List of Publications


Awards received

Received ‘Young Scientist’ award for the year 2004 in the field of ‘Medical Genetics’ by Indian Society of Human Genetics for the paper ‘Status of some of the genes involved in viral clearance and host pathogenesis in chronic hepatitis B infection’.

Presentations made

Platform presentation made for the paper title ‘Status of some of the genes involved in viral clearance and host pathogenesis in chronic hepatitis B infection’ at 29th Annual Conference of Indian Society of Human Genetics (ISHG 2004) held in Bangalore.

Same paper was also presented at Biosparks 2004, 2nd Annual Research Festival, School of Life Sciences, JNU.
Novel variations in the signal peptide region of transforming growth factor β1 gene in patients with hepatitis: a brief report from India

V. Gupta,* R. Arora,* A. Saha,* A. Dhir,* P. Kar† & R. Bamezai*

Summary

Mutations in the signal peptide region, in general, are known to inhibit cleavage by signal peptidase or cause a defective secretion of the protein to extracellular location. A preliminary analysis of genotypic status of the signal peptide region of TGF-β1 in 53 viral hepatitis patients and 90 controls using SSCP and/or direct sequencing showed a significant difference in C/C-genotype frequency at +29 position (codon 10) between a range of viral hepatitis patients and controls (P = 0.009, OR = 3.15, CI = 1.29-7.678). It was observed that out of a heterogenous pool of hepatitis patients, this significant difference between patients and controls was contributed by those who were infected with HBV alone or HBV + HDV infection (P = 0.003, OR = 5.0, CI = 1.78-13.97). Two novel variations, +29T > G in two and 36–37ins(CTG) in one patient were detected. These were not detected in 90 control samples. It is proposed that the observed variations in signal peptide region could lead to a change in the expression levels of TGF-β1. Because TGF-β1 is an important candidate for liver apoptosis, immunomodulation and liver regeneration following viral infection, a variation in genotype affecting expression could affect an individual’s susceptibility to HBV related hepatitis.

Introduction

Virtually all prokaryotic and eukaryotic proteins that are destined for export from the cytoplasm are synthesized with N-terminal peptide extensions. These extensions, known as signal peptides, are essential for the initial membrane translocation step of export (Saier et al., 1989). The signal peptide earmarks a protein for transport; its physical properties dictate several interactions between the exported protein and the lipids and proteins of the export pathway. Mutations in human signal peptides have been correlated with defective secretion and a consequent pathological state in a few reported cases (Brennan et al., 1990; Daly et al., 1990; Arnold et al., 1990; Warsz et al., 1991; Racchi et al., 1993; Regis-Bailly et al., 1995; Hughes et al., 2000; Yamagishi et al., 2003; Watanabe et al., 2003; Christensen et al., 2004). An analysis therefore of the variation in the signal peptide region of a candidate gene with a possible involvement in a disease development could provide useful information about the role of the gene in the pathogenesis of the disease.

There have been reports of association of TGF-β1 in viral hepatitis. In hepatitis C, TGF-β1 C/C-genotype at +29 position (codon 10) has been shown to be associated with resistance to antiviral therapy (Vidigal et al., 2002) and variations at codon 10 and 25 associated with fibrotic progression in hepatitis C (Gewaltig et al., 2002). TGF-β1 is a pleiotropic cytokine with activities ranging from arrest of liver cell regeneration and induction of apoptosis in liver diseases (Lin & Chou, 1992; Oberhammer et al., 1992; Takiya et al., 1995; Cain & Freathy, 2001) to liver necroinflammation, fibrosis (Nagy et al., 1991; Marwaki et al., 1998) and immunomodulation (Chen et al., 1999; Mouri et al., 2002) in viral hepatitis. We have chosen TGF-β1 as a candidate gene to study its signal peptide region for polymorphisms and a possible association with a susceptibility to a range of viral hepatitis with different independent phenotypes related to liver as an organ.

Materials and methods

Study subjects

A total of 90 (31 females and 59 males in the age range of 21–53 years) healthy controls with no previous reports of liver-related disorders and 53 (21 females and 32 males in the age range of 23–60 years) patients with viral hepatitis (i.e., the patients who showed present or previous evidence of liver disease characterized by increased serum ALT levels and/or presence of other liver-related disorders like jaundice, cirrhosis, portal hypertension, hepatic encephalopathy and ascites) from India with similar ethnic backgrounds were chosen for the study. Out of these, five were positive for HAV (hepatitis A virus), 21 for HBV (hepatitis B virus), three for HCV (hepatitis C virus), five for coinfection with HBV and HDV (hepatitis delta virus) and 19
for HEV (hepatitis E virus). These cases were confirmed to belong to different categories on the basis of the specific ELISA (enzyme-linked immunosorbent assay) or PCR (polymerase chain reaction)-based assays used commercially. Blood samples of 5–10 mL were collected from the subjects with informed consent. Genomic DNA extraction was carried out with a standard protocol (Kunkel et al., 1977) used routinely in our laboratory.

