2.1 Subjects and Samples

2.1.1. Prostate cancer patients

The present case-control study comprised of ninety-five histologically confirmed prostate cancer patients from South India. The patients were from the Urology department of Sri Ramachandra Medical College and Research Institute, Chennai. The age of prostate cancer patients ranged from 44 to 91 years with mean age of 67.1 years. Relevant clinical and pathological data were collected from all the patients. The PSA levels of the patients at diagnosis were recorded. The PSA ranged from 4ng/ml to 150 ng/ml with a mean of 49 ng/ml. Pathological grading of the tumors represented as Gleason scores (GS) (23) were obtained by the histopathological examination. The pathologic grades were classified into three groups: well differentiated (GS 2-4), moderately differentiated (GS 5-6) and poorly differentiated (GS 7-10). The well and moderately differentiated groups were considered as low grade and thus patients were stratified as low grade if their Gleason scores were less than 7 and high grade if their Gleason scores were greater than or equal to 7. The GS was less than 7 in 50 patients and greater than or equal to 7 in 45 patients.

All the patients underwent androgen ablation therapy. Of the 95 patients, 28 of them had relapse of cancer after androgen ablation therapy, while the remaining 67 patients had just begun therapy. Among the 28 patients with relapse of cancer 18 had GS ≥ 7 while 10 had GS < 7. Their PSA levels were ≥ 49 ng/ml

2.1.2. Control subjects

The study comprised of 120 age-matched male control subjects. Among the 120 controls, 80 were healthy, unrelated individuals with normal serum PSA levels (≤ 4ng/ml), digital rectal examination showing no abnormality and with no history of cancer and 40 subjects had asymptomatic benign prostatic hyperplasia (BPH) with normal serum PSA levels (≤ 4ng/ml) and with no history of cancer. Age of the BPH patients ranged from 55 to 77 years with mean of 65.5 years and that of the normal healthy controls ranged between 50 to 81 years with mean of 66.5 years.
The principal characteristics of the subjects have been tabulated (Table 2.1). The patients and controls were similar in ethnicity and nutritional habits. Occupationally neither the patients nor the controls were exposed to any specific toxic chemical. So, the effects, if any, of confounding factors such as ethnicity, diet and occupation was similar in patients and controls. The study was approved by the Institutional Medical and Ethics Committee. Blood samples were collected from both the patients and controls with an informed written consent.

Table 2.1: Principal characteristics of study subjects

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Parameters</th>
<th>Prostate Cancer Patients (N=95)</th>
<th>Controls (N=120)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean±SD</td>
<td>Healthy controls (N=80)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>Mean±SD</td>
<td>67.1±8.6</td>
<td>66.5±6.7</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>44.0-91.0</td>
<td>50.0-81.0</td>
</tr>
<tr>
<td>PSA (ng/ml)</td>
<td>Mean±SD</td>
<td>49.5±38.9</td>
<td>-</td>
</tr>
<tr>
<td>Gleason score</td>
<td>2</td>
<td>3.4%</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8.0%</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>21.8%</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>12.6%</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>9.2%</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>24.1%</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>14.9%</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>4.6%</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.4%</td>
<td>-</td>
</tr>
</tbody>
</table>
2.2 Reagents and buffers for Genomic DNA isolation from peripheral blood

2.2.1 Tris-HCl- 1M (pH 7.5/8.0): 121.1 g of Tris-base was dissolved in 900 ml of double distilled water and the pH was adjusted to 7.5 /8.0 with concentrated HCl. The final volume was made up to 1000ml with double distilled water and sterilized by autoclaving.

2.2.2 Potassium chloride- 1M: 7.45g of potassium chloride was added to 80 ml of double distilled water, after complete dissolution of the salt, the final volume was made up to 100 ml and sterilized by autoclaving.

2.2.3. Magnesium chloride- 1M: 40.6g of magnesium chloride was dissolved in 80 ml of double distilled water; the final volume was made up to 100 ml and sterilized by autoclaving.

2.2.4 EDTA di- sodium salt – 0.5M (pH 8.0): 18.6g of di-sodium EDTA was dissolved in 80 ml of double distilled water and 15.20g of sodium hydroxide pellets were added to increase the pH towards 8.0. When the sodium hydroxide pellets dissolved completely, 1N HCl was added to bring pH to 8.0. The final volume was made up to 100ml with double distilled water and sterilized by autoclaving.

