4. MATERIALS AND METHODS

4.1 Collection of Blood Samples

For the present study, the foremost requirement was collection of blood samples from prostate cancer cases as well as from disease free subjects who served as controls. The blood samples from 157 North Indian males with prostate cancer were collected in sterile EDTA-K2 coated vacutainer tubes (Becton Dickinson, San Jose, CA). The samples were collected from Department of Urology of various reputed hospitals of North India, i.e. Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, GMCH, Sector 32 Chandigarh as well as Government Rajindra Medical College & Hospital, Patiala during the period of April 2006 to December 2009. All the patients had been histologically diagnosed with carcinoma of prostate. Besides these, 170 samples of patients already diagnosed with Benign Prostate Hyperplasia (BPH) and equal number (170) of healthy control samples were collected from normal population randomly, who did not have any cancer as well as any other ailment to avoid misdiagnosis. Cases and control samples were matched with age. Although all the samples did not belong to the same hospital, all of them were from general population of North India. No chemo- or radiotherapy had been given to any of the subjects before collecting the samples, which had been confirmed from the hospital record as well as from the patients. A written consent and ethical clearance was obtained from the concerned authorities of hospitals to collect blood samples. Samples were collected by the clinical staff of the concerned hospital. A detailed questionnaire designed on the format of Indian Council of Medical Research (ICMR), containing all the information about the disease outcome, family history, age, smoking and drinking status was recorded at the time of collection of samples as per the Proforma (Annexure-I). A consent form (Annexure-II) was obtained from the patient indicating objective of the study. Besides this, the pathological grading and staging of cancer was confirmed from the pathological record of the hospital. The study proposal and ethics procedure were approved by the Ethics Review Committees of Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh and Panjab University, Chandigarh.
4.2 Collection of Tissue Samples

Tumour tissues were obtained from 100 prostate cancer and 50 BPH patients of North Indian origin. None of these had received chemo- or radiotherapy prior to tumour resection. The diagnosis was confirmed by the Pathologist. The tissues were immediately frozen in liquid nitrogen and stored at -80°C.

4.3 Procurement of Chemicals

All the chemicals used in the study were of molecular grade. Sodium chloride, Sodium citrate, Sodium acetate, Sodium dodecyl sulphate and Tris (hydroxymethyl) amino methane, Hydroquinone, Sodium hydroxide, Sodium bisulfate, etc. were procured from Himedia. Taq DNA polymerase, Magnesium chloride, dNTPs were procured from Ms Fermentas (GmbH Germany). Primers and restriction enzymes were procured from Sigma Aldrich, Texas, USA and New England, Biolabs, Ontario, Canada respectively. Agarose, bisacrylamide and Tris base were obtained from Ms. Boehringer (Manheim, Germany). For purification of Bisulfite treated DNA, Wizard kit from Promega was used and in the final stages of study RedTaq from Sigma Aldrich was also used.

4.4 Isolation of DNA from Blood Samples

Genomic DNA from blood samples was isolated by standard phenol-chloroform protocol (Sambrook et al., 1989).

Extraction of DNA

1. All the blood samples were obtained in EDTA coated vacutainer tubes and stored at -80°C.
2. Frozen blood samples were thawed and buffy coat was shifted into 2ml eppendorf tubes.
3. Then 0.8ml of 1X SSC was added to it and vortexed. Contents were centrifuged at 12K rpm in cooling centrifuge for one minute.
4. One ml of supernatant was removed and discarded into disinfectant.
5. One ml of 1X SSC was added, vortexed and centrifuged at 12K rpm in cooling centrifuge for one minute.
6. The supernatant was then removed to get a pellet at the bottom.

7. 375μl of 0.2M sodium acetate (pH5.0) was added to each pellet and vortexed briefly. It was followed by addition of 10% SDS (25μl) as well as 5μl of proteinase K (20mg/ml). The mixture was vortexed and incubated for one hour at 55°C.

