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Single base variation in DNA fragment joined to a GC-clamp can be detected by temporal temperature gradient gel electrophoresis

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Abstract:

Temporal Temperature gradient gel electrophoresis (TTGE)[2] in combination with PCR and 'GC-clamping' has proven highly efficient as a method for detection of DNA sequence differences. Duplex DNA fragments differing by single base substitutions can be separated by electrophoresis in Temporal Temperature gradient gels. To increase the number of single-base changes that can be distingished by TTGE, we used the polymerase chain reaction to attach a 50-base-pair G+C-rich sequence, designated a GC-clamp, to one end of amplified DNA fragments that encompass regions of the human bFGF promoter region. The melting properties and electrophoretic behavior of a 215 base pair DNA fragment containing a bFGF promoter are changed by

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[2] Abbreviations: bFGF: basic Fibroblast Growth Factor; PCR: Polymerase Chain Reaction; TTGE: Temporal Temperature Gradient Gel Glectrophoresis

attaching a c1amp. We show that this GC-clamp allows the detection of mutations within the bFGF gene that were previously indistinguishable by TTGE. With this approach, DNA fragments derived of human genomic DNA can be detected by silver staining of the gel, obviating the need for radioactive probes. These improvements extend the applicability of TTGE for the detection of polymorphisms and mutations in genomic DNA. We demonstrated that the use of GC-clamp in TTGE as a rapid and efficient method for detection of substitutions in the promoter fragment of bFGF gene.

Key words: Myopia, Polymerase Chain Reaction (PCR), Temporal Temperature Gradient Gel Electrophoresis (TTGE) and Sequencing.

Introduction:

DNA molecules can be separated by denaturing gels on the basis of their sequence and mobility of each fragment becomes a function of its linear length and mass-to-charge ratio. The mobility of a fragment drops abruptly when encounters a characteristic concentration in a denaturing gradient, and hence not constant as observed in conventional electrophoresis. This allows the separation of fragments with identical size, but differing sequences though by a single base. The single base substitution in specific regions of genomic DNA has been extremely useful for detection of mutations associated with human disease. The majority of base changes in a genome have been identified by the restriction fragment length polymorphism, which recognizes the DNA sequence variation caused by substitution either by a loss or gain of a restriction enzyme cleavage site. However, many single base changes do not alter a restriction enzyme cleavage site and so cannot be detected by the restriction fragment length polymorphism method. One alternative method that makes it possible to detect a base changes in a DNA fragment is temporal temperature gradient gel electrophoresis [1-4].

Temporal temperature gradient gel electrophoresis was first introduced by Yoshino, in 1991. The detection of mutation by TTGE is based on size dependent electrophoretic mobility of double stranded DNA fragmaent in a polyacrylamide gel containing a constant concentration of urea. During electrophoresis, temperature is increased gradually and uniformly. The result is a linear temperature gradient over the electrophoresis run. Thus, a denaturing environment is formed by the constant concentration of urea in the gel in combination with the temporal temperature gradient. In a denaturating environment double stranded DNA is subjected to increase in denaturant environment and will melt in descrete domains, referred to as "Melting Domains". The melting temperature (Tm) of these domains is sequence specific. When the melting temperature of the lowest domain is reached, the DNA will become partially melted reducing its mobility when compared to wild type DNA. The DNA containing mutations will encounter mobility shifts at different positions in a denaturating gel than the wild type [5-8].

However, TTGE will not separate DNA fragments differing by a base change in the highest temperature melting domain due to loss of sequence-dependent gel migration upon complete strand separation. Because of this limitation, it is estimated that on average, only 50% of all the possible single-base changes in DNA fragments between 50 base pairs (bp) and several hundred base pairs long can be detected by TTGE. We were able to overcome this problem by attachment of a high-temperature melting G+C-rich sequence, designated a GC-clamp [9, 10], into a original genomic sequence.

The polymerase chain reaction [11-13] uses two opposing oligonucleotides to amplify fragments of genomic DNA manyfold. The 50-bp GC-clamps can be attached to amplified genomic DNA fragments by using the PCR. Using the bFGF genomic DNA from humans with high myopia, we show that such GC-clamps allow the separation of single-base mutations

by TTGE that otherwise cannot be separated. The huge amplification of the genomic DNA fragments by the PCR also allows the signals to be detected by silver staining of the gel, making the use of radioactive probes unnecessary.