**Polymorphisms studied**

Primers were designed to amplify partial 5'UTR and partial coding sequence containing full signal peptide region of TGF-β1. The forward primer: 5'-cac acc acc gct gtt cgc-3'(20-mer) and the reverse primer: 5'-agg cgc agg tgg gac agg at-3'(20-mer) amplified a region between −55 to +176 (as per HUGO nomenclature from the translation initiation site) and generated an amplicon of 231 bp.

**Genotyping**

A PCR-SSCP-based protocol (Hongyo et al., 1993) was carried out for the preliminary mutation analysis. Silver stained gels were assessed for the variant bands. The normal and variant DNA bands were eluted, amplified and cloned in pGEM-T cloning vector (Promega, USA). The cycle sequencing of clones was carried out using the ABI Prism BigDye Terminator cycle sequencing system (PE Applied Biosystems, Foster City, CA, USA). Direct sequencing of the PCR products of samples showing variant bands during SSCP analysis further confirmed the variations. Both forward and reverse primers were used for sequencing of PCR products.

**Statistical analysis**

The differences in genotype frequencies of TGF-β1 between patients with viral hepatitis and age and sex matched controls were assessed using Fisher's test. The relative risk was assessed by calculating the odds ratio. For all statistical tests, the significance level adopted was $P < 0.05$. The statistical analysis was carried out by using the software, spss Version 10 (SPSS, Chicago III, Illinois, USA). Powers of the associations were calculated for one-sided test using the software available at the Internet site http://www.pasteur-kh.org/~glaziou/samspizes/s3.html#ccp.

**Results and discussion**

The amplified region involving the partial 5'UTR and the complete signal peptide region of TGF-β1 was screened for the presence of variations in a range of viral hepatitis patients (as mentioned in Materials and methods section) and controls. The details of the genotypes in controls and patients are shown in Table 1. Controls as well as patients showed known polymorphisms (Cambien et al., 1996), $T > C$ at position +29 (codon10, Leu > Pro) and $G > C$ at position +74 (codon25, Arg > Pro) (Fig. 1). Statistical analysis revealed a significant increase in C/C-genotype frequency in viral hepatitis patients as compared to controls ($P = 0.009$, Odds ratio = 3.15, CI = 1.29–7.69) (Table 1). The power of association calculated was $82.56\%$. The change of Leu to Pro at codon 10 in leader peptide has been shown to be associated with increased expression (Dunning et al., 2003). The observation of an association of the high producer genotype of TGF-β1 is further supported by an observation of increased TGF-β1 serum levels in hepatitis (Murawaki et al., 1998). Because, TGF-β1 is known as an immunosuppressor (Chen et al., 2001; Mouri et al., 2002), it could help in the establishment of viral infection in cases of viral hepatitis. This preliminary observation possibly suggests that individuals with 'high producer'-TGF-β1 genotype may be more susceptible to viral hepatitis. Apparently, it was intriguing to find that a diverse group of liver disease phenotypes showed a significant correlation with the C/C-genotype at Codon-10, the signal peptide region, of TGF-β1 gene. It was pertinent therefore to examine the association separately in each group of hepatitis, which showed a significant difference only for patients infected with either HBV or HBV + HDV ($P = 0.003$, odds ratio = 5.00, CI = 1.78–13.9) (Table 1) with the power of association as 90.94%. The observation, however, needs to be established on a larger sample size in future.

A novel variation $T > G$ at position +29 (codon10, Leu > Arg, Acc.-No-AY576687) was observed in a hepatitis C and a hepatitis E patient (Fig. 1). Further, an insertion of (CTG), was observed in a hepatitis B patient at position +36 leading to a change in trinucleotide repeat region from (CTG)$_n$ to (CTG)$_{n+1}$, and an insertion of two Leu residues in the signal peptide region of TGF-β1 gene (Acc.-No-AY576688). The functional implications of these novel variations found in the present study are yet to be elucidated. However, there are some indirect evidences of the role of these variations. The variation, Leu > Arg, is known to result into the substitution of a hydrophobic amino acid by a charged residue in the hydrophobic core region of the signal peptide of TGF-β1. Further, decreasing the hydrophobicity of the hydrophobic region has

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Controls (90)</th>
<th>Viral hepatitis patients (53)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. (%)</td>
<td>No. (%)</td>
</tr>
<tr>
<td>+29, T/T</td>
<td>32 (35.6)</td>
<td>14 (26.4)</td>
</tr>
<tr>
<td>+29, T/C</td>
<td>49 (55.3)</td>
<td>21 (39.6)</td>
</tr>
<tr>
<td>+29, C/C</td>
<td>10 (11.1)</td>
<td>15 (28.3)</td>
</tr>
<tr>
<td>+29, C/G</td>
<td>0</td>
<td>2 (3.7)</td>
</tr>
<tr>
<td>36–37ins(C/T)</td>
<td>0</td>
<td>1 (1.8)</td>
</tr>
<tr>
<td>+74, G/G</td>
<td>77 (85.6)</td>
<td>43 (81.1)</td>
</tr>
<tr>
<td>+74, C/G</td>
<td>13 (14.4)</td>
<td>10 (18.9)</td>
</tr>
</tbody>
</table>