2.2.5 Sodium chloride- 5M: 29.2g of sodium chloride was added to 80ml of double distilled water and warmed to assist dissolution. The final volume was made up to 100 ml with double distilled water.

2.2.6. Triton X100: commercially available Triton X100 was used.

2.2.7. Sodium dodecyl sulphate (SDS) - 10% (pH 7.0): 10g of SDS was dissolved in 80 ml of sterile double distilled water gently by slow mixing to avoid frothing. The solution was kept in a water bath at 65°C to assist complete dissolution. The final volume was made up to 100ml and the solution was filter sterilized.

“A study on genetic polymorphisms associated with prostate cancer risk in South Indian men”
2.2.8 **Buffer Saturated Phenol:** Buffer saturated Phenol was procured commercially and stored under refrigerated conditions.

2.2.9 **Phenol: Chloroform: Isoamylalcohol (PCI) mix-(25:24:1):** 25ml of buffer saturated phenol was mixed with 24ml of chloroform and 1 ml of isoamylalcohol to get a final volume of 50ml PCI stock solution. The solution was stored in clean airtight dark bottle at 4°C.

2.2.10 **Chloroform: Isoamyl alcohol (CI) (24:1):** 48ml of chloroform was mixed with 2ml of Isoamylalcohol. The solution was stored in clean airtight dark bottle at 4°C.

2.2.11 **Sodium acetate- 3M (pH 5.2):** 24.6g of sodium acetate was dissolved in 80ml of double distilled water and pH was adjusted to 5.2 with glacial acetic acid. The final volume was made up to 100 ml with double distilled water and sterilized by autoclaving.

2.2.12 **Absolute Ethanol:** Absolute ethanol was commercially procured and stored under refrigerated conditions.

2.2.13 **Ethanol-70%**-70ml of absolute ethanol was mixed with 30ml of sterile double distilled water and stored at 4°C.

2.2.14 **Red Blood Cell lysis buffer**

The buffer is also termed as non-nucleated cell lysis buffer or low salt buffer.

- **Tris- HCl**: 10mM
- **KCl**: 10mM
- **MgCl₂**: 10mM
- **Na₂EDTA.2 H₂O**: 2mM

All components were mixed with sterile double distilled water. The buffer was stored at room temperature (25°C).
2.2.15 White Blood Cell lysis buffer

The buffer is also known as nucleated cell lysis or high salt buffer.

- Tris HCl 10mM
- KCl 10mM
- MgCl2 10mM
- Na2.EDTA.2 H2O 2mM
- NaCl 400mM

All the components were mixed with sterile double distilled water and stored at room temperature (25°C)

2.2.16 TE buffer (Tris-EDTA buffer) – (pH 8.0)

- Tris HCl (pH 8.0) - 10mM
- Na2.EDTA.2 H2O (pH 8.0) - 1mM

The components were dissolved in sterile double distilled water.

All the chemicals were from Hi Media laboratories. The solvents were from Qualigens.

2.3 Reagents and buffers for agarose gel electrophoresis

2.3.1 TAE buffer (Tris- acetate EDTA buffer) - 50X (pH 7.2)

- Tris base – 2M
- Glacial acetic acid - 1N
- Na$_2$ EDTA.2 H$_2$O - 0.05M

Tris base and disodium EDTA were dissolved in sterile double distilled water. Using glacial acetic acid, the pH was adjusted to 7.2. The final volume was made up to 1000 ml and sterilized by autoclaving. The solution was stored in a clean sterile reagent bottle at room temperature (25°C)
2.3.2 Ethidium bromide- 10 mg/ml: To 1 ml of sterile double distilled water, 10mg of ethidium bromide was added and mixed well for complete dissolution of the dye. The stock solution was stored in aliquots in air tight containers wrapped with aluminum foil.

2.3.3 DNA sample loading dye- 6X

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ficoll 400</td>
<td>- 6%</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>-0.12%</td>
</tr>
<tr>
<td>Xylene cyanol FF</td>
<td>-0.12%</td>
</tr>
<tr>
<td>Tris- HCl (pH 7.5)</td>
<td>-12Mm</td>
</tr>
<tr>
<td>Na2 EDTA.2 H2O</td>
<td>-120mM</td>
</tr>
</tbody>
</table>

All the components were dissolved in sterile double distilled water and stored at room temperature (25°C)

2.3.4 Agarose Low EEO: Commercially available Agarose was used Bangalore Genei/Sigma

2.4 Reagents for DNA Polyacrylamide Gel Electrophoresis

2.4.1 Acrylamide: Bisacrylamide–30%: 29.2g of acrylamide and 0.8g of bisacrylamide were mixed and the volume was made up to 100ml with sterile double distilled water. The solution was filter sterilized with 0.45µm sterile filter and stored in clean airtight dark bottle.

