dot-blot membranes (strips) have been reported that allow printing of large numbers of strips with higher-density arraying and hence commercialization of the entire process (8). Today, a number of commercially available mutation detection assays for different disease mutations are available (4).

Enzyme-assisted genotyping, using nucleases, DNA ligase or DNA polymerase, are also employed. Restriction endonuclease analysis (RFLP) and allele-specific mutation amplification are the early and most widely used techniques to detect known gene mutations based on enzymatic reaction. In case the mutation fails to create or abolish a restriction site, the latter can be artificially created by incorporating the necessary nucleotide change(s) in the amplification primer trough the PCR-mediated site directed mutagenesis (PSDM) (9, 10).

The allele-specific amplification using the amplification refractory mutation system (ARMS) is probably one of the most popular detection methods for point mutations, having the advantage of being able to detect virtually all known sequence variations (11,12). False-negative results due to amplification failure can be easily monitored using an internal control of an irrelevant genomic region, while the single-tube assay allows for the simultaneous detection of both wild-type and mutant alleles (4, 13).

The oligonucleotide ligation assay (OLA) relies on the hybridization of two allele-specific oligonucleotide probes (one specific for the wild-type allele and the other specific for the mutant allele) plus a fluorescent common probe coupled with DNA ligase reaction (14). This method have been improved to genotype a large panel of informative biallelic markers through multiplexing PCR and ligase reactions and chemically modifying oligonucleotide probes. Probes bear non-nucleotide tails which allow the mobility of each ligation product to be arbitrarily defined, regardless of oligonucleotide length or sequence (sequence-coded separation) (15, 16).

A more recently developed method based on DNA polymerases is single nucleotide extension (SNE) (also single base extension –SBE- or minisequencing). In this case, the distinction between genotypes of the SNPs is based on the high accuracy of nucleotide incorporation by the DNA polymerases (17). The primer extension reaction is robust, allowing specific genotyping of most SNPs at similar reaction conditions. These features are advantageous for high throughput applications because the effort required for assay design and optimization are minimized.

Since the enzyme-assisted methods have proven to be more robust and to provide more specific allele distinction than allele-specific oligonucleotide hybridization (18), these methods have been multiplexed, automated and adapted to various detection strategies, and they provide most of the current high-throughput SNP-genotyping platforms (19).

Finally, real-time PCR has recently emerged for rapid genotyping without the need for post-PCR sample manipulation (20, 21). The method is based on fluorescence resonance energy transfer (FRET). FRET occurs when two fluorescent dyes are in close proximity to one another and the emission spectrum of one fluorophore overlaps the excitation spectrum of the other fluorophore (22). Commonly used FRET-based technologies include the LightCycler and TaqMan assays and molecular beacons (16). Whit this approach, each fluorescently labelled hybridization probes, specific for each mutation, yields a different melting curve, and genotyping is performed on the basis of a melting-curve analysis. This allows to quickly assign hetero- or homozygosity for the wild-type and/or mutant allele and, at the same time, to monitor for false-positive or negative results. Although it is expensive and difficult to standardize, the assay is very fast, simple, and with a high throughput, and allows the reliable detection of several mutations simultaneously (4).

There has been enormous development in assay formats and labeling and detection strategies. Miniaturization and multiplexing of the mutation scanning and genotyping assays is a key element for bringing down costs and increasing throughput (5).

SCANNING METHODS FOR UNKNOWN GENE SEQUENCE ALTERATIONS

Denaturing gradient gel electrophoresis (DGGE) and single-stranded conformation polymorphism (SSCP) analysis are two of the most commonly used methods for screening for both known and unknown mutations in human genes (23, 24). The hallmark of both techniques is their high discriminatory potential between the wild-type and different mutant alleles, since even a single base difference anywhere in the amplified DNA fragment will theoretically yield a different electrophoretic pattern (4). The DGGE, if optimised, displays a very high mutation detection rate (about 95%) compared with other scanning methods. The technique has been further improved by superimposing a porous gradient on the denaturing gradient [double-gradient DGGE (DG-DGGE)], which minimizes band broadening, even in prolonged runs, and permits more accurate band separation (25). Additionally, temporal temperature gel electrophoresis (TTGE), which relies on a temporal temperature gradient instead of the chemical gradient used in DGGE, has also been reported to be easier to perform and more reproducible, when compared to DGGE (4, 26).