* Nomenclature used according to HUGO. * Novel variations; * Comparison with controls gave Fisher's exact $P$-value as 0.009, OR = 3.15, CI = 1.29–7.69; * Comparison with controls gave Fisher's exact $P$-value as 0.003, OR = 5, CI = 1.78–13.97.
Genotype at codon 10

<table>
<thead>
<tr>
<th>T/C</th>
<th>T/T</th>
<th>T/C</th>
<th>C/C</th>
<th>C/G</th>
<th>T/C</th>
</tr>
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Genotype at codon 25

<table>
<thead>
<tr>
<th>G/G</th>
<th>G/G</th>
<th>G/C</th>
<th>G/G</th>
<th>G/C</th>
<th>G/C</th>
</tr>
</thead>
</table>

(a)

* +29 T

* +29 G

* +74 G

* +29 C

* 36-37(CTG)2

* +74 C

(b)

Figure 1. Variations (*) in the signal peptide region of TGF-β1 gene as observed in (a) SSCP and (b) after sequencing, shown through the partial chromatograms.

Variations in the signal peptide region of TGF-β1 gene as observed in (a) SSCP and (b) after sequencing, shown through the partial chromatograms.

been suggested to nullify signal peptide capability and result in an export defect (Briggs & Gierasch, 1986; Storms & Rutishauser, 1998). Similar findings in other proteins such as humanin, the Leu9-Leu12 region has been shown to play a functional role as a hydrophobic core and substitution of Leu by Arg leads to defects in self-secretory activity of humanin (Yamagishi et al., 2003). The functional role of the insertion/mutation of two Leu resides in the signal peptide region observed in this study could further be supported by citing an example of an insertion/deletion polymorphism in the signal peptide of the human apolipoprotein B gene that predicts two apolipoprotein B signal peptides: one that encodes a peptide of 27 residues and the other a peptide of only 24 residues (Visvikis et al., 1990). This variation has been shown to confer a secretion defective phenotype in apoB (Talmud et al., 1996). In addition to causing a defective protein transport, the length variations in the signal peptide may affect the mRNA stability. Such a phenomenon has been shown in case of ermC gene where a mutation in the leader peptide region showed a change in mRNA stability, leading to a change in its translation efficiency (Hue & Bechhofer, 1991). It is quite likely that the variations observed in this study in the signal peptide region of the TGF-β1 gene could provide susceptibility to suffer from viral hepatitis at least in some cases, although the mechanism involved in the pathogenesis needs further elucidation.

Acknowledgements

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References


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Gel-Based Nonradioactive Single-Strand Conformational Polymorphism and Mutation Detection

Limitations and Solutions

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Summary

Single-strand conformation polymorphism (SSCP) for screening mutations/single-nucleotide polymorphisms (SNPs) is a simple, cost-effective technique, saving an expensive exercise of sequencing each and every PCR reaction product and assisting in choosing only the amplicons of interest with expected mutation. The principle of detection of small changes in DNA sequences is based on the changes in single-strand DNA conformations. The changes in electrophoretic mobility that SSCP detects are sequence-dependent. The limitations faced in SSCP range from the routine polyacrylamide gel electrophoresis (PAGE) problems to the problems of resolving mutant DNA bands. Both these problems could be solved by controlling PAGE conditions and by varying physical and environmental conditions such as pH, temperature, voltage, gel type and percentage, addition of additives or denaturants, and others. Despite much upgrading of the technology for mutation detection, SSCP continues to remain the method of choice to analyze mutations and SNPs in order to understand genomic variations, spontaneous and induced, and the genetic basis of diseases.

Key Words: SSCP (single-strand conformation polymorphism); PCR (polymerase chain reaction); PCR-SSCP; PAGE (polyacrylamide gel electrophoresis); polymorphism; chemical mutagenesis; SNP (single-nucleotide polymorphism); mutation detection.

1. Introduction

1.1. What is SSCP?

The study of genomic variations has become important in understanding human history and disease susceptibility. A variety of techniques are described in the literature that can detect single base mutations, insertions, and deletions, and all of these have one or the other advantage. In recent years, high-throughput methods like solid
phase minisequencing, detection of dissimilarly sized extension fragments by matrix-assisted laser description ionization-time of flight mass spectroscopy (MALDI-MS), mismatch cleavage detection, oligoarray hybridization, molecular beacon signaling, fluorescence monitoring of polymerase chain reaction (PCR), electronic dot-blot assay, and denaturation high-performance liquid chromatography are already in use (for review, see ref. 1). Nevertheless, one of the commonest, simplest, and also reasonably sensitive methods for rapid detection of gene mutations is that of single-strand conformation polymorphism (SSCP). It has been useful in finding associations with a number of diseases ranging from hemophilia A (2), Parkinson’s disease (3), hereditary spastic paraplegia (4) to schizophrenia (5) and cancer (6). Also, the technique has been useful in detecting induction of mutations in DNA/cDNA in toxicology studies. Chemically induced mutagenesis has been studied using the SSCP technique in rats (7–10), humans (11–13), and cell lines (14).

