MATERIALS AND METHODS
IV. Materials and Methods

IV.1 Sample Collection

201 patient and 201 control samples were collected from hospitals in the two places of interest, namely Chennai and Kanyakumari (KK) district of Tamil Nadu state of India (Fig. 11 – page 51). Hospitals were approached only after prior knowledge that they had all the necessary equipment for the diagnosis of primary open angle glaucoma.

IV.1.1 Hospitals involved

The research proposal was explained in detail to the chief doctors and permission to collect the blood samples was obtained. Then the criteria for the diagnosis and other details were discussed. The various hospitals involved in the study were:

In Chennai
- Government Ophthalmic Hospital, Egmore
- Kumaran Hospital, Kilpauk
- Rajan Eye Care Hospital, Valluvar Kottam
- Sri Ramachandra Medical College and Research Institute, Porur
- Prem’s Eye Clinic, Saidapet

In Kanyakumari district
- Bejan Singh Eye Hospital, Nagercoil
- Mission Hospital, Neyyoor

IV.1.2 Criteria for Selecting Patients

The criteria for the selection of patients was fixed based on previous literature (Morissette et al., 1995) and after discussions with the doctors involved in the study. The diagnostic criteria followed were:

1. The patients had been initiated onto therapy prior to their involvement in the study.
Fig. 11. Map of India showing the close up view of Tamil Nadu, with the two regions (Kanyakumari District and Chennai) involved in this study highlighted with the "a." symbol (a. http://mapsofindia.com/maps/india-politicdmap.htm; b. http://mapsofindia.com/maps/tamilnadu/tamilnadu-district.htm).
2. At least two of the following features were observed;
   a) An IOP of $\geq 22$ mmHg,
   b) An increased optic cup/disc ratio (where the normal value is 0.3, and 
glaucomatous cupping is $\geq 0.5$),
   c) Visual field abnormalities with no other apparent cause for the loss in 
vision.

Individuals with only an increased IOP or an increased cup/disc ratio were not 
included in the study. If there were any other apparent cause for the development of the 
glaucomatous characteristics i.e., as a secondary cause, then those patients were also 
excluded. Patients consistently with a normal IOP but with the optic cupping (increased 
cup/disc ratio) and VF abnormalities (with no other apparent cause for it) were also 
included in the study.

The two types of POAG namely juvenile onset and adult onset POAG were 
distinguished based on the onset of the disease before or after the age of 40 yrs, 
respectively. This value was fixed based on available literature (Morissette *et al.*, 1995) 
and after discussions with the opthalmologist.

The measurement of IOP was carried out using the tonometer. The type of 
tonometer varied in the different hospitals. The two types used in the various hospitals 
were the Shiotz tonometer and the applanation tonometer. The anterior chamber angle 
was visualized with the gonioscope. In majority of the hospitals, the Goldman single 
mirror goniolens was used, while in Kumaran hospital the sussman 4 mirror lens was 
used. The grading of the iridocorneal angle was based on the method of Shaffer 
(1960). The optic cup/disc ratio was checked with the direct ophthalmoscope while in 
a few of the hospitals the 90D lens was used. A fundoscopic camera was used to 
photograph the optic nerve head in some private hospitals. The VF tests were carried 
out by using the Bjerrum’s screen (manual method) in the Government and Mission 
hospitals. The other hospitals used either of the two automated perimeters, Octopus or 
Humphreys.
Controls: Equal number of age and sex matched controls were selected based on the following criteria:

1. Absence of the disease or any associated character in the individual.
2. Absence of a family history of the disease.
3. Absence of related eye/systemic disease which could play a role in the causation of glaucoma.

IV.1.3 Counseling

Once the selection criteria were defined and the patients selected, the patients were informed in detail about the disease. It was found that though the awareness about glaucoma is increasing at a fast rate, many patients were unaware of the real facts about the disease. Many patients especially those in private hospitals had some information about glaucoma. However, they were not aware of most of the important necessary details. The facts about glaucoma such as:

a) That glaucoma is an incurable but preventable disease was made clear to the patients i.e., the vision loss once occurred cannot be presently reverted to normal, however, the progression of the damage could be stopped or slowed drastically.

b) The fact that prevention of the disease was more feasible at the early stage of the disease was emphasized to the patients and regular check up was advised to them.

c) The fact that glaucoma is a hereditary disease and that a family history of the disease increased the risk factor by 7-10 times was made clear to the patients and hence relatives of probands were advised for regular check ups.

d) That the disease was mostly asymptomatic and that it affects the middle to older age groups at a high frequency was made clear and hence again regular check up after middle age was advised.

e) The fact that even after therapy was initiated, the progression of damage could continue and that this has to be monitored on a regular basis for better therapeutic regimens such as combinatorial drugs or to look for the need for surgical therapy was made clear.
The patients were also informed about the present research study. They were informed explicitly that this research work was in no way directly related to the therapy or cure of the disease in the patients to be involved in the study. They were informed that this particular study is just a preliminary step in the long run to decipher the genetics of glaucoma and was in no way directly helpful to the individual involved in the study. Those who were interested were explained about the experiments to be carried out. Others were generally informed about the sample volume and the mode of collection.