In this report we demonstrate the usefulness of TTGE analysis to detect possible variations in the promoter region of bFGF. An important aspect of this work is the use of the MELTMAP computer program to rationally design amplification primers. We analyzed 300 subjects (200 subjects and 100 control subjects) with high myopia, and found 1 polymorphism in promoter region.

Materials and Methods:

Subjects:

A total of 300 subjects (200 with high myopia of ≥ - 6D and 100 healthy control subjects with a spherical equivalent ranging from −0.5 D to +1.0 D) are recruited from local hospital (Ophthalmology unit) as well from our ophthalmologist's clinic. Informed consent was obtained from the study subjects after an explanation of the nature and possible consequences of the study. Criteria for selection included a history of onset of myopia in all affected subjects. Individuals were excluded if there was known ocular disease such as retinopathy, cataract or if they had a known genetic disease associated with myopia, such as stickler or Marfan syndrome. An ophthalmic examination of the participating subjects was performed by our ophthalmologist. Ophthalmic evaluation included measuring visual acquity, keratometry, retinoscopy, slit lamp examination of the anterior segment, fundus examination and measurement of axial length. Auto-refraction was taken and A- scan was done on both eyes. Subjects were encouraged to narrate all the details relevant to this study. This included age of subject, history of onset of myopia, any associated ocular complications

and information regarding close work. The study was approved by Research Ethics Committee. Informed consent in accordance with the Declaration of Helsinki was obtained from all participants or their parents and controls.

Polymerase chain reaction

Genomic DNA was extracted from venous blood samples using standard protocols. Primer pairs for individual exons were designed using the Primer-3 program. Amplifications were performed in 50 µl reaction volume containing 50-100 ng of genomic DNA, 400 nM of each primer, 250 µM of dNTPs, 2.5mM MgCl2, and 0.2 U Taq DNA polymerase in the standard PCR buffer provided by the manufacturer (Sigma Aldrich). The primer sequences used for amplification are shown in **Table 1**.

Table 1: PCR Primers for bFGF

Primer	Sequence (5' ---> 3')
Primer A [a]	GCATG GCCTT TTGAA ACCTA
Primer B [b]	CAGCG TCTCA CACAC TGAGG
Primer C [c]	CGCCGCCGCCGCCCG CAGCG TCTCA CACAC TGAGG
Primer D [d]	CGCCCGCCGCGCGCCGCGCCCGTCCCGCCGCCGCCCGCCGCCGCCGCCCG

[a] Primer A is the bFGF forward primer, Both Primer A and Primer B are complementary to the genomic DNA sequence to be amplified. [c] Primer C is extended with a 15-bp GC-rich linker sequence that is not complementary to the target sequence. [d] Primer D is a 50-bp G + C-rich primer and also contains this 15-bp linker sequence.

PCR amplification consisted of a denaturation step at 96 °C for 5 min followed by 20 cycles, each consisting of 96 °C for 45 s followed by 55 °C for 45 s and 72 °C for 1 min. Expected PCR product of 239 bp was generated successfully. The actual size of fragment is 15bp longer because of the linker sequence of Reverse Primer. A GC-clamped PCR product was generated by second round of PCR amplification. One microliter of PCR product was transferred to a second round of amplification with Forward Primer and GC-Primer using conditions as described

above for 35 cycles. PCR products were analyzed on 1.5% agarose gel and purified using purification kit or NaI.

Temporal Temperature Gradient Gel Electrophoresis (TTGE):

TTGE was performed on the D CodeTM mutation detection system and temperature range for TTGE for each PCR fragment was determined empirically with the aid of computer simulation using WinMeltTM (Bio-Rad Laboratories). PCR products were analyzed on 8% polyacrylamide (acrylamide:bis = 37.5:1) gels prepared in 50x Tris acetate-EDTA buffer (2mol/L Tris Base, 1 mol/L glacial acetic acid and 50mM EDTA, pH 8.0) containing 6 mol/L urea. Approximately 15µl purified PCR product and 5 µL of 2x gel loading dye (700 mL/L glycerol, 1 g/L bromphenol blue, 1 mL/L xylene cyanol) were applied to each lane of the gel. The electrophoresis was performed at 150V for 4h at an initial temperature of 68°C and a ramp rate of 3°C/h. The gels were stained in a silver stain.

