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Screening of Mutations/SNPs through Spectrophotometric Melt-Curve Analysis: A Preliminary Study on Non-Electrophoretic and No-Dye Method of Mutation Detection

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Abstract

Presently available methods for screening of mutation require florescent labeled probe/dye or electrophoresis or both that make these methods tedious, expensive and time consuming. Here we described a method of screening of mutation/SNP without need of probe/dye and electrophoresis. The technique requires only PCR product and spectrophotometer with peltier. We screened mutation by monitoring DNA melting profile and transition temperature of homoduplex and heteroduplex by recording absorbance of UV by melting duplex. Absorbance for each duplex was measured at 260 nm from 60°C to 85°C at an increment of 1°C temperature using spectrophotometer having peltier with a heating rate of 1°C /min. In the heteroduplex samples there is rapid increase in the absorbance of UV at transition temperatures of 70°C. While in homoduplex sample it is reached after 75°C. Mutation in the sample was detected by observing the decrease of transition temperature of heteroduplex samples compared to homoduplex.

Keywords: Spectrophotometer; Mutation; Heteroduplex; Homoduplex

Introduction

Initial screening methods of mutation detection were mainly based on conformational changes in DNA fragments arise due to change in nucleotide sequences. These conformational changes were detected by mobility shift of DNA fragments in gel electrophoresis. The techniques based on this principle include denaturing gradient gel electrophoresis (DGGE) [1] temperature gradient gel electrophoresis (TGGE) [2], heteroduplex mobility assay (HMA) [3] and single-strand conformation polymorphism (SSCP) [4] and others. These techniques are increasingly being utilized for mutation detection. However, as these techniques are based on electrophoretic mobility of the DNA molecule, the movement of which during electrophoresis depends upon the physical and chemical properties of the electrophoretic medium including the temperature, concentration of ions, quality of gel and solvent, and the applied voltage. This greatly affects the reproducibility and sensitivity of these techniques.

The reliance on gel electrophoresis for mutation detection is overcome by recent advances in mutation detection techniques involving screening of DNA duplex stability of PCR products by thermal denaturation and monitoring the melting profile by florescent dyes or a fluorescein-labeled oligonucleotide probes. Mutation detection by real-time PCR and the high resolution melting curve analysis (HRMA) based on fluorescently labeled probe [5] are methods of choice for genotyping and variant scanning. Although major differences between PCR products can be screened by melting temperature (Tm), slight sequence variation may be missed out by fluorescent melting analysis. These techniques involve complicated probe designs, use of fluorescent dyes, high cost equipments, and software for DNA melting analysis. To avoid the use of fluorescent dyes Nasef et al. [6] introduces a new method for label less electrochemical melting curve analysis using methylene blue as an electroactive indicator of hybridization and denaturation. Though the technique is useful for detection of specific mutation it is unable to detect unknown mutation and also requires immobilize probe. While presently available methods for screening of mutation successfully overcome the need of gel electrophoresis, they still require fluorescent dye or probe. We therefore, interested to develop a technique of mutation detection that neither requires electrophoresis nor any dye or probe.

Exploiting the two basic properties of DNA, one is lowering of melting temperature of DNA duplex having mismatched base pairing and other increase in absorbance of UV light in transition from double stranded to single stranded DNA, we have developed a simple PCR-based high-resolution melt curve analysis method for detection of mutation without the use of any dye or probe and gel electrophoresis. Mutants were identified based on difference in the transition temperature of the DNA denaturation of the heteroduplex and the homoduplex. In the present study, we have standardized the technique of screening mutation in exon-2 of the *p16* (CDKN2A) gene in cervical cancer samples.

Methodology

DNA extraction and PCR amplification

Genomic DNA was extracted from 105 cervical cancer and 35 normal tissue biopsies (The Institutional, ethical committee approved the study and informed consent was obtained from all patients) by phenol/chloroform method as described by Sambrook et al. [7]. The primer sequences of a segment of exon-2 of p16 gene are obtained from

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Lee et al. [8]. The primers sequence F 5` AGCCCAACTGCGCCGAC 3` and R 5` CCAGGTCCACGGGCAGA 3` for PCR amplification is of a segment of exon-2 of the *p16* gene having amplicon size of 147 bp [8]. PCR amplification was performed in 25 µL reaction volume containing 1x PCR buffer, 1.5 mM $MgCl_2$, 25 µM of each dNTPs, 10 pmol of each primer, 50-100 ng of genomic DNA and 0.5 U Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA). Standard temperature profile used for amplification of genomic DNA constituted an initial denaturation at 95°C for 4 min followed by 35 cycles. Each cycle consists of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s, which was extended for 4 min in the final cycle.