III.4 Consent form and Questionnaire

After explaining about all the necessary facts in detail, the individuals' consent signature was obtained on the consent form and then the questionnaire was filled. The consent form was in conformity with the Institute ethical committee's requirements (Appendix I). The consent form for SRMC & RI was designed considering the requirements of their ethical committee (Appendix II).

The questionnaire had two major components: the clinical details and the personal details (Appendix III).

Clinical details – This included all the diagnostic features namely; IOP levels, cup/disc ratio and visual field. These were obtained with the help of the concerned ophthalmologists. The other clinical details such as therapy were obtained directly from the ophthalmologist or from the patients' records. Other clinical details like presence of diabetes, BP were also obtained from the records.

Personal details – These included the details such as name, age, address, marital status (if married, consanguineous or not). Further, a detailed family history was also collected. This was represented in the form of a pedigree. This was essential to know, if there was a family history of the disease.

IV.1.5 Blood Collection

Once all the initial formalities were completed, peripheral venous blood from the branchial vein at the elbow joint was withdrawn using a 5 ml disposable syringe.
- The blood was transferred to the sterile 15 ml centrifuge tubes with a pinch of Na₂EDTA. The tube was inverted for a few times and was stored at 4°C till further processing.

In KK district, blood was collected by organizing a ‘camp’. First POAG cases were selected from the hospital records and then they were either personally informed about the research program by the doctor or they were informed by post. A duration of three days was fixed during which the patients were requested to arrive to the mentioned hospital. The clinical details were recorded and blood was withdrawn. The blood samples were stored at 4°C temporarily and transported in ice to Chennai and processed immediately.

**IV.2 DNA Isolation**

DNA was isolated from the blood samples using the ‘salting out’ protocol of Miller *et al.* (1988).

**Reagents Required:**

All the chemicals used for this study were obtained from reputed vendors like Qualigens, SRL, HiMedia and Sigma Chemical Co.

a) RBC lysis buffer

- **Ammonium chloride – NH₄Cl**
  - Dissolved 7.462 g in 800 ml of double distilled water.

- **Tris – hydroxymethyl amino methane**
  - Dissolved 2.06 g in 100 ml of double distilled water

Made upto 1 litre with autoclaved double distilled (dd) water. The solution was autoclaved @ 15 psi/15 minutes and stored at room temperature (RT).

b) 1 M Tris – hydroxymethyl amino methane

12.114 g of Tris was dissolved in 80 ml of dd water. The pH was adjusted to 8.0 with 1 N HCl and the final volume was made upto 100 ml with dd water. Autoclaved the solution @ 15 psi/15 min and stored at RT.
c) 1 M Sodium chloride (NaCl)

5.844 g of NaCl was dissolved and made upto 100 ml with dd water. Autoclaved at 15 psi/15 min. Stored at RT.

d) 0.5 M Na$_2$EDTA

18.612 g of Na$_2$EDTA was dissolved in dd water. The final volume was made upto 100 ml. Autoclaved @15 psi/15 min. Stored at RT.

e) WBC lysis solution

| 1 M Tris | 1 ml |
| 1 M NaCl | 40 ml |
| 0.5 M Na$_2$EDTA | 0.4 ml |

41.4 ml

Made upto 100 ml using autoclaved dd water and stored in autoclaved reagent bottles at RT.

f) 10% Sodium Dodecyl Sulphate (SDS)

2.5 g of SDS was dissolved in autoclaved dd water and the final volume was made upto 25 ml. Stored in an autoclaved reagent bottle at RT.

g) 6 M NaCl

35.064 g of NaCl was dissolved in autoclaved dd water and the final volume was made upto 100 ml. Stored at RT in autoclaved reagent bottles.

h) Ethyl alcohol (absolute)

This was commercially purchased from Tedia, USA and stored in the refrigerator.
i) 70% Ethyl alcohol

<table>
<thead>
<tr>
<th>Ethyl alcohol (absolute)</th>
<th>- 70 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoclaved double distilled water</td>
<td>- 30 ml</td>
</tr>
</tbody>
</table>

This was stored in autoclaved bottles at 4°C.

j) Tris –EDTA (TE) buffer

| Tris | - 121.14 mg |
| Na₂EDTA | - 37.2 mg |

121.14 mg of Tris and 37.2 mg of Na₂EDTA were dissolved in 80 ml of dd water. The pH was adjusted to 8.0 and the total volume was made upto 100 ml with dd water. The solution was autoclaved @15 psi/15 min and stored at RT. The centrifuge tubes, microcentrifuge tubes and micropipette tips were autoclaved @15 psi/15 min.

