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EXPERIMENTAL / LABORATORY STUDIES

Mutation Analysis By The Use of Temporal Temperature Gradient Gel Electrophoresis

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Today, technological development continues in the mutational analysis area of molecular medicine. In the past few years, the necessity to screen amplified DNA products to identify mutations or polymorphisms has assumed increasing importance in population genetics, as well as in medical genetics. As a well-known molecular genetic topic, mutation analysis refers to the identification of changes in DNA which produce disease or dysfunction. Detecting mutations in DNA (or RNA) requires various combinations of (a) physical mapping, (b) cloning or amplifying DNA, (c) screening methods to identify DNA changes involving one to a few nucleotide bases, (d) electrophoretic separation of DNA fragments, and (e) sequencing. The 'gold standard' in terms of DNA mutation analysis is the sequencing so that an exact mutation can be defined. There are two types of mutation analysis performed which require completely different and rarely overlapping methods. The first is the more difficult search for a mutation in a piece of DNA for the first time, "unknown mutations" and the second one is the search for previously identified mutations, "known mutations" in a family or a population.

However, most of the time, the genes are too large to make sequencing a practical diagnostic approach to do a large screening for a common disorder. There are several commonly used screening methods for unknown mutations. These include denaturing gradient gel electrophoresis (DGGE) (1); temporal temperature gradient gel electrophoresis (TTGE) (2); temporal gradient gel electrophoresis (TGGE) (3); single strand conformational polymorphism (SSCP) (4); heteroduplex analysis (HA)(5); chemical mismatch cleavage (CMC) (6); and protein truncation test (PTT) (6). Based on reported research studies, there are several factors that these techniques have handicaps. For example, SSCP and HA have low sensitivity (60-90%) for detecting mutations (7,8), TGGE and DGGE have difficulty in casting gradient gels, and cost effective for the synthesis of GC clamped primers, CMC has high background and has the significant disadvantage of using toxic chemicals during the process, and PTT has the disadvantage of the elimination of unstable mutant transcripts. One important disadvantage of using the SSCP, HA and CMC techniques is the difficulty in detection of homozygous mutations (9,10).

It is hard to make a decision for making an ideal choice of applying one of the techniques above. However, SSCP, HA, TTGE, and DGGE are widely used for the large scale screening of unknown mutations as well as polymorphisms. All four of the techniques have variable detection rates. There are several factors for the reduced mutation detection rate, in the application of long as well as short amplicons. Several research studies have been made on several large genes confirming that the mutation

detection rate is relatively low (11) and aimed to perform confirmation-sensitive methods for large scale screenings. Recently, a fluorescent based high throughput mutation detection method has been developed based on SSCP (known as F-SSCP) and heteroduplex analysis. This method is at least as sensitive as the radioactively labeled method. However, it is reported that some mutations may be missed, but the method can be improved by running at different temperatures and conditions, such as injection time and electrophoresis voltage. It is more reliable than conventional SSCP/HA analysis, but more expensive in the sense of using fluorescent labeled primers for PCR and the disposable materials (12,13). On the other hand, DGGE is a more reliable method when compared to conventional SSCP/HA analysis, but has almost the same reliability of using TTGE. It has the difficulty of casting the different gradient gels, the usage of clamped primers, labour-intensive, and time consuming process.

Temporal Temperature Gradient Gel Electrophoresis (TTGE)