Heteroduplex analysis

For heteroduplex analysis, 12.5 µl PCR product of sample to be screened for mutation was mixed with equal volume of PCR product of the normal control sample in 1 ml PCR tube. The PCR mixture was diluted 20 times with 1x PCR buffer to remain within the upper limit of concentration of DNA to be read by spectrophotometer. Heteroduplex was formed by hybridizing the PCR mixture in the thermal cycler with initial denaturation at 95°C for 1 min, followed by 10 cycles of denaturation and renaturation for 30 s each at 94°C, 45°C and 10°C. Absorbance for each duplex was measured at 260 nm from 60°C to 85°C with a heating rate of 1°C/min by using Jasco V560 spectrophotometer having peltier for heating the samples. (In the manuscript, we have shown the graph for screening of mutation in the temperature range of 20-85°C with temperature set to increase at 1°C/min just to show that there is no change in absorbance of UV before the transition temperature). Absorbance versus temperature was plotted to obtain the melting profile and transition temperature of heteroduplex. Similarly melt-curves were obtained for homoduplex. Mutation was detected by observing the decrease in transition temperature of heteroduplex sample compared to homoduplex of normal sample.

Single strand conformation polymorphism (SSCP)

Screening of mutations in the *p16* gene was also done by SSCP method as described by Orita et al. [4] and was performed according to the modification as suggested by Katiyar et al. [9]. The PCR products were radiolabeled by regular amplification of 30 cycles with an additional 10 cycles where dCTP was replaced with [α-^{32} P] dCTP (specific activity, 4000 Ci/mmol) (BARC, India) in the PCR mixture. Thus for a typical PCR mixture of 100 µl meant for 10 samples (10 µl each) 1 µl (10 µCi) of [α-^{32} P] dCTP was added with other reaction component being the same. 1 µl radio-labeled PCR product was diluted with 9 µl of denaturing solution (95% formamide, 20 mM EDTA pH 8.0, 0.05% xylene cyanol and 0.05% bromophenol blue) and heat denatured for 5 min at 95°C and immediately chilled on ice for 5 min. 3 µl of each sample was loaded in 6% polyacrylamide gel with 10% glycerol. Gel was run in 0.5X TBE buffer for 16 h at 200 V in Base Ace™ sequencing gel apparatus (Stratagen, GmbH, Germany) at 17 ± 1°C ambient temperature. The gel was dried on gel drier (BioRad, USA) and exposed to X-ray film within an intensifying screen at -70°C overnight to develop the autoradiograph of the band positions. Alteration in electrophoretic mobility shift in single stranded DNA bands were determined by comparing their position with that of normal controls. The control PCR products used in the SSCP for comparison of band migration of sample DNA were amplified from the DNA of normal individuals and matched with wild type sequence available from GenBank prior to use.

DNA sequencing

Automated DNA sequencing of all the PCR products was performed to confirm and determine the type of mutation. The PCR products were commercially sequenced (Macrogen Inc., Korea) by automated sequencer.

Results

In SSCP method for screening of mutation, the mutant PCR products are sorted out from normal samples by the shift of band position in the polyacrylamide gel while in heteroduplex analysis method mutants are identified by the decrease in transition temperature of mutant samples compared to normal samples (Figure 1) Single mutation was found in cervical cancer samples by both SSCP and Heteroduplex analysis screening methods. The mutation was confirmed by direct sequencing

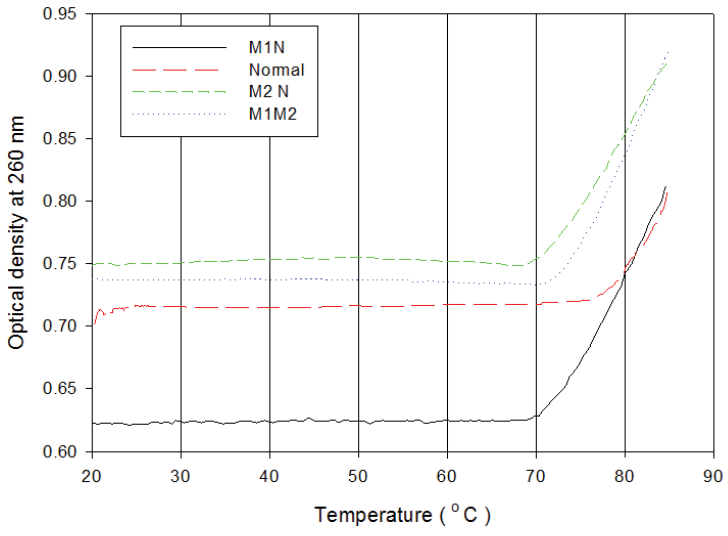

Figure 1: The melt curves of normal and mutant PCR products at absorbance of 260 nm and temperature range of 20-85°C with an increment of temperature at the rate of 1°C/min. M1N, M2N and M1M2 are Heteroduplex PCR products having mutation while '*Normal*' is homoduplex. In normal homoduplex melting of dsDNA PCR products starts at ~75°C while in all the three heteroduplex PCR products melting starts early at temperature of ~70°C. This shows the clear difference between a mutant and a normal sample.