Methodology:

- The blood samples were centrifuged in a Remi-R8C- swing out rotor centrifuge at 3000 rpm for 30 min.
- The 'buffy coat' (WBC layer) was removed carefully using a plastic pasteur pipette and transferred to another 15 ml centrifuge tube.
- 10 ml (double the initial volume of blood) of RBC lysis buffer was added to the buffy coat and aspirated well with the help of the plastic pasteur pipette.
- Incubated in a 37°C water bath for 10 min.
- Centrifuged the suspension at 3000 rpm for 20 min.
- Discarded the supernatant and the RBC lysis step was repeated till a clear white pellet was obtained.
- To the pellet, 3 ml of WBC lysis solution was added and mixed thoroughly to get an even suspension.
- 0.2 ml of 10% SDS was added. The tube was gently invert mixed for 10 min.
- Incubated this mixture for 16±2 hours in a 37°C water bath.
- 1 ml of 6 M NaCl was added. The tube was gently inverted for several times in 20 seconds.
- Centrifuged at 3000 rpm for 20 min. The supernatant was carefully transferred devoid of the pelleted proteins to another sterile centrifuge tube with the help of a 1 ml Micropipette.
- Double the volume of chilled absolute ethanol was added to the supernatant. The solutions were gently inverted. DNA precipitated as ‘fluffy fibers’.
- The DNA was carefully transferred to a 1.5 ml microcentrifuge tube by spooling to a 1 ml micropipette tip.
- 70% ethanol was added to the DNA precipitate.
- Inverted gently couple of times. Centrifuged at 10,000 rpm for 10 min.
- Discarded the supernatant and added 1 ml of 70% ethanol and stored in -20°C till further use.
- The tubes were centrifuged at 10,000 rpm for 10 min.
- Supernatant was discarded. The tubes were left open at RT (away from direct air currents) to air dry the pellet.
- When the DNA pellet was semi dried, then 200 µl of TE was added and the tubes were left at RT overnight for the DNA to dissolve thoroughly.
- The tubes were labeled properly and stored at -20°C.

IV.3 Spectrophotometric Analysis of the DNA

This was carried out to find out the purity of the isolated DNA and also to quantify the DNA. The spectrophotometer used was the double beam model (Shimadzu UV-Vis 300).

Methodology:
- 975 µl dd water was taken in tubes and 25 µl of each sample was added to each tube and mixed well.
- Absorbance at λ260 nm and at λ280 nm for the samples was noted.
- The concentration of DNA was calculated using the formula
  \[ \text{Quantity in } \mu g = \text{OD}_{260} \times 50 \times \text{dilution factor} \]
[where 50 = standard quantity of double stranded (ds) DNA/1OD\(_{260}\)

dilution factor = 40 (25 \(\mu\)l made upto 1000 \(\mu\)l)]

Total quantity isolated = Conc in \(\mu\)g/\(\mu\)l \times Total DNA vol. (200 \(\mu\)l)

Based on the calculated concentration, working solutions of 0.1 \(\mu\)g/\(\mu\)l were prepared.

- The purity of the DNA was calculated by the formula

\[
\text{Purity} = \frac{\text{OD}_{260}}{\text{OD}_{280}}
\]

IV.4 Agarose Gel Electrophoresis

IV.4.1 Reagents Required:

a) TBE buffer Stock (normally stocks of 5 X were prepared)

5 X TBE Stock

- Tris - 54 g
- Boric Acid - 27.5 g
- 0.5 M EDTA - 20 ml (3.724 g)

Dissolved all the above, adjusted pH to 8.3 and made up the final solution to 1000 ml with dd water. Autoclaved at 15 psi /15 min and stored at RT.

b) 1 X TBE (working solution)

Diluted the 5 X stock solution with autoclaved dd water (1 ml stock + 4 ml autoclaved dd water).

c) Loading dye

- Bromophenol Blue - 50 mg
- Glycerol - 3 ml
- TE (pH 8.0) - 7 ml

Dissolved the above in autoclaved 15 ml centrifuge tubes and stored as 1.5 ml aliquots at 4 °C.
d) Ethidium bromide (EtBr)

EtBr - 10 mg
Autoclaved dd water - 1 ml

Dissolved the above and stored at 4°C in sterile 1.5 ml micro-centrifuge tubes.