2.4.2 TBE Buffer (Tris - borate EDTA) - 10X (pH 8.3)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>0.89M</td>
</tr>
<tr>
<td>Boric acid</td>
<td>0.89M</td>
</tr>
<tr>
<td>Na2. EDTA.2 H2O</td>
<td>0.04M</td>
</tr>
</tbody>
</table>

Tris base and EDTA di sodium salt were dissolved in 800ml of sterile double distilled water. Boric acid was added to adjust the pH to 8.3 and the volume was made up to 1000ml with distilled water. The solution was sterilized by autoclaving and was stored in clean sterile glass bottle at room temperature.
2.4.3. TEMED -100% - commercially available TEMED.

2.4.4. Ammonium per sulphate APS - 10%: 1g of APS was dissolved in 10 ml of sterile double distilled water and stored at 4°C.

2.5. REAGENTS FOR SILVER STAINING

2.5.1 Acetone: 50% acetone was prepared by mixing 30ml of acetone with 30 ml of distilled water.

2.5.2 Tricholoro Acetic Acid (TCA): 50% TCA was prepared by dissolving 12.5 g TCA in 25 ml of sterile distilled water.

2.5.3 Sodium thiosulphate (Na$_2$S$_2$O$_3$): 10% Na$_2$ S$_2$ O$_3$ was prepared by dissolving 2.5 g Na$_2$S$_2$O$_3$ in 25 ml of sterile distilled water.

2.5.4 Silver nitrate: 20% silver nitrate was prepared by dissolving 5g silver nitrate in 25 ml of sterile distilled water.

2.5.5 Formaldehyde: Commercially available 37% formaldehyde was used.

2.5.6 Sodium carbonate: 1.4g was used for preparing the developer.

2.5.7 Glacial Acetic acid: 1% prepared from commercially available 100% glacial acetic acid (Merck) by diluting 1m acetic acid with 99 ml sterile distilled water.

2.6. Reagents for Polymerase Chain Reaction

All the PCR fine chemicals were commercially procured from Bangalore Genei Pvt.Ltd.

1. 10 X PCR Taq DNA Polymerase buffer.
2. Taq DNA Polymerase (3Units/μl)
3. d NTP mix (10mM).
4. Primers: Primers were obtained as lyophilized powder of different OD values and were reconstituted with TE buffer.
2.7 Reagents for Restriction Fragment Length Polymorphism

Restriction enzymes and Buffers were procured from New England Biolabs and Bangalore Genei Pvt.Ltd.

2.8. Reagents for DNA sequencing and processing

Big Dye™, 50% HiDi Formamide, 70% ethanol, 3M Sodium acetate pH 5.2

2.9 Reagents for Immunohistochemistry (IHC)

2.9.1 Citric Buffer: 01M Citric acid (pH 6): 2.1g citric acid and 7 pellets of sodium hydroxide are dissolved in 1000ml of distilled water.

2.9.2. Tris Buffer: 6.05g Tris, 8.7g Nacl and 37ml of HCl were dissolved in 1000ml of distilled water and the pH was adjusted to 7.2-7.6 with HCl

2.9.3 Primary antibodies:
   a. Monoclonal mouse anti-Human p53 protein, Clone: DO-7 from Dakocytomation
   b. Monoclonal mouse anti-Human bcl2oncoprotein, Clone: 100 from Biogenex

2.9.4. Secondary antibody detection: Supersensitive™ Link-label IHC detection System was procured from Biogenex.

2.9.5 Hematoxylin stain
2.9.6 Xylene
2.9.7 Isopropyl alcohol
2.9.8 DPX mount
2.10 High molecular weight genomic DNA isolation from blood sample by Phenol-chloroform Isoamyl alcohol method

Blood samples were collected from prostate cancer patients and controls in sterile EDTA vacutainers. High molecular weight genomic DNA was isolated from the peripheral blood by Phenol-chloroform Isoamyl alcohol method with modifications from Sambrook et al 1989 (143).

