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METHODS
Chapter 2

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MATERIALS AND METHODS

1.1 Study subjects

This case-control study consists of 100 patients suffering from lung cancer and 76 population-selected controls. Lung cancer cases were recruited from patients undergoing bronchoscopy at the Department of Pulmonary Medicine, Post-Graduate Institute of Medical Education and Research, Chandigarh and from the Department of Chest and Infectious Diseases, Government Medical College, Chandigarh. Informed consent was obtained from all the cases and controls before proceeding for the collection of samples from each subject.

1.2 Epidemiology

The patients were asked to fill up a questionnaire (Appendix-2) regarding their smoking, other habits and medical history, including clinicopathological features, life style and occupation. Bidi smokers were defined as individuals who had smoked a minimum of 1000 bidis during their lifetime. The smoking index or Brinkman index of the smokers was calculated by multiplying the total number of bidis smoked per day into number of years smoked. Information was also collected on the brand of bidis and the mode of consumption of tobacco by chewing.

2. Cyp1a1, cyp2d6, cyp2e1 gstm1 and gstt1 genotype studies

2.1 Genomic DNA isolation from blood samples from lung cancer patients and controls

Genomic DNA was isolated according to the protocol of Fields et al. (1999) with some modifications.

1) One ml of blood was washed with equal amount of washing buffer containing 320mM Sucrose, 5mM MgCl2, 1% TritonX-100 and 10mM Tris Cl (8.0) and centrifuged at 4000 R.P.M. for 3 minutes
2) The supernatant was discarded and the pellet washed again with washing buffer and the above step was repeated thrice.

3) The pellet was then lysed with lysis buffer containing 40mM Tris Cl (8.0), 1% SDS, 1mg/ml Pronase, 20mM EDTA and 10mM NaCl and kept for 12 hours at 42°C with constant shaking.

4) After incubation, 500μl of phenol: 480μl chloroform and 20μl isoamyl alcohol, was added to the lysed pellet, then centrifuged at 6500 R.P.M. for 5 minutes.

5) The supernatant was taken without disturbing the interface layer and again subjected to phenol: chloroform: isoamyl alcohol treatment as in the above step.

6) The supernatant was taken and to it chloroform : isoamyl alcohol was added in the ratio of 24:1 and centrifuged at 6500 R.P.M. for 5 minutes.

7) After centrifugation, the supernatant was taken in a fresh eppendorf and equal amount of ice-cold isopropanol was added for precipitation of DNA.

8) DNA was pelleted by centrifugation at 10,000 R.P.M. for 5 minutes.

9) DNA pellet was given a washing with 70% ice-cold ethanol and centrifuged at 10,000 R.P.M. for 5 minutes.

10) DNA pellet was dried and then dissolved in 30-50μl of TE buffer (10mM Tris Cl (8.0) and 1mM EDTA (8.0) and stored at −20°C.

2.2 Cyp1a1 genotyping

Cyp1a1 genotyping for msp1 and ile/val polymorphism was done by PCR- based Restriction Fragment Length Polymorphism (RFLP) and Allele-specific PCR methods (Drakoulis et al., 1994; Sivaraman et al., 1994). The primers used for the msp1 RFLP were P79 5-AAG AGG TGT AGC CGC TGC ACT and P80 – 5-TAG GAG TCT CTC ATG CCT which amplified a 335 bp fragment. Briefly genomic DNA was amplified in 1X reaction buffer
(Sigma, St Louis, MI), 2mM MgCl₂, 100µg BSA, 200µm dNTPs, 1.5U Taq (MBI, Fermentas), 0.5µM of each primer. Initial denaturation was performed at 95°C for 5 min, followed by 30 cycles of denaturation at 1 min, annealing at 56°C, extension at 72°C and final extension at 72°C for 3 min in thermal cycler (Minicycler, MJ Research). The PCR products were digested with 15 units of msp1 restriction enzyme (Sigma, St Louis, MI) for three hours at 37°C, and then subjected to electrophoresis on a 2.5% agarose gel (USB, Cleveland) prepared in TAE buffer along with DNA marker and electrophoresed at 60V. The gel was stained with ethidium bromide (0.5µg/ml) and visualized under a UV transilluminator. The wild type allele (wt1/wt1) showed a single band of 335 bp, the variant allele (m1/m1) resulted in two fragments of 206 and 129 bp, and the heterozygous allele (wt1/m1) in three bands of 335, 201 and 129 b.p.