IV.4.2 Gel Casting

0.7% agarose was apt for electrophoresis of human genomic DNA.

Composition:

30 ml Gel

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>210 mg</td>
</tr>
<tr>
<td>1X TBE</td>
<td>30 ml</td>
</tr>
</tbody>
</table>

Methodology:

- Platforms of desired size and appropriate combs (6 well/8 well/13 well and 18 well) were cleaned well and the two ends of the platform were sealed with a tape.
- Appropriate combs were fitted into the slots provided.
- Weighed the corresponding amount of agarose into the respective volume of 1 X TBE, melted the suspension in a microwave oven to get a clear solution.
- 3 µl of EtBr was added to 30 ml gel mix and swirl mixed.
- When the temperature of the gel mix reached ~50°C, the gel mix was poured onto the sealed platform with the appropriate comb.
- The gel was left undisturbed for ~ 45 minutes (to be polymerized fully).
- The comb(s) and sealing materials were carefully removed and the gel + platform were placed in the buffer tank filled with 1 X TBE buffer such that the gel was submerged completely.
- Samples were loaded in the ratio of 5 µl or 3 µl of sample + 2 or 3 µl dye accordingly.
- Electrophoresis was carried out normally for at least 1½ - 2 hours.
- The gels were viewed under the UV-transilluminator (GLW, Germany).
- Photographs if required were taken using Nikon coolpix 4500 digital camera.
IV.5 PCR

IV.5.1 Sequence of the gene

The sequence of the TIGR/MYOC gene was obtained from the web site, www.ncbi.nlm.nih.gov.

IV.5.2 Primer Design

Primers were designed for the region of interest (hot spot for mutations) namely the entire exon III and the initial part of the exon I (till aa 71). The criteria that were considered during primer design included:

- Primers were designed such that they amplified overlapping regions. This was to ensure that no sequence of the region of interest was missed from the screening procedure.
- Primers extended into the 3'UTR, the 5'UTR and the II intronic region for the same reason.
- Primers were designed such that the amplification products were < 300 bp. This is the upper range of fragment size for SSCP analysis (http://europium.csc.mrc.ac.uk/WebPages/Database/Methods/pcrpract.htm).

The primers designed were custom synthesized from Sigma Aldrich (genosys), Bangalore, India. Of the ten primers, primers 1 and 2 corresponded to exon I and the rest 8 primers were for exon III.

IV.5.3 Reconstitution of Primers

All ten primers were obtained in the lyophilized form. The primers were reconstituted as per our requirements.

Methodology:

- Centrifuged the tubes (with the lyophilized primers) briefly at 10,000 rpm for ~ 1 min.
- Added 100 µl of TE.
- Vortexed the tubes and kept at RT for 30 min.
- Centrifuged briefly @10,000 rpm, stored at -20°C.

The concentration of individual primers was spectrophotometrically determined.

Primer concentration and quantity:
- Spectrophotometer reading at \( \text{OD}_{260} \) was taken with 2 µl of primer stock diluted to 1 ml (i.e., dilution rate of 500) with autoclaved dd water.
- The concentration was calculated using the formula
  \[
  \text{Conc. in } \mu\text{g/ml} (X) = \text{OD}_{260} \times 33 \times \text{dilution factor} \quad [\text{where } 33 = \text{standard quantity of ssDNA in } \mu\text{g for } 1 \text{OD}_{260}, \text{and dilution factor } = 500].
  \]
- The stocks were diluted to get working concentration of 250 ng/µl.

The details of the primers along with sequences are furnished in the form of a table (Table 3 - page 63) (Figs. 12 a & b - page 64).

**IV.5.4 Reagents for PCR**

a) Primers – The working solutions were prepared as mentioned above and stored at -20°C.

b) dNTPs – These were purchased from Abgene, UK. The concentrations supplied were 10 mM/µl of each of the 4 dNTPs. Equal volumes of the four were mixed together to yield 2.5 mM of each dNTPs and stored at -20°C.

c) Taq DNA polymerase - These were also obtained from ABgene, UK. The concentrations supplied varied from 5 U/µl to 3 U/µl. 0.5 U/20 µl reaction was used.