Temporal Temperature Gradient Gel Electrophoresis is a sensitive and effective method both for identifying and characterizing genetic polymorphisms. Successful application of TTGE method in the detection of mutations in both nuclear and mitochondrial genes has been documented. When compared to other techniques TTGE has many advantages both for the application and its cost. Temporal temperature gradient gel electrophoresis was first introduced by Yoshino, in 1991. It has the same working principle as the DGGE technique, based on the melting behavior of DNA molecules. When compared to DGGE, it is applicable without the requirement of using a chemical denaturing gradient (2). PCR-based TTGE is a non-radioactive protocol. Once a mutation is detected, the PCR product can be easily isolated for its subsequent sequencing analysis to identify the mutation. Unlike DGGE, it does not require specially synthesized GC clamped primers, and it can efficiently be applied to PCR products as large as 1 kb. It eliminates the process of pouring a chemical gradient gel. TTGE uses an acrylamide gel that contains a constant concentration of chemical denaturant (urea). The temperature is increased gradually and uniformly (0.5-3 °C/hour) during electrophoresis (9). The result is a linear temperature gradient over the time course of the electrophoretic run. Thus, a denaturing environment is formed by the constant concentration of denaturants in the gel in combination with the temporal temperature gradient. Since, the denaturant in TTGE is the temperature; it is easier to modulate the temperature during electrophoresis and to provide a broader separation range that results in much higher sensitivity of detection. It can detect single nucleotide substitutions, small size deletions, and insertions. Like all other techniques, the detection rate of TTGE is not 100 %, but it is by far the most sensitive mutation detection method and the easiest to use. In general, by optimization TTGE running conditions such as temperature range, temperature increment rate, gel percentage, and running time, the detection rate can reach 100%. One must keep in mind that mutations deep in introns not included in PCR, or PCR failure due to deletion or primer mismatch, will also result in reduced detection rate not related to the TTGE technique itself (9,14).

The PCR based TTGE mutation detection method is a powerful tool when applied to the detection of heterozygous nucleotide variants. It is based on the difference in the sequence-specific melting behaviour of the normal and mutant DNA in a temporal temperature gradient that gradually increases in a linear fashion over the duration of the electrophoresis. Denaturation and reannealing of PCR products allows the formation of homoduplexes as well as hybrid heteroduplex molecules. A total of four bands will be observed in a heterozygous mutation; two homoduplexes and two heteroduplexes at optimal separation conditions (Figure 1). Single band shift up or down will be observed in a homozygous mutation DNA fragment (Figure 2).

As an example, TTGE has the sensitivity of detecting approximately 92 to 97.5% of all mutations of the cystic fibrosis transmembrane regulator (*CFTR*) gene by screening all 27 exons. Its specificity is estimated to be about 99%, with a false negative rate of about 1%, without any false positives (9,15), whereas DGGE has 98% sensitivity of detecting the mutations of *CFTR* gene (16). On the other hand, with only the use of two buffer polyacrylamide gel electrophoresis (PAGE) system based SSCP/HA analysis results in a detection rate of 97.5% for all 27 exons of the *CFTR* gene. This general protocol is optimized by varying the temperature, gel composition,