Sequencing:

Samples that showed presence of heteroduplex bands were sent for sequencing to confirm the presence of sequence variations.

Sequence analysis:

Sequence results obtained in fasta and pdf formats were analysed using ClustalX version 2 software (Thompson JD et al, 1997 & Larkin MA et al, 2007) and by Chromas Pro version 1.49 beta 2 software for the detailed inspection of individual chromatograms.

Statistical analysis:

Genotypes were obtained by direct counting with subsequent calculation of allele frequencies. Statistical analysis was undertaken using the $\chi 2$ test and significance value (p). A p value of <0.05 was considered significant. Adherence to the

Hardy-Weinberg equilibrium constant was tested using the $\chi 2$ test with one degree of freedom. Odds ratio and confidence interval was also calculated.

Results:

Attachment of GC-Clamp for Screening:

The promoter region fragment of bFGF was analysed by a WinMelt software, a computer algorithm that uses the nucleotide sequence of a DNA fragment to predict melting behavior indicated that this DNA promoter fragment melts in three distinct melting domains (Fig 2A). The melting map, which is a plot of tm (the temperature at which each base pair in a molecule has a 50% chance of being helical or melted) versus the nucleotide sequence of the molecule predicted that the 5' half of the promoter melts as a single domain with a tm of =69°C (designated domain 1), while the 3' half of the promoter melts in two domain with a tm of 73°C (designated domain 2) and 70°C (designated domain 3) (Figure 2A). On the basis of melting predictions, promoter fragments carrying mutations in domain 3 probably should not separate by TTGE experiments, confirmed by running the samples on TTGE gel (Figure 2B).

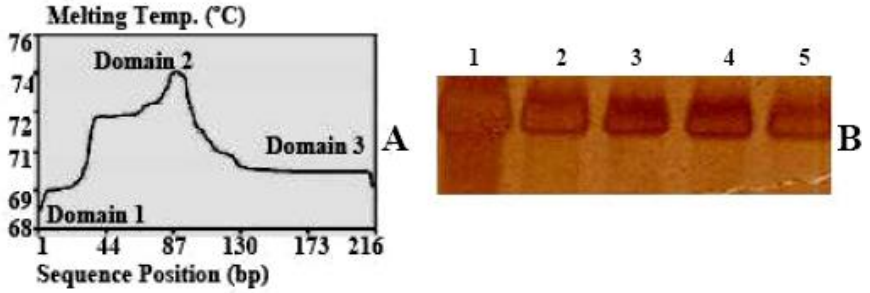

Figure 2: Theoretical melt maps and experimental TTGE analysis for *bFGF* promoter region. (A) Melt map of a 214 bp fragment without GC-clamp shows three stable temperature domains. (B) TTGE analysis of the 214 bp fragment without the GC-clamp. The mutation was not detected in the promoter region of the bFGF promoter fragment due to complete strand dissociation.

The attachment of a 50-bp GC-Clamp to 5' end results in ideal two melting domain profile, a high-temperature melting domain with a tm > 90°C designated as Domain 1 and a single flat lower melting with a tm 70°C designated as domain 2 (Fig 4A). The addition of the GC-clamp to the promoter fragment should allow the detection of mutations in domain 2 by TTGE (Fig 4B).