d) Magnesium Chloride (MgCl₂) - This was diluted to get a working concentration of 1.5 mM in the PCR mixture.
Table 3. Details of the primers including primer name, sequence, binding site and expected amplicon size for each primer set.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
<th>Forward/Reverse</th>
<th>Binding Site #</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>RR1</td>
<td>5'AgAgCTTTCCAgAgAaAg 3' - 18 mer</td>
<td>Forward</td>
<td>-36 to -19 (5'UTR)</td>
<td>248 bp</td>
</tr>
<tr>
<td>RR2</td>
<td>5' ATgACTgACATggCCTgg 3' - 18 mer</td>
<td>Reverse</td>
<td>c.195 to c.212 (exon I)</td>
<td></td>
</tr>
<tr>
<td>RR3</td>
<td>5' TggATTAAGTggTgCTTC 3' - 18 mer</td>
<td>Forward</td>
<td>c.731-40 to c.731-23 (Intron II)</td>
<td></td>
</tr>
<tr>
<td>RR4</td>
<td>5' TggCTATgAggTCATAC 3' - 18 mer</td>
<td>Reverse</td>
<td>c.900 to c.917 (exon III)</td>
<td>227 bp</td>
</tr>
<tr>
<td>RR5</td>
<td>5' ggATgTCgCgCCAggTTT 3' - 17 mer</td>
<td>Forward</td>
<td>c.879 to c.895 (exon III)</td>
<td>260 bp</td>
</tr>
<tr>
<td>RR6</td>
<td>5' CAATgTCgTGgTAGCCAC 3' - 18 mer</td>
<td>Reverse</td>
<td>c.1121 to c.1138 (exon III)</td>
<td></td>
</tr>
<tr>
<td>RR7</td>
<td>5' TggCTACCACgAgACAgT 3' - 18 mer</td>
<td>Forward</td>
<td>c.1089 to c.1106 (exon III)</td>
<td>243 bp</td>
</tr>
<tr>
<td>RR8</td>
<td>5' gAggTgTAgCTgCTgAC 3' - 17 mer</td>
<td>Reverse</td>
<td>c.1315 to c.1331 (exon III)</td>
<td></td>
</tr>
<tr>
<td>RR9</td>
<td>5' CCTTCATCATCTgTggCA 3' - 18 mer</td>
<td>Forward</td>
<td>c.1286 to c.1303 (exon III)</td>
<td>248 bp</td>
</tr>
<tr>
<td>RR10</td>
<td>5' gTACAgCTTggAggCTT 3' - 17 mer</td>
<td>Reverse</td>
<td>*5 to *21 (3'UTR)</td>
<td></td>
</tr>
</tbody>
</table>

# Numbering of nucleotides to represent the primers’ binding sites was followed as per the nomenclature of the Human Genome Variation Society (HGVS) (http://www.hgvs.org/mutnomen/recs-DNA.html; http://www.hgvs.org/mutnomen/recs.html#general).
Fig. 12a. TIGR gene structure and the “hot spot” region that was screened with the respective primers as represented by the arrowheads. The primers are represented by their corresponding numbers on arrowheads.

CTGAGATGCCAGCTGTCCAGCTGCTGCTTCTGGCCTGCCTGGTGTGGGATGTGGGGGCCAGG
ACAGCTCAGCTCAGGAAGGCCAATGACCAGAGTGGCCGATGCCAGTATACCmCAGTGTGG
CCACTCCCAATGAAATCCAGTGGCCCAGAGCAAGAG

Fig. 12b. The sequence of the ‘Hot Spot’ of the TIGR gene with the primers differentially highlighted.
e) Template - Working solution of the templates (DNA samples) were prepared at the concentration of 0.1 μg/μl and this amount was used per 20 μl reaction.

IV.5.5 Methodology

The various components of a PCR mixture and the sequential order of addition are shown in the table 4.

Table 4. Components of a PCR reaction, their respective volumes and order of addition.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Component of the Reaction mixture</th>
<th>Volume to be added (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>dd Water</td>
<td>12.3</td>
</tr>
<tr>
<td>2.</td>
<td>10 X PCR Buffer with 15 mM MgCl₂</td>
<td>2.0</td>
</tr>
<tr>
<td>3.</td>
<td>2.5 mM dNTPs</td>
<td>0.8</td>
</tr>
<tr>
<td>4.</td>
<td>Primer (forward)- 250 ng/μl</td>
<td>0.4</td>
</tr>
<tr>
<td>5.</td>
<td>Primer (reverse)- 250 ng/μl</td>
<td>0.4</td>
</tr>
<tr>
<td>6.</td>
<td>Taq DNA Polymerase (5 U/μl)</td>
<td>0.1</td>
</tr>
</tbody>
</table>

| Total Volume without template | 16 |
| Total Volume - 0.1 μg/μl      | 4  |

Total Volume

20

All the mixing of PCR reagents was carried out on ice and centrifuged briefly at 4 °C when required.