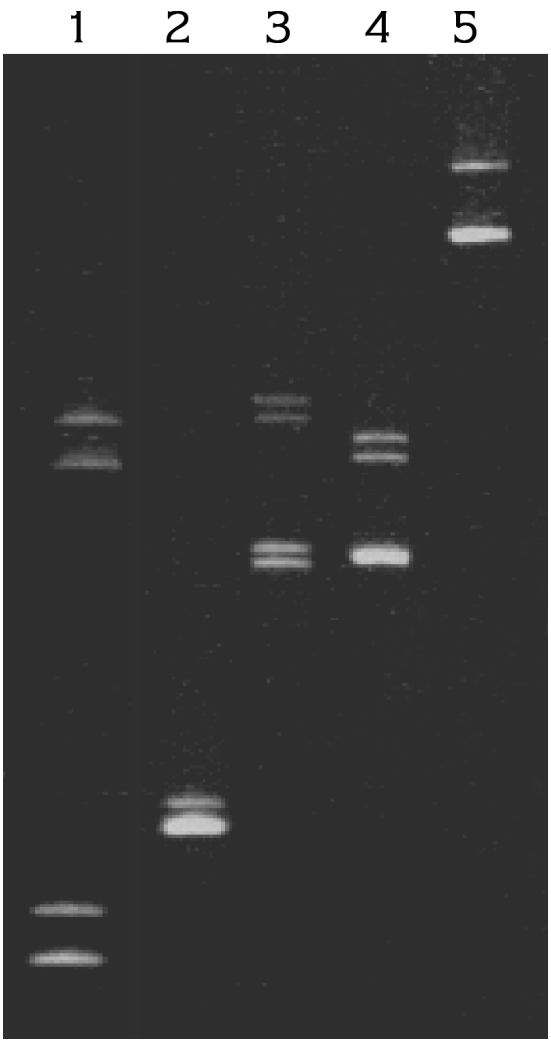

Figure 1. TTGE analysis of different exons related with the *CFTR* gene running under a common condition. TTGE was performed on a 6 % PAGE gel containing 6M urea at a ramp rate of 1.5 °C/ hour from 45 to 52.5 °C. Lanes 1-5 are the PCR products containing heterozygous frameshift mutations or polymorphisms in the exons of 1 (amplicon size 265bp, mutation 124-146del23bp), 3 (amplicon size 309bp, mutation 360-365insT) , 8 (amplicon size 456bp, mutation 1288insTA), 10 (amplicon size 491bp, polymorphism Met470Val/1525-61A>G, both heterozygous) and 13 (amplicon size 862bp, mutation 2289-2295del9bp) of the *CFTR* gene. A good example of running the different amplicon sizes in the common TTGE condition.

ionic strenght and the use of additives (17). On the other hand, DNA fragments with similar melting behavior can be run under the same conditions and this can be time and cost effective in TTGE (Figure 1). This is a great

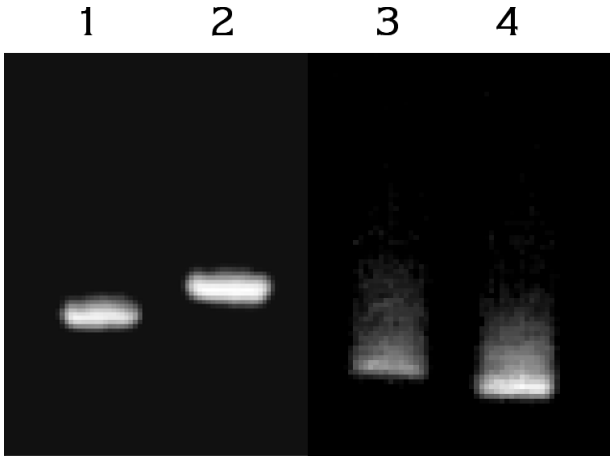

Figure 2. Detection of mutations and polymorphisms in *CFTR* gene by TTGE analysis. Lanes 1 and 3 are wild type DNA and lanes 2 and 4 are mutant DNAs. Lane 2 homozygous 1288insTA mutation in exon 8. Lane 4 homozygous polymorphism 296+28A>G in intron 2.

advantage when working with a gene with a large number of exons (14).

Up to date, the TTGE technique has been used to detect unknown mutations in both nuclear genes, including *CFTR* (14,15,18), *COL2A1* gene (19), *TP, FGFR2* genes (unpublished data), and mitochondrial genes (20,21).

Even with the use of denaturing high performance liquid chromatography (DHPLC) and chip technologies in today's life, only 87% and 85% of the mutations are detected (false positives were 40% and 45%, respectively (10). It is the same for the F-SSCP technique, which has the sensitivity of 95%, and specificity of 97%. All these automated methods were highly reproducible, and the only advantage is the time.

In the Turkish population, only several studies have been performed related with the identification of the unknown mutations or the polymorphisms for most of the genetic diseases. It is important to screen for unknown mutations in order to confirm a diagnosis and to provide accurate genetic counseling. Identification of the nuclear gene mutations spectrum for the genetic diseases like deafness (22), cystic fibrosis (23-25), and craniosynostosis (unpublished data) in the Turkish population will improve the diagnosis and allow proper genetic counseling including carrier detection and

prenatal diagnosis. We believe that TTGE can be easily used both in a diagnostic setting and in large scale screening research studies for the detection of nuclear and mitochondrial gene mutations.

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