8. After one hour, 120μl of phenol chloroform isoamyl alcohol mixture (25:24:1) was added and vortexed for 30 seconds.

9. The samples were centrifuged for two minutes at 12K rpm in cooling microcentrifuge.

10. The aqueous layer was carefully removed in a fresh 1.5ml eppendorf tube without disturbing the interface layer.

11. Chloroform: iscamyl alcohol in the ratio of 24:1 was added to it, vortexed for 30 seconds and centrifuged at 12K rpm in cooling centrifuge for two minutes.

12. The upper layer was separated out again in fresh 1.5ml eppendorf without disturbing the interface layer. An equal amount of chilled (100%) ethanol was added and DNA was precipitated out.

13. It was centrifuged at 12K rpm in microcentrifuge for one minute.

14. Then supernatant was decanted off and pellet was rinsed with ethanol (80%). Centrifugation was again performed for one minute at 12K rpm in a cooling microcentrifuge.

15. Pellet was then dried at 37°C and dissolved in 30-50μl of TE buffer (pH8.0) and stored at -20°C.

4.5 Running on Agarose Gel

1. Agarose (0.4gm) was weighed and dissolved in 50ml of TAE buffer by heating for one minute at 80°C in microwave. Then 2μl of ethidium bromide (0.5 μg/ml) was added to it. Gel was poured in casting tray. It was kept at cool place for sometime till it solidifies.

2. The solidified gel was kept in running tank containing TAE buffer (pH 8.0).

3. DNA sample (2μl) was mixed with 0.4μl of gel loading dye (bromo-phenol blue) and sample was loaded into a well of 0.8% agarose mini gel.

4. Electrophoresis was carried out at 100V, until bromophenol blue had migrated approximately two centimeters down.
5. Gel was observed and photographed in Gel doc, in which short wavelength ultraviolet irradiations were used as fluorescent medium (Fig. 4.1; 4.2).

6. The intensity of fluorescence of unknown DNA was compared with that of DNA standards and its quality was estimated in the sample.

Fig. 4.1: Extracted DNA from blood samples of prostate cancer patients
Lanes 1-6: Extracted DNA

Fig. 4.2: Extracted DNA from blood samples of controls
Lanes 1-7: Extracted DNA
4.6 Quantification of DNA by Spectrophotometer

Quantitatively DNA was measured by Spectrophotometer. Readings were taken at wavelength of 260nm and 280nm. One microlitre of DNA was diluted to 100 times to get 100μl of DNA. Then OD was noted and the concentration of DNA was calculated. Ratio between these two ODs helped to calculate the purity of nucleic acid.

4.7 Amplification of DNA for Polymorphic Genes

All the genes under study were amplified by standard protocol (Table 4.1). The sequences of various primers used to amplify genes under study with their cyclic conditions are given in Table 4.2. The primers were procured from Ms. Sigma-Aldrich (Texas, USA).

Table 4.1 Constituents of PCR Reaction Mixture (50 μl)