Once the conditions for the different primer sets were standardized, the reactions were carried out for all the samples.
The typical amplification cycle is represented as below:

1. Initial Denaturation @ 94°C for 1 minute \\
   ↓
2. Denaturation @ 93°C for 30 seconds \\
   ↓
3. Annealing @ X°C for 30 seconds \\
   ↓
4. Extension @ 72°C for 30 seconds \\
   ↓
5. Repeat steps 2 to 4, 29 times \\
   ↓
6. Final extension @ 72°C for 2 minutes \\
   ↓
7. Hold the tubes at 4°C

The PCR amplification conditions for the different primer sets were the same except the annealing temperature (Ta).

**Primer set 1, 2 (RR1 and RR2)**

For this primer set, the annealing temperature (Ta) of 64°C was seen to be best suited for the amplification and all the samples were amplified at this Ta. Each time, a 2% agarose gel was run to check for the presence of the amplified product.

**Primer set 3, 4 (RR3 and RR4)**

For this primer set, the Ta of 58°C was seen to be best suited and all the samples were amplified at this Ta. The amplicons were checked each time by electrophoresing on a 2% agarose gel.

**Primer set 5, 6 (RR5 and RR6)**

For this primer set, the Ta of 60°C was seen to be best suited and all the samples were amplified at this Ta. A 2% agarose gel was run each time to check for
the presence of the required amplicons.

**Primer set 7, 8 (RR7 and RR8)**

For this primer set, the Ta of 62°C was seen to be best suited and all the samples were amplified at this Ta. The amplicons were checked each time by electrophoresing on a 2% agarose gel.

**Primer set 9, 10 (RR9 and RR10)**

For this primer set, the Ta of 64°C was seen to be best suited for amplification and all the samples were amplified at this Ta. Each time, a 2% agarose gel was run to check for the presence of the required amplicons.

**IV.6 Single Strand Conformation Polymorphism (SCCP) Analysis**

The PCR-SSCP methodology is an ideal technique to detect mutations at a high level of sensitivity (as high as 90%) in any fragment of DNA of < 300 bp (http://europium.csc.mrc.ac.uk/WebPages/Database/Methods/pcrpract.htm). The major advantage of this technique is that compared to the numerous methodologies of DNA mutation detection, it is the easiest and the cheapest way to detect even unknown mutations. The technique involves three steps:

- Denaturing of the amplicons.
- Native PAGE (Polyacrylamide gel electrophoresis) to analyse the denatured DNA.
- Silver staining to detect the amplicons.

The first step was achieved by keeping the samples in boiling water for 5 min. and plunging the tube into ice flakes. The denatured amplicons were electrophoresed on polyacrylamide gel (PAG) for the separation of the two single strands. Silver staining identified the position of the single strands.

A composite gel i.e., acrylamide – N,N’-methylene bisacrylamide and agarose was seen to be best suited to analyze the amplicons effectively in our laboratory. This was standardized at our laboratory using the protocol of Peng et al. (1995).
IV.6.1 Reagents required

a) Acrylamide; N,N'-methylene bisacrylamide

The quantity varied with the percentage of the gel. The percentage of the gel varied for each primer set. The ratio of acrylamide: N,N'-methylene bisacrylamide was always in the ratio of 39:1.

b) Agarose

0.1% or 0.27% was used for the analysis of the different amplicons as required.

c) 10% Ammonium per sulphate (APS) (w/v)

1 g of APS was weighed and dissolved in 10 ml of autoclaved dd water. Stored in the refrigerator.

d) N,N,N',N' tetramethylethylenediamine (TEMED)

Purchased commercially from Sigma Chemical Co., Bangalore, India and stored at 4°C.

e) Sample loading dye

0.1% Bromophenol blue
0.1% Xylene cyanol
90% formamide
20 mM Na$_2$EDTA

Weighed 10 mg of BPB and 10 mg of xylene cyanol and added to 9 ml of formamide in a sterile centrifuge tube. Then 74.448 mg of Na$_2$EDTA was added then made up to 10 ml with dd water, stored at 4°C.

f) Running buffer (0.5 X TBE)

1 X TBE was mixed at 1:1 (v/v) ratio with autoclaved dd water and used as running buffer for the SSCP analysis.
g) 2.5% Glycerol

Glycerol may be added especially when electrophoresing at RT (> 25°C). For a 30 ml gel, 0.75 ml was added. No vast difference in resolution of bands was observed in gels without glycerol and with glycerol.