For all these approaches, careful adjustment of the experimental conditions is, however, required in order to obtain reproducible results between different runs, particularly for the purposes of prenatal diagnosis.

In recent years, denaturing HPLC (DHPLC) has been gradually adopted for use in several diagnostic laboratories because it provides a semiautomated, fast, and reliable alternative to DGGE. DHPLC uses an ion-pair chromatography separation principle, combined with accurate control of the column temperature and optimized mobile phase gradient for separation of mutant DNA molecules (3). Different experimental protocols have been described for diagnostic purposes, showing ~98%

sensitivity and specificity in detecting point mutations or even large deletions (27, 28). Considering the above-mentioned advantages, together with the high initial investment costs for purchasing the DHPLC set up, this method seems the most appropriate for diagnostic laboratories that have large test volumes and are involved in routine carrier identification and mutation screening.

It should be noted that ideally the above-mentioned methodologies should be coupled with DNA sequencing, for either the definitive identification of unknown DNA sequence variations or the confirmation of inconclusive results, such as neutral gene variants, or ambiguous chromatograms and/or electrophoretic patterns (4).

Direct sequencing approach is the benchmark for genotyping, but costs and throughput are the key limitations. Many new sequencing methods are being explored at present (sequencing by hybridisation, pyrosequencing, base-by-base sequencing by synthesis, sequencing by ligation, nanopore technology) and their integration in high throughput and automated platform may help in overcoming sequencing limits (29). An alternative to direct sequencing is the employment of mass spectrometry for the identification of both known and unknown DNA variations (2, 30).

MICROCHIP

In the last few years, technology rapidly improved and new laboratory tools became available. Among them DNA chips have the potential for sample detection in integrated systems. Through miniaturization of the test platform, microchip-based nucleic acid technologies allow assay development for rapid detection of a large variety of SNPs and mutations in a large population sample, thus reducing time and manual work. Many important molecular biological analyses will be improved by the introduction, in both research and clinical diagnostic laboratories, of this new powerful technique that can be proved faithful for a variety of applications (1)

Two principally different approaches underlie the new miniaturized assays: development of highly parallel assays in solid-phase microarray formats and homogenous assays performed in individual channels in microfluidic devices (5).

DNA chips are referred to as high-density oligonucleotides or cDNA molecules attached to a solid support. The fundamental principle of most of them is the highly selective nature of DNA double helix hybridization. In particular, the immobilized nucleic acids are interrogated through hybridization with a fluorescent nucleic acid molecule.

The biochip technology for SNP typing mainly uses two different approaches to perform allelic discrimination: either allele specific nucleotide incorporation based on enzymatic reaction (SNE, SBE and arrayed primer extension – see above) or allele specific hybridization (1, 31).

FUTURE TRENDS

In practice, the requirement of a PCR amplification step to achieve sensitive and specific SNP genotyping is the principal factor that limits the throughput of assays today. New PCR instruments that use microcapillaries instead of microtitre plate formats have been devised and offer increased PCR throughput and reduced reagent costs as they use extremely short amplification times and small reaction volumes. Fully automated SNP analysis systems could then be designed based on homogeneous detection or by streamlining the PCR and the subsequent genotyping procedure in microfluidic ‘lab-on-chip’ devices that operate with submicrolitre reaction volumes. Such microfluidic devices are now under development in several biotech companies. Additionally, recent developments of composite materials and fluorescence detection strategies offer increased detection sensitivity and specificity for SNP-genotyping assays. Hybrid gold and silver nanoparticles have been used, instead of fluorophores, as labels on allele-specific oligonucleotide probes (32). In another, very promising strategy for multiplexing bioassays, multicolour optical coding is accomplished by embedding different sized “quantum dots” into polymeric microbeads at accurately controlled ratios (32): this technology has the potential for several-thousand-fold multiplexing.

Despite the numerous technical advances in detection and multiplexing strategies, no technique, however, represents the final benchmark approach.

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