<table>
<thead>
<tr>
<th>Reagent Used</th>
<th>Concentration</th>
<th>Amount (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taq buffer IX</td>
<td>05.0</td>
<td></td>
</tr>
<tr>
<td>BSA 0.1%</td>
<td>05.0</td>
<td></td>
</tr>
<tr>
<td>Forward primer</td>
<td>10μl</td>
<td>02.0</td>
</tr>
<tr>
<td>Reverse primer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dNTPs 10mM</td>
<td>02.0</td>
<td></td>
</tr>
<tr>
<td>Taq DNA Polymerase</td>
<td>5units/l</td>
<td>00.4</td>
</tr>
<tr>
<td>MgCl₂ 2mM</td>
<td>02.0</td>
<td></td>
</tr>
<tr>
<td>Water (Molecular biology grade)</td>
<td>--</td>
<td>31.6</td>
</tr>
<tr>
<td>Template DNA 100ng</td>
<td>02.0</td>
<td></td>
</tr>
</tbody>
</table>
### Table 4.2: List of Primers and Thermocycling conditions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genotyping</th>
<th>Primer Sequence</th>
<th>PCR Cycling Conditions</th>
<th>Reference</th>
</tr>
</thead>
</table>
| XPC (PvuII) | Product:220bp | F – GATGCAGGAGTGACTCTCT  
R – GTAGTGGGGAGCAGCAACT | 95°C - 5 min  
94°C-30s  
65°C - 30s | Ye et al. (2006) |
| XPD (PstI) | Product:436bp | F – GCCGCCTCTGGATTATACG  
R – CTATCATCTCCTGGCCCCC | 95°C - 5 min  
94°C-30s  
62.6°C-30s | Spitz et al. (2001) |
| GSTP1 (exon 5) (Alw261) | Product:433bp | F – GTAGTTGGCCCAAGGTCAAG  
R – AGCCACCTGAGGGATGAG | 95°C - 5 min  
94°C-30s  
65°C - 30s | Ishii et al. (1999) |
| GSTP1 (exon 6) (AciI) | Product:365bp | F – GGGAGCAACAGAGAGGAGTA  
R – CAGGGTGTAGTCAGGACAGGAG | 95°C - 5 min  
94°C-30s  
63°C -30s | Ishii et al. (1999) |
4.8 PCR-RFLP Analysis of Repair Genes

4.8.1 Polymorphism Analysis of XPC

For the study of XPC polymorphism, the PCR amplification was carried out in BioRad i-cycler with denaturation at 95°C followed by annealing at 65°C and extension at 72°C for 30 cycles followed by final extension for one minute at 72°C in 50 μl of reaction volume. 15μl aliquots of the PCR product (220 bp; Fig.4.3) was digested overnight with 2 units of restriction enzyme PvuII (MBI, Fermentas, USA) at 37°C. Products were separated on 2.5% agarose gel. Three types of genotypes were obtained showing two bands of 190 bp and 30 bp for homozygous wild, three bands of 220, 190 and 30 bp for heterozygous and 220 bp band for homozygous mutant (Figs. 4.4 and 4.5).

Fig. 4.3: PCR products of XPC gene

Lanes 1-7: PCR amplified product (220bp)
Lane M: 100bp DNA marker
Fig. 4.4: RFLP analysis of XPC gene digested with PvuII
Lanes 2, 5: Heterozygous Mutant (220, 190, 30bp)
Lanes 3, 4: Homozygous Mutant (220bp)
Lanes 1, 6: Homozygous Wild (190 and 30bp)
Lane M: 100bp DNA marker

Fig. 4.5: RFLP analysis of XPC gene digested with PvuII
Lanes 4, 5, 6: Heterozygous Mutant (220, 190, 30bp)
Lanes 1, 2: Homozygous Mutant (220bp)
Lanes 3, 7: Homozygous Wild (190, 30bp)
Lane M: 100bp DNA marker
4.8.2 Polymorphism Analysis of XPD

For the study of XPD polymorphism, the PCR amplification was carried out in BioRad i-cycler with denaturation at 95°C followed by annealing at 62.6°C and extension at 72°C for 30 cycles followed by final extension for one minute at 72°C in 50µl of reaction volume.

Fig. 4.6: PCR products of XPD gene
Lane 1-7: Amplified product of PCR (436bp)
Lane M: 100 bp DNA ladder

Fig. 4.7: RFLP Analysis of XPD gene digested with PstI
Lanes 3,4: Heterozygous Mutant (290, 227, 146, 63bp)
Lanes 1,7: Homozygous Mutant (227, 146, 63bp)
Lanes 2,5,6: Homozygous Wild (290, 146bp)
Lane M: 100bp DNA marker
15μl aliquots of the PCR product (436bp; Fig.4.6) was digested overnight with 10 units of restriction enzyme PstI (MBI, Fermentas, USA) at 37°C. Products were separated on 2.0% agarose gel. Three types of genotypes were obtained showing two bands of 290 bp and 146 bp for homozygous wild, four bands of 290, 227, 146 and 63 bp for heterozygous and 227, 146 and 63 bp bands for homozygous mutant (Fig 4.7).