RBC lysis (non- nucleated cell lysis)
1. To the blood sample collected double the volume of RBC lysis buffer and 0.1% of Triton X100 were added and mixed gently.
2. The tube was incubated at 37°C water bath for 5 minutes.
3. After incubation, it was centrifuged at 2000rpm for 15 minutes at 4°C.
4. The supernatant was discarded and to the pellet, 10 ml of RBC lysis buffer was added and the pellet was suspended well.
5. The sample was centrifuged at 2000 rpm for 15 minutes at 4°C.
6. The wash was repeated until a white pellet free of hem was obtained.

WBC lysis (nucleated cell lysis)
7. To the pellet 1 ml of WBC lysis buffer was added. The pellet was suspended well with a sterile 1 ml wide mouthed micropipette tip to lyse the cells.
8. 10% SDS was added to a final concentration of 0.2% and incubated in a water bath at 55°C for 60 minutes.

Purification and Precipitation of DNA

9. Following incubation, the solution was transferred to a sterile 2 ml microcentrifuge tube and mixed well with equal volume of Phenol Chloroform Isoamyl alcohol mix.
10. It was centrifuged at 10,000 rpm for 15 min at 4°C.
11. The upper aqueous phase was carefully transferred into another tube and equal volumes of CI was added, mixed well and spun at 10000 rpm for 15 min at 4°C.
12. The upper phase was then transferred into another tube and to this 1/10th volume of
Materials and Methods

3M sodium acetate pH 5.2 was added, mixed gently and double the volume ice cold absolute ethanol was added. The contents were mixed gently by inverting the tube to precipitate the DNA.

13. The precipitated DNA was scooped out into another sterile microcentrifuge tube and washed with 500µl of 70% ethanol at 2000 rpm for 5 minutes at 4°C.

14. The tube was centrifuged at 2000 rpm for 5 minutes at 4°C.

15. The DNA pellet was air-dried and dissolved in 200 µl of TE buffer. It was refrigerated until dissolution.

16. DNA was aliquoted and stored in frozen condition until further use.
Materials and Methods

High molecular weight genomic DNA isolation from blood sample by Phenol-Chloroform Isoamyl alcohol method

To the blood sample double the volume RCLB and 0.1% Triton X 100 were added

Incubated at 37°C for 5 min

Centrifuged at 2000rpm for 15 min at 4°C

Supernatant discarded and to the pellet 10ml RCLB was added

Pellet dispensed well and centrifuged at 2000 rpm for 15 min at 4°C

Supernatant discarded and to the pellet 1ml WBC lysis buffer + 0.2% SDS were added

Incubated at 55°C for 1 hr

The sample was transferred to another tube and equal volume of PCI was added

Centrifuged at 10000 rpm for 15 min at 4°C

Aqueous layer transferred to another tube and equal volume of CI added

Centrifuged at 10000 rpm for 15 min at 4°C

To the supernatant 1/10th volume sodium acetate and double the volume ice-cold absolute ethanol were added

The precipitated DNA was scooped into another tube, washed with 70% ethanol centrifuged at 2000rpm, 5 min

Supernatant discarded, the DNA pellet was air dried and dissolved in 200µl TE Buffer
2.11 Qualitative and quantitative analysis of DNA

2.11.1 Agarose gel electrophoresis

The quality of the DNA samples were checked in 0.8% agarose gel.

1. 0.8g of agarose was dissolved in 100ml of 1X TAE buffer by boiling.
2. The solution was allowed to become lukewarm followed by which ethidium bromide was added to a final concentration of 0.1mg/ml.
3. The gel was poured on a gel-casting tray and allowed to solidify.
4. The gel was placed in an electrophoresis tank with 1X TAE buffer.
5. The samples were mixed with bromophenol blue dye and loaded on the gel.
6. The gel was electrophoresed at 2 volts/cm and was visualized in a gel documentation system (Bio Rad) (Fig 2.1)

2.11.2 Spectrophotometric analysis

The quality and quantity of the DNA samples were checked by spectrophotometer. 10 µl of the DNA was diluted with 990 µl of TE buffer. The OD was read at 260 nm and 280 nm. The concentration of the samples was determined based on the OD value at 260 nm, where 1 OD corresponds to 50µg of double stranded DNA. Samples with 260/280 OD ratio between 1.7-1.9 were selected for PCR. Samples with lesser than 1.7 OD were reprecipitated and then used for PCR.