SSCP relies on the ability of a single (or multiple) nucleotide change(s) to alter the electrophoretic mobility of a single-strand DNA molecule under native (nondenaturing) conditions. Under nondenaturing conditions, most single-stranded DNA molecules assume one or more stable 3D conformations that depend on the nucleotide sequence. This change in a single nucleotide leads to a conformational change that is reflected in the electrophoretic mobility of the polymorphic sequence in comparison with the more common “wild-type” sequence (Figs. 1 and 2).

Mutations/polymorphisms occur within many different regions of a gene (promoter, 5' untranslated region [5' UTR], exons, introns, exon–intron junctions, 3' UTR, and others), and are screened by the following steps: (1) designing primers complementary to the region flanking the specified region; (2) subjecting the amplicons produced by PCR to SSCP analysis; (3) eluting the variant bands for characterization through sequencing; and (4) using these bands (DNA) for comparison in further analysis. A large number of samples can be prescreened for mutation(s) and single-nucleotide polymorphisms (SNPs).

The basic protocol of SSCP described by Orita in 1989 was based on PCR (15) or restriction fragment length polymorphism (16)-based detection of mutations in radiolabeled fragments on a sequencing apparatus. This basic protocol has undergone drastic changes since then, and nonradiolabeled (17) and fluorescence-based detection (18) has subsequently been used for SSCP analysis.

The advantages of the SSCP technique are that it is easy and cost-effective and requires low technical input. It can be used as a basic tool to identify variations before sequencing. If properly standardized, it can also be applied to find loss of heterozygosity (LOH) and microsatellite instability (MIS). Another advantage is the ease of separation, direct elution, and cloning of the variant band in both heterozygous and homozygous mutant situations. However, among its disadvantages are that it is at times difficult to standardize. SSCP results may be misleading if nonspecific bands are seen in some gels, a problem that can be overcome by repeating and obtaining reproducible results. Some mutations may not be resolved at all using SSCP. Also, it is a low-throughput technique: at the most, a worker can analyze 30–40 samples in a day.

In the literature, a variety of parameters have been found to improve the efficiency of the SSCP technique. Additives (such as sucrose, glycerol [19], urea [20,21],
formamide \[20\], and \[22\] polyethylene glycol \[23\], use of shorter length fragments for analysis \[24\], varying acrylamide percentage \[25\] and acrylamide to bis-acrylamide ratio \[20\], optimization of PCR conditions \[26\], use of acrylamide substitutes (such as the mutation detection enhancer \[MDE\] \[27\], PHAST SYSTEM \[28,29\], and agarose \[30\]), varying pH, current, and voltage \[31\], use of multitemperature SSCP \[32\], use of cold SSCPs \[33\], and use of combined SSCP/duplex analysis by capillary electrophoresis \[34\] have all been proposed to improve the resolution of single strands of the denatured double-stranded DNA.

1.2. Standardizing SSCP

It is important to remember that the optimum SSCP conditions for a particular fragment are determined by sequence, i.e., the length, base composition, and type of mutation to be studied. At least 90\% of single-base pair substitutions can be detected if the fragment length is within the optimal size range of 130–320 bp. Also, 80\% of single base pair substitutions can be detected if the PCR products are kept under 400 bp in length \[35\]. Low pH greatly increases the sensitivity of the mutation detection and allows the screening of fragments of 800 bp in length \[20\], although in our experience, amplicon length remains a limiting factor.

Factors important for standardization are:

1. **Gel temperature**: temperature is an important factor as it affects the stable DNA conformation as well as the mobility. As the temperature increases, the mobility also increases, and the total run time decreases. Low temperature is maintained either by running the gel in a cold room or by circulating cold running buffer in the gel apparatus. Running the same sample at different temperatures maximizes the chances of detecting mutations, as the variations missed under one temperature may be picked up at another. The same sequence can show two absolutely different profiles at two different temperatures. Temperature is one of the most important factors affecting the resolution of the single-strand DNA bands in the gels. The temperature range of 4°C (cold room) to 25–30°C (room temperature) shows the best differentiation of the single-stranded (ss)DNA bands. Migration of the bands is directly related to the increase in temperature.

2. **Gel pH**: generally, SSCP gels are run in 1X TBE solution, pH 8.3. However, TBE concentrations can be varied from 0.089 M to 134 M TBE. pH can also be lowered by adding 5–10\% glycerol. Low pH can enhance sensitivity for longer fragments \[20\]. pH can also be varied by the addition of sucrose, formamide, polyethylene glycol (PEG), and so on. Running buffer pH can also be lowered to 6.8.

3. **Voltage**: high voltage increases mobility and hence lowers the run time. However, the heat produced at higher voltage can lead to uneven heating of the gel matrix, which can result in band smiling or frowning, if the heat dissipation system is not effective.

4. **Gel matrix**: bands move more slowly and resolve better at higher acrylamide concentrations. The gel % is mainly determined according to the size of the sequence to be analyzed. Sometimes the use of alternative matrixes like MDE, gene amp, Hydrolinks, agarose, or PHAST can also increase the possibility of finding a mutation.