IV.6.2 Methodology

- Required amount of acrylamide and N,N'-methylene bisacrylamide were weighed and dissolved in required amount of water (depending on the gel percentage and total gel volume).
- 2.5% Glycerol was also added to the acrylamide–N,N'-methylene bisacrylamide solution and mixed well.
- Agarose (of the required percentage) was prepared by melting in 1 X TBE
- On cooling, both the agarose gel mix and the acrylamide solutions were mixed.
- 260 μl of 10% APS was added for a 30 ml gel volume. 13.1 μl TEMED was added to the 30 ml gel volume and mixed well.
- The mixture was immediately poured between the plates and comb (1 mm) was inserted and left undisturbed for ~1 hour.
- Clamps and lower spacer were removed and the gel + plates were clamped to the electrophoresis unit.
- Comb was removed carefully and then the wells and the lower portion of the gel (in contact with the buffer) were cleaned well with the help of a syringe, to remove the air bubbles and the adhering vaseline/grease/unpolymerised acrylamide.
- A pre-run @150 V for 45 minutes was performed.
- 16 μl of dd water, 2 μl of 10x loading dye, 2 μl of PCR product were mixed and placed in boiling water for 5 min.
- After denaturation, the tubes were immediately plunged into ice (to prevent renaturation). The samples were then loaded onto the gel.
- Non-denatured DNA sample (17 μl of double distilled water + 2 μl of loading dye + 1 μl of PCR product) was prepared fresh just prior to loading.
- A high initial voltage was given, then the voltage was reduced according to the primer set being analysed.
- The gels were electrophoresed for 13 – 14 hrs.
- The gels were carefully removed after removing the glass plate.
- They were stained using silver nitrate.

The silver staining techniques followed was the modified protocol of that mentioned by Wallace (1997).

**IV.6.3 Silver Staining**

Reagents required:

a) **Solution A**

   Acetic acid – 10%
   Ethanol – 0.5%

   20 ml of acetic acid + 1 ml of ethanol was made up to 200 ml using dd water. Prepared fresh before use.

b) 0.1% Silver nitrate (AgNO₃) (solution B)

   Weighed 200 mg and dissolved in 200 ml of dd water. This solution was reused at least three times.

c) 1.5% Sodium hydroxide (NaOH) + 0.15% formaldehyde (solution C)

   3.0 g of NaOH was weighed and dissolved in 200 ml of dd water. The formaldehyde (0.30 ml) was added just prior to use.

d) 0.75% Sodium carbonate Na₂CO₃ (solution D)

   1.5 g was dissolved in 200 ml of dd water.

Methodology:

- Placed the gel in solution A for 3 min on an orbital shaker.
- Replaced the old solution with fresh solution A and kept for three more min.
Then solution B was added and left for ~12 min. The time was increased when silver stain was reused.

- Then rinsed twice for ~20 sec each with dd water.
- Solution C was added and the gel was left to develop for about 5-7 min.
- Stopped the development using solution D. The gels were photographed under white light transillumination and they were left in solution D or dried and stored.

All the steps from the fixative stage were carried out in the dark (silver nitrate is light sensitive). Care was taken to wear gloves while handling gels for silver staining (as the reduction of silver to metallic silver at the place of contact lead to black spots on the gel). Similarly the quality of dd water used was ensured to be free of salt as that would lead to rapid reduction of the silver nitrate and result in blackening of the gel.

The protocol of SSCP varied for the different amplicons. The composite gel percentage and the run time (electrophoresis time) varied for amplicons of each primer set. Though the difference was not too drastic but it was essential for the good resolution of the two single strands of amplicons of each primer set.

**IV.6.4 SSCP analysis of amplicons of primer set RR1 and RR2**

The composite gel used was 8.7% acrylamide and 0.1% agarose. Glycerol was also added. The composition is depicted in the following table;

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume/Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>2.5467 g</td>
</tr>
<tr>
<td>N,N' -methylene bisacrylamide</td>
<td>0.0653 g</td>
</tr>
<tr>
<td>dd water</td>
<td>13.977 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.750 ml</td>
</tr>
<tr>
<td>Total acrylamide Volume</td>
<td>14.727 ml</td>
</tr>
<tr>
<td>Agarose</td>
<td>30 mg</td>
</tr>
<tr>
<td>1X TBE</td>
<td>15.00 ml</td>
</tr>
<tr>
<td>10 % Ammonium persulphate</td>
<td>0.260 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.013 ml</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>30.00 ml</strong></td>
</tr>
</tbody>
</table>
Running conditions:

150 V – 1 hr; 100 V – 1 hr; 50 V – ~13 hrs.

SSCP analysis of amplicons of primer set RR3 and RR4

The composite gel used was 9% acrylamide and 0.1% agarose. The composition is depicted in the following table:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume/Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>2.632 g</td>
</tr>
<tr>
<td>N,N’-methylene bisacrylamide</td>
<td>0.067 g</td>
</tr>
<tr>
<td>dd water</td>
<td>13.977 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.750 ml</td>
</tr>
<tr>
<td>Total acrylamide Volume</td>
<td>14.727 ml</td>
</tr>
<tr>
<td>Agarose</td>
<td>30 mg</td>
</tr>
<tr>
<td>1X TBE</td>
<td>15.00 ml</td>
</tr>
<tr>
<td>10% Ammonium persulphate</td>
<td>0.260 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.013 ml</td>
</tr>
<tr>
<td>Total Volume</td>
<td>= 30.00 ml</td>
</tr>
</tbody>
</table>

Running conditions:

150 V – 45 min; 100 V – 1 hr; 50 V – 10- 11 hrs; 100 V – 1 hr.

SSCP analysis of amplicons of Primer set RR5 and RR6

The composite gel used was 8.7% acrylamide and 0.27% agarose. The composition is depicted in the following table:
### Component | Volume/Quantity
--- | ---
Acrylamide | 2.5467 g
N,N'-methylene bisacrylamide | 0.0653 g
dd water | 13.977 ml
Glycerol | 0.750 ml

**Total acrylamide Volume**
14.727 ml

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume/Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>81 mg</td>
</tr>
<tr>
<td>1X TBE</td>
<td>15.00 ml</td>
</tr>
<tr>
<td>10 % Ammonium persulphate</td>
<td>0.260 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.013 ml</td>
</tr>
</tbody>
</table>

**Total Volume** = 30.00 ml

Running conditions:

150 V – 1 hr; 100 V – 2½ hrs; 40 V – 12 hrs; 100 V – 1 hr.

**SSCP analysis of amplicons of Primer set RR7 and RR8**

The composite gel used was 9% acrylamide and 0.1% agarose which was the same as that used for the amplicons of the primer set RR3 & RR4.

Running conditions:

150 V – 1 hr; 100 V – 1 hr; 35 V – 15 hrs; 100 V – 30 min.

**SSCP analysis of amplicons of primer set RR 9 and RR10**

The composite gel used was 8.7% acrylamide and 0.27% agarose. The composition was same as that used for the amplicons of the primer set RR 5 & 6

Running conditions:

150 V – 1 hr; 100 V – 3½ hrs; 50 V – 13 hrs.

**SSCP analysis for confirmation of deviant samples**

A total of ten amplicons (of three different primer sets) showed a consistent deviant pattern. The samples with mobility shift were analyzed at least twice more. Further, the samples were reamplified from a fresh working solution of template and
also analyzed by SSCP to confirm the deviant banding pattern. These samples that consistently showed a deviant pattern were then column/spin filter purified for sequencing.

IV.7 PCR purification

The samples with a consistent deviant banding pattern were reamplified. The samples were from three primer sets and from each of these primer sets one control sample was also reamplified. Thus a total of 13 PCR products were purified. The PCR purification was carried out using the PCR purification kit from ABgene, UK.

IV.8 Sequencing

The purified samples were sent for sequencing to a commercial sequencing center namely, Genotypic Technology Pvt. Ltd., Bangalore. Sequencing was carried out using Dye termination chemistry and read using 96 capillary 3730x1 DNA analyzer, Applied Biosystems, USA.

IV.9 Bioinformatics Analysis

The sequences obtained from the above samples were checked using Basic Local Alignment Search Tool (BLAST) to see if the sequence actually corresponded to the gene/region of the gene, of interest. Both the nucleotide and protein sequence (derived from the DNA sequence) were searched.

Methodology:
- The web site www.ncbi.nlm.nih.gov was opened.
- BLAST link was clicked on the home page.
- The nucleotide blast (nBLAST) was opened.
- The query sequences were pasted in to the required box one at a time.
- Then the query was searched using BLAST.

Then the protein sequence was obtained from the DNA sequence by translating it into the corresponding amino acid sequence in the correct reading frame using the Software tool BioEdit. The protein sequence was searched in BLAST using a similar methodology but instead of nucleotide blast, protein blast (pBLAST) was given.
BLAST not only confirmed that the sequences were the required sequences but also showed us the regions of non-homology thereby indicating variants.

Motifs/domains/sites for phosphorylation, glycosylation were predicted using the tools PROSITE (http://kr.expasy.org/prosite/) and PATTINPROT (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_pattinprot.html). The secondary structures of the 6 mutant amino acid sequences were predicted using at least two different tools namely, GOR (Garnier, Osguthorpe and Robson) secondary structure prediction (http://molbiol.soton.ac.uk/compute/GOR.html) and Chou-Fasman secondary structure prediction (http://fasta.bioch.virginia.edufasta/chofas.htm).