2.12 In-vitro amplification of gene of interest by Polymerase Chain Reaction (PCR)

Amplification of the gene of interest is performed using specific primers under appropriate cycling conditions of denaturation, annealing and extension in a Thermal cycler (Master Cycler gradient- Eppendorf).

2.12.1 PCR: 20µl reaction was set up in the following concentration (Table 2.2)
Materials and Methods

A study on genetic polymorphisms associated with prostate cancer risk in South Indian men

Table 2.2: PCR Mix

<table>
<thead>
<tr>
<th>S.No</th>
<th>Reagents</th>
<th>Stock Concentration</th>
<th>Working Concentration</th>
<th>Working Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Taq DNA Polymerase Buffer</td>
<td>10 X</td>
<td>1 X</td>
<td>2 µl</td>
</tr>
<tr>
<td>2</td>
<td>dNTP mix</td>
<td>10mM</td>
<td>200µM</td>
<td>0.4µl</td>
</tr>
<tr>
<td>3</td>
<td>Primer Forward</td>
<td>100µM</td>
<td>50 pM</td>
<td>0.5µl</td>
</tr>
<tr>
<td></td>
<td>Primer Reverse</td>
<td>100µM</td>
<td>50 pM</td>
<td>0.5µl</td>
</tr>
<tr>
<td>4</td>
<td>Taq DNA polymerase</td>
<td>3U/µl</td>
<td>1.5Units</td>
<td>0.5µl</td>
</tr>
<tr>
<td>5</td>
<td>Water (nuclease free)</td>
<td>-</td>
<td>-</td>
<td>15.1µl</td>
</tr>
<tr>
<td>6</td>
<td>Template</td>
<td>-</td>
<td>100ng</td>
<td>1µl</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td></td>
<td>20µl</td>
</tr>
</tbody>
</table>

A master mix comprising of all components except the template was prepared and aliquoted into separate tubes. The template DNA was then added, the tubes were placed in the thermal cycler and subjected to the standardized PCR conditions. The PCR conditions were standardized for each gene by gradient PCR.

2.12.2 Confirmation of PCR amplification

PCR amplification was confirmed by 2% agarose gel electrophoresis. 100bp molecular weight marker was used confirm the amplicon size. Electrophoresis was carried out at 4V/cm and the gel was visualized in the gel documentation system.

2.12.3 Purification of PCR amplicons

The PCR amplicons were purified by reprecipitation with ethanol.

1. To the PCR product thrice the volume TE buffer was added.
2. This was followed by the addition of one tenth the volume 3M sodium acetate pH 5.2
3. The product was then precipitated by the addition of double the volume ice-cold absolute ethanol and incubation at 0°C overnight or -70°C for 1 to 2 hours.

“A study on genetic polymorphisms associated with prostate cancer risk in South Indian men”
4. It was then centrifuged at 12000rpm for 30 minutes at 4° C.
5. The precipitated product was washed once with 70% ethanol at 12000rpm for 30 minutes at 4° C.
6. The pellet was then air dried and resuspended in 20 µl of TE buffer. The samples were stored at 0°C until further use.

2.13 PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)

The purified PCR amplicon was subjected to restriction digestion with the appropriate restriction enzyme and incubated for 1-2 hours at the optimum temperature for the enzyme. A 20µl restriction digestion reaction was set up as follows (Table 2.3):

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Stock Concentration</th>
<th>Working Concentration</th>
<th>Working Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 X Restriction Buffer</td>
<td>10 X</td>
<td>1 X</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>Restriction enzyme</td>
<td>*U/µl</td>
<td>*U</td>
<td>*µl</td>
</tr>
<tr>
<td>Water (nuclease free)</td>
<td>-</td>
<td>-</td>
<td>7.9 µl</td>
</tr>
<tr>
<td>PCR amplicon</td>
<td></td>
<td></td>
<td>10 µl</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>20 µl</td>
</tr>
</tbody>
</table>

*Depends on the enzyme

Analysis of PCR-RFLP products

a. Agarose Gel Electrophoresis: Analysis of PCR-RFLP products was done by a 2% or 3% agarose gel electrophoresis based on the restriction fragment size. The gel was electrophoresed at 4V/cm and visualized in the Gel documentation system. Genotypes were assigned based on the restriction pattern

b. Polyacrylamide Gel electrophoresis: To resolve restriction fragments which differ in size by few base pairs a polyacrylamide gel electrophoresis was performed. The acrylamide gel mix for 15 ml was prepared and casted (Table 2.4).
10µl of the sample, 2µl of 6X BPB dye were mixed and loaded onto the gel. Electrophoresis was done at 4V/cm. Silver staining was done to observe the DNA.