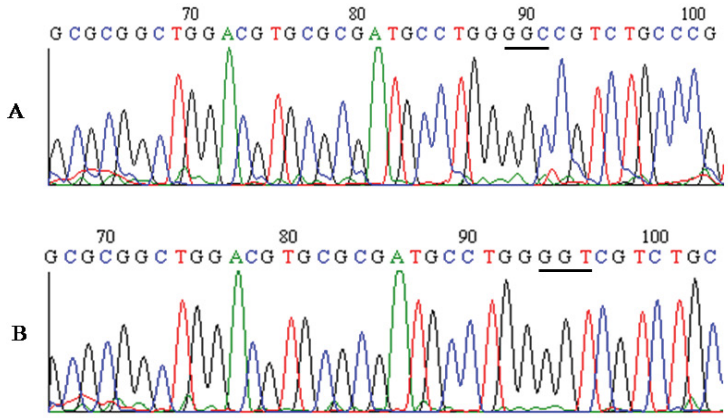

Figure 2: Partial pherogram of automated DNA sequencing of PCR amplified products of exon-2 of the p16 gene in (A) DNA from Normal control sample showing p16 exon-2 normal sequence and (B) DNA from cervical cancer tissue showing C→T transition substitution mutation leading to codon change GGC→ GGT at codon 111 of p16 gene.

as the silent mutation due to transition of CT at nucleotide number 604, codon number 111, led to codon change GG**C**→GG**T** (Figure 2). The other point mutation was found in head and neck cancer (data not shown). In the heteroduplex samples M1N, M2N and M1M2 there is rapid increase in the absorbance at temperatures beyond 70°C. While in homoduplex sample N (normal) it is attained at 75°C. Therefore, the transition temperature of mutant samples is 70°C and that of normal is 75°C (Figure 1). In the melt-curve analysis method the curve obtained by plotting absorbance of UV verses temperature is linear and parallel to x-axis before melting of the duplex DNA and there is steep slope after the transition temperature.

Discussion

In this study we have described a simple PCR-based high-resolution melt curve analysis method for the identification of mutation/polymorphism without the use of any dye or probe and gel electrophoresis. Previous studies on mismatched DNA mainly focused on nearest-neighbor model to predict the stability and thermodynamics of DNAs with Watson-Crick pairs [10,11]. Here, we exploited this characteristic of DNA for detection of mutation and polymorphism by melt curve analysis of PCR products using spectrophotometer. The method is based on the differential melting temperature of PCR products of homoduplex and heteroduplex formed by melting and reannealing pairs of homologous DNA fragments with fragments having mutation. The difference in melting temperature can be measured by differences in the amount of absorbance of UV light at 260 nm during melting of the double stranded DNA. The increase in absorbance of UV light (A_{260}) by dsDNA after melting is because of the phenomenon of the hypercromicity that is dsDNA is hypochromic relative to ssDNA. Nucleic Acid absorbs UV light due to the conjugated aromatic nature of the bases. The wavelength of maximum absorption of light by both DNA and RNA is 260 nm. The absorbance at 260 nm is greatest for isolated nucleotides, intermediate for single stranded DNA or RNA and least for double stranded DNA. This effect is caused by the fixing of the bases in a hydrophobic environment by stacking of the bases [12].

The heteroduplex has a lower melting transition temperature compared to the homoduplex because of the instability induce by mismatched base pairing. Denaturation is a highly cooperative process, where the conformational states of base pairs exhibit a strong dependence on the states of their neighbors [13]. Thus in heteroduplex, melting of dsDNA starts at point of mismatch site and proceed to complete separation of DNA strands. DNA fragments containing a single mismatch have lowering of transition temperature by 1 to 5°C in comparison to its homologous DNAs with complete Watson-Crick base pairing. Both the bases at the mismatch site and neighboring stacking interactions influence the destabilization caused by a mismatch [14]. Since we have not use any dye or probe and also the PCR products are of short length requiring less amount of energy for denaturation, the melting of DNA shows precipitous transition in temperature at which double stranded DNA is converted into single strand which is evident by the steep slope in the melt curves.

One limitation of our study is that the mutations that we have reported are not located in the terminal position of the PCR product therefore we were unable access the effect of mutation located on 5' and 3' end of the amplicon on the melting behavior of the duplex DNA. However, at both ends of amplicon are located the primers and if there is any mismatch at primer binding site then there will not be any amplification.

Conclusion

Presently described method does not require radioactively labeled reagents or probe and is therefore, suitable for use both in research and clinical laboratories. It can be performed for screening of both known and unknown mutations and polymorphism. No previous normalization or standardization of the curve is required. The curves obtained are sharp and single line without any unspecific signals. The method neither requires any florescent/other dye or probe nor any gel electrophoresis. It needs only PCR products and the buffer as reagents and spectrophotometer with peltier as instrument. The method is rapid, simple, inexpensive and sensitive and sensitive enough for the molecular diagnosis of cancer and other genetic diseases occur due to genetic alterations.

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