4.9 PCR-RFLP Analysis of Metabolic Genes

4.9.1 Polymorphism Analysis of GSTP1 exon 5

PCR amplification (433 bp; Fig.4.8) was carried out in BioRad i-cycler with denaturation at 95°C followed by annealing at 65°C and extension at 72°C for 30 cycles followed by final extension for one minute at 72°C in 50μl of reaction volume. 15μl aliquots of the PCR products were digested for 16 hours with 2 units of restriction enzyme Alw261 (New England Biolab) at 37°C. Products were separated on 12 % PAGE and three types of genotypes were obtained showing 329 and 104 bp for homozygous wild, 329, 222, 107 and 104 bp for heterozygous and 222, 107 and 104 bp for homozygous mutant (Figs. 4.9, 4.10).

![Fig. 4.8: PCR products of GSTP1 (exon 5) gene](image)

Lanes 1-7: Amplified products of PCR (433bp)
Lane M: 100bp DNA marker
Fig. 4.9: RFLP analysis of *GSTP1* (exon 5) gene digested with *Alw261*

Lanes 4,6: Heterozygous mutant (329, 222, 107, 104bp)
Lanes 2,3: Homozygous mutant (222, 107, 104bp)
Lanes 1,5: Homozygous wild (329, 104bp)
Lane M: 100bp DNA marker

Fig. 4.10: RFLP analysis of *GSTP1* (exon 5) gene digested with *Alw261*

Lane 1: Heterozygous mutant (329, 222, 107, 104bp)
Lane 2: Homozygous mutant (222, 107, 104bp)
Lanes 3-6: Homozygous wild (329, 104bp)
Lane M: 100bp DNA marker
4.9.2 Polymorphism Analysis of \textit{GSTP1} exon 6

PCR amplification (365 bp; Fig. 4.11) was carried out in BioRad i-cycler with denaturation at 95°C followed by annealing at 63°C and extension at 72°C for 30 cycles in 50 µl of reaction volume.

![Fig. 4.11: PCR products of \textit{GSTP1} (exon 6) gene](image)

Lanes 1-7: Amplified product of PCR (365bp)

Lane M: 100bp DNA marker

![Fig. 4.12: RFLP analysis of \textit{GSTP1} (exon 6) gene digested with \textit{AcI}](image)

Lanes 6,7: Heterozygous mutant (365, 247, 118, 55bp)

Lanes 2,5: Homozygous mutant (365, 55bp)

Lanes 1,3,4: Homozygous wild (247, 118, 55bp)

Lane M: 100bp DNA marker
15µl aliquots of the PCR products were digested overnight with 2 units of restriction enzyme AciI (MBI, Fermentas, Vilnius, Lithuania) at 37°C. Products were separated on 12% Polyacrylamide Gel Electrophoresis (PAGE) and three types of genotypes were obtained showing 247, 118 and 55 bp for homozygous wild; 365, 247, 118, and 55 bp for heterozygous wild and 365 and 55 bp for homozygous mutant (Fig.4.12).

4.10 Methylation

4.10.1 Bisulfite Conversion of blood DNA

DNA (2µg) in a volume of 50µl was denatured by NaOH (final concentration, 0.2 M) for 10 min at 37°C. 30µl of 10mM hydroquinone (Ms. Sigma-Aldrich, Texas, USA) and 520µl of 3M Sodium bisulfite (Ms. Sigma-Aldrich, Texas, USA) at pH 5, both freshly prepared, was added and mixed, and samples were incubated under mineral oil at 50°C for 16 hrs. Modified DNA was purified using the Wizard DNA purification resin according to the manufacturer protocol (Promega, Madison WI USA) and was eluted into 50µl of water. Modification was completed by NaOH (final concentration, 0.3M) treatment for 5 min at room temperature, and was followed by ethanol precipitation. DNA was suspended in water and was stored at -20°C.