5. **Additives**: sucrose (10\%), urea, glycerol (5–10\%), PEG, ethylene glycol, or formamide may be added to gel. It has also been suggested that 5–10\% glycerol stabilizes the 3D DNA conformations, especially at higher temperature, although in our experiments we have not found any difference in resolution of bands. Glycerol enhances the mutation
Fig. 1. Schematic representation of hypothetical conformation differences adopted by the mutation-bearing ssDNA molecules (♀) in comparison with the wild-type (nonmutant) ssDNA molecule, thus resulting in the mobility shifts of the two strands of DNA. The heterozygous profile in some cases may only resolve into three bands instead of four.

Detection and interpretation of bands, as it reduces deformer formation and is known to affect pH in the TBE buffer system (19).

6. Another option is to label both strands. Using different colors for each strand allows the detection of the residual double-stranded molecules remaining after denaturation and also overlapping peaks in the two colors may confirm the presence of unresolved bands in the three-band situation. Dedicating a color to each fragment allows one to confirm the origin of extra peaks.

It is difficult to predict optimum conditions for a particular fragment even if its complete sequence is known. Optimum conditions have to be standardized by trial-
Snap chilling and gel analysis

Homozygous profile
(two DNA bands)

Heterozygous profile
(four DNA bands)

Fig. 1 (Continued)
Fig. 2. SSCP profile seen for two different amplicons. The conditions are: 15% acrylamide, 160 V, 1X TBE. The gels were run at room temperature for 22 h. (A) 231-bp-long fragment of TGF-β1 5' UTR showing variations in all the lanes. Lane 1, a three-band pattern heterozygous profile; lanes 3, 5, and 6, four-band pattern heterozygous profiles; lanes 2 and 4, homozygous profiles. (B) A different region: a 231-bp fragment for the TGF-β1 promoter. Lane 1, a four-band heterozygous profile; lanes 2 and 3, homozygous profiles.

and-error methods, trying out various permutations and combinations of the parameters influencing the outcome.

To start with, 200–325-bp DNA fragments can be run in 12–15% gels at 100–200 V. The total run time increases as the gel percentage and fragment size increases, and it decreases as the temperature and voltage decrease. Sometimes increasing the run time can also resolve some bands not resolved in less time. A major percentage of the fragments can be resolved this way. For the rest, various additives and different physical conditions (as already discussed) can be varied.

2. Materials

2.1. Genomic DNA Isolation

1. Lysis buffer: 0.32 M sucrose (autoclaved), 5 mM MgCl₂, 0.01 M Tris-HCl, pH 8.0, 1% Triton X-100 (e.g., Sigma, St. Louis, MO, or Qualigen, Mumbai, India). Store at room temperature.

2. Digestion buffer: 100 mM NaCl, 25 mM EDTA, 0.5% sodium dodecyl sulfate (SDS), 10 mM Tris-HCl, pH 8.0.

3. 20 mg/ml Proteinase K in solution.

4. Tris-HCl-saturated phenol, pH 8.0 (store at 4°C) and chloroform.

5. TE buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0.

6. Gel loading dye: 0.25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol use gloves and store at 4°C.
7. TBE buffer: 80 mM Tris-base, 40 mM boric acid, 2 mM EDTA. Make as 10X stock solution and store at room temperature.
8. Ethidium bromide: 10 mg/mL stock solution in autoclaved distilled water (use gloves and protect from direct light; store at 4°C).
9. 1% Agarose in 1X TBE buffer.

2.2. Polymerase Chain Reactions
Use gloves to handle.
1. Sterile 0.5-mL PCR tubes and microtips.
2. PCR kit (reaction buffer, enzyme, dNTP mix; store at -20°C).
3. Primers for the region to be studied (store at -20°C until use and after).
4. Mineral oil (store at room temperature).
5. Thermal Cycler (e.g., MJ Research, Miami, FL).

2.3. Polyacrylamide Gel Preparation and Gel Electrophoresis
1. Autoclaved distilled water.
2. Glass plates of 20 x 18 cm, spacers, and combs of 1-mm thickness each, with a well size of 3.5 mm.
3. Gel electrophoresis system.
4. Acrylamide: bis-acrylamide stock (29:1); store at 4°C.
5. 10% APS solution and TEMED:
6. TBE running buffer: see Subheading 2.1., item 7.
7. SSCP dye: 0.15 g Ficoll, 0.0025 g bromophenol blue, 0.0025 g xylene cyanol, 950 μL formamide, and 50 μL 1X TBE.
8. Power pack (e.g., Bio-Rad PAC 3000, Hercules, CA).

2.4. Silver Staining of SSCP Gel
1. Fixative: 10% ethanol and 1% acetic acid solution.
2. Staining solution: 0.1% silver nitrate solution.
3. Developing solution: 1.5% NaOH solution.
4. 0.75% Sodium carbonate and X% acetic acid solution.

2.5. PAGE Elution of DNA
1. Elution buffer preparation (for 25 mL): add 0.9365 g ammonium acetate and 0.0536 g magnesium acetate to 10 mL water and autoclave. Add to this 125 μL of 2 M EDTA and 250 μL of 10% SDS and make up the volume to 25 mL.