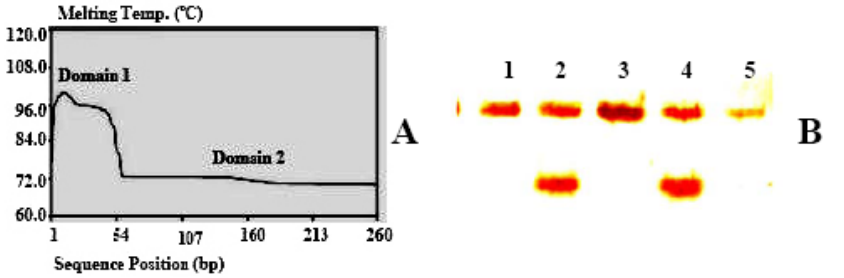

Figure 4: Theoretical melt maps and experimental TTGE analysis for GC clamped *bFGF* promoter fragment. (A) Melt map of a 264 bp fragment of GC-clamped bFGF promoter fragment shows three stable temperature domains. (B) TTGE analysis of the 264 bp fragment with detected mutation. Lane 1, 3 & 5 represents normal sample, whereas lane 2 and lane 4 represents as heterozygous mutation.

To test the feasibility of this approach, the amplification of these fragments was carried in two rounds of PCR with three primers (referred to as 1, 2, and 3). Primer 1 was a 20-mer primer and primer 2 was a 35-mer primer composed of a 35-bp sequence complementary to the respective gene sequences and the 15-bp linker sequence that amplified the non-clamped 239 bp fragment in initial round of DNA synthesis. Primer 3 is a 50-bp G + C-rich primer and also contains this 15-bp linker sequence, at the 3' end. In the next round of amplification, with Primer 2 and Primer 3, the entire 50 nucleotides were incorporated into the fragments, resulting in amplified promoter fragments with the GC-clamp attached at one end. The analysis of the generated DNA fragments on a 1.5%

agarose gel demonstrates that the second round of amplification yields a single DNA fragment of the expected size (Figure 3). The bFGF promoter fragment is 215 bp (Fig 1), un-clamped GC-clamped gene fragment is 239bp in size, whereas the GC-clamped gene fragment is 264 bp (Fig 3), indicating that the 50-bp GC-clamp is attached to this fragment.

Figure 1: Ethidium bromide staining of amplified DNA fragment size separated on a 1% (wt/vol) agarose gel. PCR products of a 214bp bFGF fragment amplified by Forward Primer and Reverse Primer.

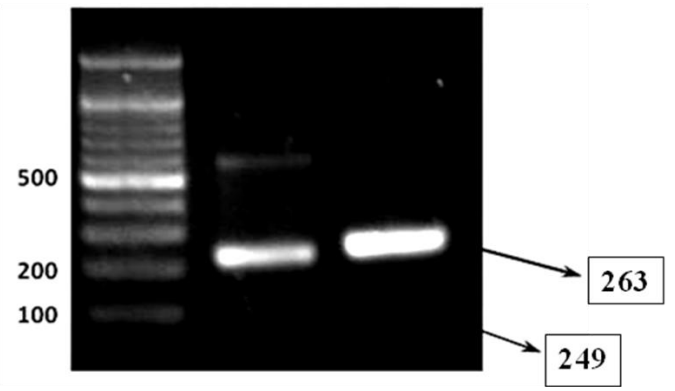

Figure 3: Ethidium bromide staining of amplified DNA fragment size separated on a 1% (wt/vol) agarose gel. PCR products of a 214bp bFGF fragment amplified by Forward Primer and Reverse Primer. The actual size of fragment is 15bp longer because of the linker sequence of Reverse Primer. GC-clamped PCR products of the *bFGF* fragments generated by two consecutive amplifications. The size marker is a 100-bp DNA ladder (FERMENTAS).

Analysis of Single-Base Substitutions in Human Genomic DNA:

In the melting map shown in Fig. 2A, the mutations lie in the highest temperature melting region of the fragment in the absence of a GC-clamp; therefore, mutations in this region should not be resolved by TTGE. However, melting calculations predict that attachment of a 50-bp GC-clamp to the 5' end of the fragment results in a single melting domain (with a tm of 70C) for the bFGF portion of the fragment, and a second domain with a tm of > 90C for the GC-clamp.