4.10.2 Methylation Specific Primers

The methylation specific primers were procured from Ms. Sigma-Aldrich (Texas, USA). The sequences of various primers and their annealing temperatures are given in Table 4.3.
Table 4.3: List of Methylation Specific Primers and Annealing Temperatures

<table>
<thead>
<tr>
<th>Name of the Gene</th>
<th>Primer Sequence</th>
<th>Annealing Temp. (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTP1(M)</td>
<td>F TTCGGGTTAGCGGTTCGT</td>
<td>69.9</td>
<td>Herman et al., 1996</td>
</tr>
<tr>
<td></td>
<td>R GCCCAATACTAAATCAGACG</td>
<td>66.1</td>
<td></td>
</tr>
<tr>
<td>GSTP1 (U)</td>
<td>F GATGTGGTTAGTGTTGGTTT</td>
<td>66.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R CCACCAATAGTTATCAACA</td>
<td>65.3</td>
<td></td>
</tr>
<tr>
<td>MGMT(M)</td>
<td>F TTTCTTCGTAAGGGTTTTCG</td>
<td>69.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R AACTCAGTCCAGAAACGAAACG</td>
<td>68.7</td>
<td></td>
</tr>
<tr>
<td>MGMT (U)</td>
<td>F TTTTGTCTTTGATGTTGTTTTGTTTGT</td>
<td>66.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R AACTCCACACTCTTCCAAAAACAAACA</td>
<td>68.8</td>
<td></td>
</tr>
</tbody>
</table>

4.10.3 Methylation Specific PCR (MSP) for GSTP1 Gene

Methylation specific PCR for GSTP1 gene was performed by specific protocol given by Herman et al. (1996). Taq polymerase was added after 5 minutes (Hot start) and the programme was run for 45 seconds at 95°C, 45 seconds at 58°C and 45 seconds at 72°C. PCR was carried out for 35 cycles.

After keeping the PCR products at 4°C for 1 hour, the methylated as well as unmethylated products were run on 3% agarose gel for 2 hrs. After successful run, the gel was transferred to Ethidium bromide (dye) to visualize the amplification bands. After that the photograph was taken in the Gel Documentation system (Fig.4.13)
4.10.4 Methylation Specific PCR (MSP) for MGMT Gene

Methylation specific PCR for MGMT gene was performed by specific protocol by Herman et al. (1996). Taq polymerase was added after 5 minutes (Hot start) and the programme was run for 45 seconds at 95°C, 45 seconds at 58°C and 45 seconds at 72°C. PCR was carried out for 35 cycles.

After keeping the PCR products at 4°C for 1 hour, the methylated as well as unmethylated products were run on 3% agarose gel for 2 hrs. After successful run, the gel was transferred to Ethidium bromide (dye) to visualize the amplification bands. After that the photograph was taken in the Gel Documentation system (Fig.4.14 & 4.15).
Fig. 4.14: Methylation specific PCR of MGMT gene in prostate cancer cases
Lanes 2,4,6,7: Methylated
Lanes 1,3,5: Unmethylated
Lane M: 100bp DNA marker

Fig. 4.15: Methylation specific PCR of MGMT gene in control samples
Lanes 4,5,7,8: Methylated
Lanes 1,2,3,6: Unmethylated
Lane M: 100bp DNA marker
4.11 PAGE (Polyacrylamide Gel Electrophoresis):

It was carried out as follows:

Requirements:

Acrylamide: bisacrylamide (29:1) 30% w/v

Ammonium persulfate 10% w/v

Ammonium persulfate was used as a catalyst for the co-polymerization of acrylamide and bisacrylamide gels. The polymerization reaction was driven by free radicals that are generated by an oxido-reduction reaction in which a diamine (e.g., TEMED) is used as the adjunct catalyst. 5X TBE electrophoresis buffer polyacrylamide gels was poured and run in 0.5X or 1X TBE at low voltage (1-8 V/cm) to prevent denaturation of small fragments of DNA by heating.