2.6. Agarose Purification of DNA Bands
1. Agarose (molecular biology grade; e.g., Sigma or Pronadisa, Rehovat, Israel).
2. Tris-HCl–saturated phenol, pH 8.0 (store at 4°C).
3. 3 M Sodium acetate, pH 5.2 (store at room temperature).
4. Absolute ethanol (e.g., Merck, Mumbia, India).

2.7. Ligation of DNA Bands for Sequencing
1. PCR product ligation kit (e.g., Promega, Madison, WI).
2. Insert DNA.
2.8. Transformation of Ligated Product and Screening of Positive Clones

1. Competent bacterial cells (e.g., DH5α).
2. Sterile culture plates.
3. Luria broth medium (e.g., Himedia, Mumbai, India).
4. Ampicillin (store at -20°C).

3. Methods (see Note 1)

3.1. Preparation of Template

1. Incubate blood (5 mL) with 4.5 mL lysis buffer for 30 min on ice. Break the clumps or clots with the help of a Pasteur pipet.
2. Centrifuge at 4°C for 20 min at 1100g.
3. Resuspend the pellet gently in 4.5 mL of digestion buffer.
4. Add Proteinase K solution to the homogenous cell suspension at a final concentration of 100 ng/mL.
5. Incubate the suspension at 65°C for 1 h, followed by overnight incubation at 37°C with gentle shaking.
6. Next day, deproteinate the cell suspension by extracting twice with an equal volume of phenol/chloroform and twice with chloroform, centrifuging at 2200-3500g for 20 min at room temperature.
7. Precipitate by adding 1/10th vol of 3 M sodium acetate and 2 vol of ethanol.
8. Spool DNA by binding on a glass rod or pick DNA in a broad-mouthed Pasteur pipet and wash with chilled 70% ethanol.
9. Dry the DNA and dissolve in TE.
10. Mix the genomic DNA with 1/6th vol of gel loading dye and load into wells of a 0.8% agarose gel. Run at low voltage of 40–45 volts. Visualize on a UV transilluminator. The high-quality genomic DNA should be seen as a band without shearing.
11. Quantitate by taking the optical density (OD) at 260 nm. Also compare the ratio of OD at 260 nm with that at 280 nm to check the quality; DNA samples having OD ratios > 1.5 are considered to have low protein contamination. Dilute the samples to the concentration of 25 ng/μL in autoclaved distilled water, and store at -20°C until analysis.

3.2. Polymerase Chain Reaction for Amplification of the Region to Be Studied

1. A 12.5 μL vol of PCR reaction for each sample includes: 6.25 pmol of each primer, 10 ng target DNA, 100 nM dNTPs, 1X PCR reaction buffer, 0.5 U Taq polymerase (e.g., Promega PCR kit).
2. PCR reaction is done for 30 cycles (denaturation at 94°C for 2 min, annealing at 55–65°C for 1 min, and extension at 72°C for 1 min) and final extension at 72°C for 10 min.
3. PCR products are checked by loading in 0.8% agarose gel at 120 V.

3.3. Preparation of Nondenaturing Polyacrylamide Gel (12–15%) for SSCP Analysis (see Notes 3–6, 8–10)

1. Clean the gel plates with detergent and rinse well with distilled water.
2. Wipe the plates with absolute ethanol.
3. Clean the spacers with absolute ethanol and place securely on the bottom plate. Secure the plates properly with clamps.
4. Clean the combs with absolute ethanol and keep it ready to be inserted between the two plates after pouring the gel mix.
5. Prepare the gel mix (12-15%) by adding a premade solution of acrylamide and bis-acrylamide at a ratio of 29:1, adding 10X TBE to make final concentration of 1X, 200 μL of 10% APS, and 50 μL of TEMED.
6. Mix all the constituents properly and pour between the two plates (see Notes 3-5).
7. Leave the assembly undisturbed until the gel becomes polymerized.
8. Prepare the samples for loading by taking 5 μL of PCR product, adding 4.5 μL 1X TBE, and 0.5 μL of loading dye.
9. Denature samples at 95°C for 5 min and chill immediately on ice for 5 min prior to loading.
10. Load all of the sample prepared into the appropriate wells of the non-denaturing gel, and electrophorese at a constant voltage at room temperature or at 4°C. (See Note 6.)

3.4. Silver Staining of SSCP Gel
1. After completion of the gel run, fix the gel in fixative for about 30 min.
2. Stain the gel in 0.1% silver nitrate solution for 15 min.
3. Wash the gel three times with autoclaved water.
4. Develop by adding 1.5% NaOH solution and wait till the bands appear.
5. Throw away the previous solution and add 0.75% sodium carbonate solution.
6. Photograph/scan the gels for record purposes.
7. For long-term storage: dry the gels in a gel dryer or store in a 1% acetic acid solution. (See Note 7.)

3.5. PAGE Elution of Bands Showing Variation (see Notes 2 and 3)
1. Cut out the band showing variation, and mince it well with the help of a microtip in an Eppendorf tube.
2. Add 400 μL of PAGE elution buffer and incubate at 37°C overnight with shaking.
3. Centrifuge at 16,000g 4°C for 1 min.
4. Take the supernatant and add 1/10th vol of sodium acetate, pH 5.2, followed by 1 mL ethanol.
5. Keep at -80°C for 1 h.
6. Centrifuge at 16,000g 4°C for 20 min.
7. Wash twice with 70% ethanol, followed by centrifugation at 16,000g, 4°C for 10 min.
8. Air-dry DNA and dissolve in 10 μL autoclaved and double-distilled water.
9. PCR-amplify the eluted fragment DNA.