The PCR procedure was used with primers 1 and 2 or primers 2 and 3 to amplify unclamped and clamped GC rich promoter fragment. The resulting amplified DNA fragments were then subjected to electrophoresis in a TTGE gel. As shown in Fig. 2B, the un-clamped bFGF amplified with primers 1 and 2, did not separate from the normal-type fragment in the gel. However, when the same fragments were amplified with primers 2 and 3, the affected DNA fragments separated from the normal-type (Fig. 4B). The gel was stained with silver stain and examined by transillumination. This data indicate that the PCR procedure can be used to attach a 50-bp GC-clamp to DNA fragments and that the GC-clamp allows the detection of single-base substitutions present at positions in the fragments that otherwise would not be detected.

Mutations Identified:

The bFGF contains 4 exons which encodes a 175 amino acid protein. Sequencing of the promoter regions of *bFGF* which show heteroduplexes revealed one nucleotide (C>G) change. Genotype analysis of individual variants revealed the presence of only heterozygous genotypes (Fig. 5).

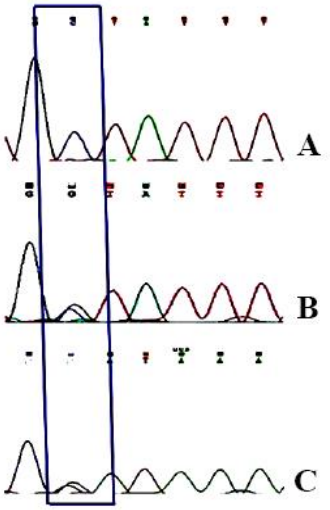

Figure 5. bFGF promoter sequence in an unaffected and affected individual: (A) Sequence chromatograms of an unaffected individual showing the wild type allele C. (B) Sequence chromatogram of an affected individual showing the variation G to C. (C) Sequence chromatogram of an affected individual using reverse primer. The position of the wild type nucleotide C and the variation G/C in an unaffected and affected individual is indicated in box.

Discussion:

By screening 300 myopic DNA samples for mutations with TTGE, we detected only one polymorphism. This approach is particularly applicable where the site of mutation or polymorphism is not known in advance and it is desirable to screen up to several hundred base pairs in a single test. The melting theory suggest that attachment of a GC-clamp to either end of almost all DNA fragments in the size range from 50 to several hundred base pairs improves the resolution of separation of most mutant DNA fragments by TTGE . The DNA sample is amplified by PCR, run on the TTGE gel and the results can be examined directly by silver staining. The strategy that we have devised requires the amplification of genomic DNA by PCR and single lane analysis on a gel. Hence, it is worthwhile to consider its application in clinical tests for

specific mutations.

When a DNA fragment consists of three melting domains (as depicted in Fig.2A) mutation detection is only possible in the lowest melting domain and is not possible in the higher (non-melted) domain. When this lowest melting domain, is on one of the ends of the fragment (Fig. 3A) a solution a stretch of G/C nucleotides is added to this end of the fragment through the short primer. This primer will increase the *T*m value of the lowest melting domain and thereby promote the establishment of the desired two-domain profile. To illustrate the result of adding G and/or C residues to the primer, we examined promoter region of the *bFGF* gene. To determine the melting behaviour of the fragment, the mutation was not detected in absence of GC clamp. The fragments without an added stretch of GC nucleotides appeared as a single sharp focused band after 4h electrophoresis at 150 V. The results can be interpreted either as the absence of mutations in this fragment or low sensitivity of TTGE to detect mutation. When the G/C nucleotides was attached to the end of the fragment, the theoretical desired two-domain profile was created and the mutation was resolved after 4–5 h electrophoresis at 150 V. Thus, based on computer calculations and practical experimentation, we propose that the attachment of a small stretch of G/C nucleotides to the short primer may improve mutation detection under these conditions.

Our results with bFGF [14] gene suggest that the combination of PCR and TTGE after computer analysis is a powerful and sensitive method for detection of nucleotide substitutions in genomic DNA. However, that the genetic polymorphisms in promoter region of *bFGF* did not play any important role in high myopia.

Competing Interests:

The authors declare that they have no competing interests.

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Author's Contributions:

Aeijaz Ul Noor: Preparation of manuscript, formulated and performed all the lab work.
Sabia Rashid: Provided High Myopia samples
Khurshid I Andrabi: Designed the work, edited the manuscript, co-ordinated the group and overall invigilator of the study.
All authors have read and approved the manuscript.

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