Procedure

- Glass plates and spacers were thoroughly cleaned. The plates were held from the edges or by wearing gloves, so that oil from the hands didn’t get deposited on the working surfaces of the plates. The plates were rinsed with deionized water and ethanol and were set aside for drying. Precautions were taken that the glass plates were free of grease spots to prevent formation of air bubbles in the gel.
- Glass plates were assembled with spacers in gel caster.
- 12% polyacrylamide gel solution was prepared according to the protocol given in Table 4.4
- Acrylamide gels were run using 0.5X TBE. The volume of 5X TBE and H₂O was adjusted accordingly. Fresh solution of 29:1 (% w/v) acrylamide: bisacrylamide was used everytime to cast polyacrylamide gel.
- After the addition of TEMED, casting of the gel was done immediately to prevent early polymerisation of the gel.
Table 4.4. Various Reagents Used to Cast Polyacrylamide Gels

<table>
<thead>
<tr>
<th>Gel %</th>
<th>30% Acrylamide (29:1)</th>
<th>H₂O (ml)</th>
<th>5x TBE (ml)</th>
<th>0% APS (µl)</th>
<th>TEMED (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 %</td>
<td>3.2 ml</td>
<td>6.4</td>
<td>2.4</td>
<td>200</td>
<td>10</td>
</tr>
<tr>
<td>10%</td>
<td>4.0 ml</td>
<td>5.6</td>
<td>2.4</td>
<td>200</td>
<td>10</td>
</tr>
<tr>
<td>12%</td>
<td>4.8 ml</td>
<td>4.8</td>
<td>2.4</td>
<td>200</td>
<td>10</td>
</tr>
</tbody>
</table>

- The comb was immediately inserted into the gel, taking care not to allow air bubbles to become trapped under the teeth of the comb. The plates were clamped in place with paper clips. The remaining acrylamide gel solution was used to fill the gel mould completely. Leaking of acrylamide solution from the gel mould was avoided.
- The acrylamide was allowed to polymerize for 30-60 minutes at room temperature.
- After complete polymerization, the comb and the top of the gel was surrounded with paper towels soaked in 1X TBE. The entire gel was sealed in Saran Wrap or plastic bag and was stored at 4°C until needed.
- For electrophoresis, the gel was removed from gel caster, carefully cleaning spilled gel from back of white plates and was inserted into vertical gel electrophoresis system (Bangalore Genei, India). Running buffer was carefully added, pulling the combs from the polymerized gel. Both of the reservoirs had same batch of electrophoresis buffer.
- The wells were flushed out using Pasteur pipette or a syringe with 1x TBE. The DNA samples were mixed with the appropriate amount of gel loading buffer. The mixture was loaded into the wells using a micropipette equipped with a drawn-out plastic tip.
- Electrodes were connected to a power pack and, then power was turned on and electrophoresis was carried out.
- The gel was run till the marker dye had migrated upto the desired distance. Power was turned off and electrophoresis buffer from the reservoirs was discarded.
Glass plates were detached. A spacer or plastic wedge was used to lift a corner of upper glass plate. The upper plate was smoothly pulled away. Spacers were removed.

- Gel was then stained with ethidium bromide.
- The picture of the gel was taken in the Gel Documentation System (BioRad).

4.12. Statistical Analysis

Multivariate logistic regression was used to obtain the OR estimate and 95% CI for the main effects of various demographic factors for each enzyme genotype. An adjustment was made for the potential confounding effects of age and sex. The interaction of smoking and each of the genes were evaluated on the additive and multiplicative scales. Comparative analysis was done with nonsmokers having the wild-type genotype as the reference category. Adjusted ORs generated in this manner were evaluated for deviation from the expected null value on the additive or multiplicative scale. In addition, interaction terms including genotype and environmental factors were evaluated for statistical significance with logistic regression. The gene-gene interaction was again done with adjusted OR to rule out any confounding effect and 95% CI was estimated by multiplicative scales. All the statistical analysis was performed using SPSS Professional Statistic software version 13 (SPSS Inc., Chicago IL, USA).