Silver Staining

1. The gel was carefully removed from the gel plates, transferred into a staining tray.
2. The gel was first fixed by adding 60ml of 50% acetone, 1.5ml of TCA stock solution and 25µl of 37% formaldehyde. The gel was rocked gently for 5 min.
3. The solution was discarded and the gel was rinsed with distilled water for 5-10 sec.
4. 60 ml of 50% acetone was then added to the gel and gently rocked for 5 min. The solution was discarded.
5. 100µl of sodium thiosulphate solution made up to 60ml with distilled water was added to the gel and rocked for 1 min. The gel was rinsed with distilled water.
6. The gel was stained with the solution containing 0.8ml of 20% silver nitrate; 0.6ml of 37% formaldehyde made up to 60ml with distilled water. The gel was rocked gently for 8min. The solution was discarded and the gel was rinsed with distilled water
7. The gel was developed by adding the developer containing 1.4g of sodium carbonate, 25µl formaldehyde and 12.5µl sodium thiosulphate made up to 60 ml with distilled water. The gel was rocked till the DNA stained.
8. The solution was then poured off and the reaction arrested by adding 1% glacial acetic acid.
9. The stained gel was immersed in distilled water and viewed on a white light transilluminator and documented in the Gel Documentation system.
2.14 Gene-Scan

For Gene scan, 3.0 μl of the PCR product was mixed with 0.2 μl of LIZ500™ and 6.8 μl of 50% Hi-Di formamide. Upon denaturation for 5 min at 95°C and cooling for 5 min on ice, the samples were run on ABI 3730 DNA analyzer (Applied Biosystems, USA) at the Centre for Cellular and Molecular Biology (CCMB), Hyderabad, India. The raw data were analyzed using Gene Mapper software to determine the number of repeats.

2.15 DNA sequencing

The genotypes of representative samples were confirmed by DNA sequencing. This was carried out in ABI 3730 DNA analyzer at the Centre for Cellular and Molecular Biology (CCMB), Hyderabad. Sequencing of the PCR amplicon involved 2 steps: The PCR amplicon to be sequenced was first subjected to a sequencing PCR followed by post PCR processing and sequencing.

**Sequencing PCR** was performed with the PCR amplicon as the template, with one of the respective forward or reverse primers. A master mix of BigDye™, primer and PCR water were prepared as given in Table 2.5 and then dispensed equally into MicroAmp96well plate. The PCR products were then added to the wells and subjected to sequencing PCR reaction.

### Table 2.5 Sequencing PCR reaction mix

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BigDye™</td>
<td>1.8</td>
</tr>
<tr>
<td>Forward or Reverse Primer</td>
<td>0.1</td>
</tr>
<tr>
<td>PCR water</td>
<td>2.1</td>
</tr>
<tr>
<td>PCR product (10ng/μl)</td>
<td>1</td>
</tr>
</tbody>
</table>

**Sequencing PCR** was carried out in the GeneAmp 9600 thermalcycler (Perkin-Elmer). The reaction conditions are as follows: 95°C for 10sec, 50°C for 5sec, 60°C for 4min, the conditions are repeated for 30 cycles, following which they were subjected to post PCR processing.

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Processing the Sequencing PCR product

(1) 3ml absolute alcohol was added to 120μl of 3M Sodium acetate pH 5.2. 25μl of this was added into each well of the plate.

(2) The plate was centrifuged at 4000 rpm for 20min at 25ºC.

(3) The plate was inverted to remove the supernatant. 100μl of 80% ethanol was added to each well and again centrifuged at 4000 rpm for 10min.

(4) The plate was once again inverted and subjected to a pop spin for few seconds at 750 rpm to remove the alcohol. The plate was covered with fresh aluminium foil.

(5) At the time of sequencing, 10 μl of 50% HiDye™ formamide was added to all the wells. The sample plates were run in the ABI Prism® 3730 DNA Analyzer.