3.6. Agarose Purification of DNA Bands
1. Run the PCR product to be eluted in a 0.8% agarose gel.
2. Excise the PCR-amplified product from the agarose gel and put it in a 1.5-mL Eppendorf tube.
3. Add 1 mL of saturated phenol, pH 8.0 into the tube, and freeze it at -80°C. Take out the frozen tube, and thaw it completely. Freeze-thaw the tube three times.
4. In the final step, take out the frozen tube and centrifuge at a high speed of 13,000g for 20 min at room temperature.
5. Take out the aqueous phase and transfer to a fresh tube. Add an equal volume of chloroform into the tube, mix, and centrifuge again at 13,000g for 10 min.
6. Transfer the aqueous phase and add 1/10th vol of 3 M sodium acetate, pH 5.2, and make up to 1 mL vol by absolute ethanol. Allow the DNA to precipitate for 30-45 min at -80°C.
7. Centrifuge at 16,000g at 4°C for 20 min for precipitating DNA.
8. Wash the DNA pellet with chilled 70% ethanol for 10 min at 4°C.
9. Dry the pellet and finally dissolve it in 10 μL of autoclaved distilled water.
10. Check the quality of DNA by running in an agarose gel and quantitate the amount for cloning.

3.7. Ligation of Gel-Eluted Product for Determination of Allele Sequence
1. Mix 2X reaction buffer, T-tailed vector (e.g., pGEM-T Promega ligation kit), enzyme (ligase), and DNA insert (gel-eluted product) in a 0.5-mL Eppendorf tube. Mix well and make up the volume to 10 μL (per instructions given in, e.g., the Promega PCR ligation kit).
2. Incubate at 16°C for 4–6 h, followed by an overnight incubation at 4°C.

3.8. Transformation of Ligated Product and Screening of Positive Clones
1. Carry out the transformation using competent cells (e.g., E.coli strains DH5α, XL-1).
2. Rescreen for positive clones through colony PCR using the same set of primers as was used for amplifying genomic DNA and at the same PCR conditions.
3. Sequence the resulting PCR product manually, or by an automated sequencer.

3.9. Recombinant Plasmid Isolation From Positive Clone for Further Use as Control in SSCP Gels (see Note 3d)
1. Inoculate 5 mL of fresh LB medium with a single colony and grow it at 37°C overnight with high-speed shaking.
2. Isolate the plasmid as in the instructions of a plasmid isolation kit (e.g., Sigma or Promega plasmid isolation kit).
3. PCR-amplify the plasmid and run it for all subsequent gels of the same amplicon.

4. Notes
1. General considerations while doing PCR-SSCP:
   a. Prepare and store the chemicals as mentioned, and handle with care.
   b. While isolating genomic DNA, handle with care to minimize the shearing of DNA.
   c. DNA is better purified with phenol/chloroform.
   d. For PCR reaction, use fresh sterile tips, tubes, and aseptic bench area.
   e. Filter the gel mix before adding APS and TEMED. APS should be freshly prepared.
   f. Any indecision in band shifts can be removed by repeating the PCR either fresh or by reamplifying the eluted variant band.
2. False bands seen: sometimes SSCP results may be misleading, and false bands may appear:
   a. At times, deformers bands appear in some lanes. Therefore, during sample preparation, ensure an equal quantity of DNA and uniform treatment of all samples. In order to confirm that the extra bands appearing in some lanes are the variant bands, these should be cut out, eluted, amplified, and run again in an SSCP gel (see Note 3).
   b. The presence of repetitive sequences causes primer slippage and hence the generation of extra bands during PCR amplification, a possible cause of false bands. To avoid this, try to keep PCR conditions as stringent as possible.
   c. The Taq polymerase used for PCR amplification can also introduce mutations. It is preferable to use high-proofreading-activity polymerase rather than other polymerases with high fidelity. PCR-SSCP should be repeated for all the samples showing variations, and their profiles should be confirmed before they are sequenced.
3. Appearance of extra bands in all the lanes: sometimes all the lanes show a number of bands, which makes the analysis difficult, as these may be confused with the mutant bands:

   a. Nonspecific bands generated during PCR, which could be owing to nonstringent conditions or primer dimers. This can be controlled by maintaining stringent PCR conditions by reducing target or salt concentration, or keeping annealing temperature high. Also, the primer concentration should be kept low. It is suggested to always check the PCR standardization results on acrylamide gel along with marker. An agarose gel is not sensitive enough to discriminate bands differing by only a few base pairs.

   b. Loading of high amounts of PCR products can also make visible the otherwise faint bands. Therefore, it would be good to optimize the amount of PCR sample to be used for loading depending on the PCR amplification, gel thickness, and well size. Sometimes reducing the PCR amount can also reduce the intensity of the extra bands.