2.16 Immunohistochemistry

Deparaffinization

1. Paraffin embedded tissue sections on Poly L Lysine coated slides were placed on a hotplate at 60ºC for 30min.

2. The sections were deparaffinized by placing them in xylene for 5min.

3. The slides were then placed in isopropyl alcohol for 5min and rehydrated in distilled water for 5min

Antigen retrieval

4. The slides were immersed in warm citric buffer and boiled in a microwave for 15 min for antigen retrieval. The slides were allowed to cool and then rinsed in distilled water for 5 min.

Endogenous peroxidase Block

5. Two drops of peroxide block was added on the section and incubated for 5min at room temperature in order to block the endogenous peroxidase activity.

6. The section was then placed in distilled water for 5 min.

Primary antibody staining

7. Slides were placed in Tris buffer for 5 min.

8. 2 drops of primary antibody was added to cover the section and incubated for 1 hour in a moist chamber.

9. The sections were then rinsed in Tris buffer for 5min.
Secondary Ab conjugation and detection

10. Multi link biotinylated secondary antibody was then added and the slides were incubated for 30 min in a moist chamber.

11. The sections were rinsed in Tris buffer for 5min, streptavidin peroxidase conjugate was then added and incubated for 30 min in the moist chamber.

12. The sections were then rinsed in Tris buffer for 5min, the substrate (DAB Chromogen+H2O2) was then added and sections were observed till the brown precipitate was formed. The sections were counterstained with hematoxylin and air-dried. Once dry, the slides were cover slipped with DPX mount.
“A study on genetic polymorphisms associated with prostate cancer risk in South Indian men”
2.18 Statistical Analysis

The descriptive measures like mean, median and standard deviation of various characteristics such as age, PSA levels and tumor grade of the subjects were calculated. The allelic and genotype frequencies of prostate cancer patients and controls were determined. The expected genotype frequency in both the patients and controls was calculated to test whether the frequencies follow Hardy-Weinberg equilibrium.

Binary logistic regression analysis was carried out to assess the age-adjusted odds ratio between cases and controls. The odds ratio (OR) and its 95% CI were used to describe the risk associated with different genotypes. The significance level was fixed at 5% for all the tests.

Fisher’s exact test results were used to test the differences whenever the cells had the expected number less than 5. For calculation of odds ratio with 0 cell frequencies, 0.5 was added to all the cells (144). One sample Chi-Square test was also performed to determine the risk attributed by the genotypes in patients with relapse of cancer after androgen ablation therapy.

The study subjects were stratified into two groups based on their mean age (<66 years vs ≥ 66 years). The genotypes were compared between patients and controls within the age groups and the risk was determined for each age group by calculating the odds ratio with 95% CI. The relation of the genotypes with the aggressiveness of cancer was assessed by stratifying the patients into two groups based on Gleason scores. Patients with Gleason score <7 were classified as low grade (less aggressive) and patients with ≥7 were classified as high grade (more aggressive). The genotypes were then compared between patients in the low grade and high grade and the risk was assessed by calculating the odds ratio with 95% CI. Further, the association between the genotypes and PSA levels of the patients was determined by stratifying the patients into two groups with mean PSA level (49) as the cut off. The genotypes were then compared between patients with low PSA and high PSA values and the risk was assessed by calculating the odds ratio with 95% CI. Further, stepwise multiple logistic regression analysis was carried out to calculate the adjusted OR for age at diagnosis, PSA and tumor grades.
For the CAG and GGN repeat analysis the mean number of repeats in cases and controls, and in two groups of Gleason Score (GS), age and PSA were compared by Student’s unpaired ‘t’ test. To assess whether the distribution of GGN repeats varied by the level of CAG repeats and the linkage between the repeats were tested by $\chi^2$-test. To test whether specific haplotypes contribute to the risk, logistic regression analysis was carried out with CAG and GGN as binary covariates.

In order to assess the interaction between genes within each pathway as well as among the different pathways logistic regression analysis was performed to determine the risk of each of the genes after adjusting for the effects of the other genes. The genotype frequencies of each gene of the present study controls was compared with the controls of other population by chi-square test.

For immunohistochemistry analysis, the relationship between the expression of p53 and bcl2 proteins by performing a kappa test for agreement.

All the statistical analysis were performed with SPSS (version 13) package.