   c. The PCR fragment adopts more than a single conformation or deformer. Adoption of more than one stable form is sequence-dependent, but varying physical conditions like pH, temperature, voltage, and so on may reduce the appearance of extra bands. This can also occur during temperature fluctuations. It is important to check for temperature fluctuations during the run. A deformer is less intense than the original band. Bands can be PAGE-eluted and PCR-amplified. Reamplified products should be electrophoresed along with the genomic control samples. A similar multiple band profile as earlier indicates the band is a deformer.

   d. Double-stranded DNA, as seen in gels of higher concentration: run two to three control samples for which the profile is known with the samples to be analyzed. Also, run a sample that has not been denatured to determine the location of double strands in the gel.

   e. Partially denatured double-stranded DNA takes a different conformation than single-stranded DNA and fully double-stranded DNA: denature the samples for at least 5 min, chill immediately, and load immediately.

4. Gel polymerization problems: sometimes the gel does not polymerize well:

   a. This could be caused by dirty plates or the use of chemicals of poor quality. It can be avoided by proper cleaning of plates using detergent, rinsing with autoclaved water followed by ethanol, wiping, and air-drying. Also, the gel solution should be made fresh and filtered, TEMED and APS should be of good quality, APS should be made fresh, and its concentration should be checked; this can also be increased (under the optimal range).

   b. Gel polymerization may take longer at low room temperatures. A table lamp or a heat convector should be used in such conditions.

5. Difficulty in resolving variant bands: sometimes variant bands do not resolve at all from the normal bands:

   a. At times, three bands appear under heterozygous conditions instead of the expected four-band profile, in which two bands take a similar conformation and hence show an identical mobility. In such cases, it is not important to resolve the three-band profile into a four-band profile.

   b. Sometimes the variant strand may not resolve from the normal band under a particular set of conditions, as both of them may have same mobility. In such cases, SSCP has to be standardized, as mentioned in Subheading 1.2.

   c. An inadequate run of the sample also may not resolve the variant bands completely.

6. Problems in sample loading: sometimes improper loading leads to formation of a diffused band pattern:
a. The presence of mineral oil may interfere with loading. Try using no mineral oil while loading.
b. The sample may not settle in the well; use Ficoll or glycerol in the denaturing dye.

7. Silver staining problems: sometimes the gel may not stain well, or ghost bands may appear in the middle section of the gel; instead of staining black, it remains clear and without stain:
   a. Improper fixing and improper staining. Gels should be fixed properly, and freshly made silver nitrate and developer should be used.
   b. Bands are not clearly visible after staining if the amplification during PCR is low. Therefore, PCR conditions and the amount loaded should be standardized by checking the efficiency of PCR amplification and its signal on agarose gel.
   c. If the signal is good enough to be detected on PAGE, then the problem is in the staining step. Reduce the number of intermediate washings. Shake the gel well, and mix all the solution with the gel for uniform staining.

8. Sample forms large streaks back toward the well:
   a. This may be owing to the dry run of the gel. Try to avoid the dry run, which might allow evaporation of the buffer or some leakage. Seal the space between the plates and the tank to avoid leakage from the upper tank to the lower tank.
   b. Try changing the gel percentage; an inappropriate percentage might lead to streak formation.

9. Uneven band patterns: sometimes bands are U-shaped, smeary, or diffused; they may show smiling or frowning:
   a. The bottom of the well may not be flat. This could be owing to improper polymerization of the gel. Take care during polymerization, as discussed in Note 1. Also, take out the combs only after the gel is properly polymerized. Always flush the well with buffer or water before loading in order to remove the residual gel solution, which might polymerize in the wells and lead to an uneven well bottom.
   b. Uneven thickness of the gels owing to a lack of compatibility between combs and spacers causes film formation in the wells and hence improper loading and band pattern. Therefore, spacers and combs should be of identical thickness to avoid uneven polymerization and film formation.
   c. At times the appearance of bubbles in the gel matrix or at the interface of the gel and the buffer interfere with the current flow and affect the quality of the run. The lanes which show the presence of air bubbles within the matrix could be avoided when running precious samples, and the bubbles at the interface could be removed by flushing with the help of a syringe and a needle.
   d. This pattern may occur because the temperature is higher at the center than at the edges as joule heating effects are more easily dissipated at the edges of the gel; since electrophoretic mobilities vary inversely with the viscosity of the solvent, DNA samples in the center of the gel migrate faster than sample near the edges. Therefore, it is important to maintain the temperature of the matrix.

10. Gel-to-gel variations are seen even under similar running conditions: sometimes two gels run at different times but in the similar conditions show varying profiles. Minor variations in gel percentage, temperature, buffer concentration, pH, and so on can give a dif-
ferent profile for the same amplicon in two different gels. The parameters influencing the outcome should be maintained as constant as possible. The following important points should be taken into account:

a. Genomic DNA should be quantitated carefully, and the amount used for PCR should be optimal.

b. The pH of the running buffer should not increase. The running buffer should not be reused again and again, although it can be used at least twice.

c. Try to maintain a constant temperature, voltage, and running time of the samples.

References