2.3 Allele specific PCR for ile/val polymorphism of cyp1a1 gene

For the ile/val polymorphism at exon 7 of the cyp1a1 gene was assessed by allele-specific PCR. For this, genomic DNA was amplified with each of the following primers IA- 5'-GAA AGG CTG GGT CCA TCT, 2A- 5'-AAG ACC TCC CAG CGG GCA AT and primer 2G- 5'AAG ACC TCC CAG GCA AC. Primers IA was commonly used each time in the presence of IX reaction buffer (Sigma, St Louis, MI), 2mM MgCl₂, 100µg BSA, 5% DMSO, 200µm dNTPs, 1.5U Taq (MBI, Fermentas), 0.5µM of each primer. Initial denaturation was performed at 95°C for 5 min, followed by 25 cycles of denaturation at 1 min, annealing at 63°C and 72°C for 1 min and a final extension at 72°C for 6 min. PCR products were analyzed in 2% agarose gel (USB, Cleveland) prepared in TAE buffer along with DNA marker and electrophoresed at 60V. The gel was stained with ethidium bromide (0.5µg/ml) and visualized under UV transilluminator. The wild type homozygous genotype (ile/ile) yielded a product of 322 bp with the combination of primers 1 and 2A. The heterozygous genotype (ile/val) yielded a product of 322 bp with combination of primers 1 and 2G and
primers 1 and 2A. The mutant genotype (val/val) gave a 322 bp product with the combination of primers 1 and 2G.

2.4 PCR-RFLP analysis of *cyp2e1* gene polymorphism

The 5-flanking region polymorphic site of the *cyp2e1* gene was analyzed according to protocol of Hayashi et al. (1992). Briefly genomic DNA was amplified using the primers SS1- 5-CCA GTC GAG TCT ACA TTG TCA and SS2- 5-TTC ATT CTG TCT TCT AAC TGG in 1X reaction buffer (Sigma, St Louis, MI), 1.5mM MgCl$_2$, 100µg BSA, 200µm dNTPs, 1.5U Taq (MBI, Fermentas), 0.5µM of each primer. Initial denaturation was performed at 95°C for 5 min, followed by 30 cycles of denaturation at 1 min, annealing at 55°C, extension at 72°C and final extension at 72°C for 4 min in thermal cycler (Minicycler, MJ Research). The PCR products were digested with 10 units *PstI* restriction enzyme (Bohinger Manhiem, Germany) at 37°C overnight and then subjected to electrophoresis in a 2.5% agarose gel (USB, Cleveland) prepared in TAE buffer along with DNA marker and electrophoresed at 60V. The genotypes of *cyp2e1* gene were classified as follows: homozygous wild (c1/c1) 410 bp, heterozygous (c1/c2) 410,290,120 bp, and homozygous mutant (c2/c2) 290,120 bp.

2.5 PCR-RFLP analysis of *cyp2d6* gene polymorphism

A PCR-RFLP assay was used for the detection of the G1934A mutation at the junction of intron 3/exon 4 of the *cyp2d6* gene, which results in a mutation responsible for the poor metabolizer allele (*cyp2d6*4). Amplification of genomic DNA was carried out in a 50µl reaction volume containing 0.5µM of each primer D1: 5-GCT TCG CAA CCA CTC CCG-3 and D2: 5-AAA TCC TGC TCT TCC GAG GC-3, 200µM dNTP, 100µg of BSA, 1X PCR buffer (Sigma, St Louis, MI), 1.5U Taq (MBI, Fermentas), 1.5mM MgCl$_2$. An initial denaturation was performed at 95°C for 5 min, followed by 30 cycles of denaturation for 1 min, annealing at 57°C, extension at 72°C and a final extension at 72°C for 4 min in thermal cycler (Minicycler, MJ Research). The PCR products were digested with 10 units *BstN1*
restriction enzyme (New England Biolabs, U.S.A) at 65°C for 16 hrs and subjected to electrophoresis in a 2.5% agarose gel (USB, Cleveland) prepared in TAE buffer along with DNA marker and electrophoresed at 60V. The wild type allele (EM) was identified by the presence of 230 and 104 bp alleles with G 1934 A mutation did not have a restriction site for BstN1 and only produced an undigested 334 bp fragment and was designated as mutant (PM), whereas heterozygous genotypes (HEM) showed three fragments of 334, 230 and 104 bp.

2.6 Multiplex PCR for gstm1 and gstt1 genotyping

The presence of gstm1 and gstt1 genes was analyzed using a multiplex PCR approach (Arand et al., 1996). Briefly genomic DNA was amplified by using six sets of primers GSTM1 (F) 5- GAA CTC CCT GAA AAG CTA AAG C, GSTM1(R) 5-GTTGGG CTC AAA TAT ACG GTG G, GSTT1 (F) 5-TTC CTG ACT GGT CCT CAG ATC TC, GSTT1 (R) 5-TCA CCG GAT CAT GGC CAG CA ALBUMIN (F) 5-GCC CTC TGG TAA CAA GTC CTA and ALBUMIN (R) 5-CTA AAA AGA AAA TCG CCA ATC 1X reaction buffer (Sigma, St Louis, MI), 2.5mM MgCl2, 100µg BSA, 5% DMSO, 200µm dNTPs, 2.5U Taq (MBI, Fermentas), 0.5µM of each GSTM1 primer, 0.3µM of each GSTT11 primer and 0.15µM of each ALBUMIN primer. Initial denaturation was performed at 95°C for 5 min, followed by 30 cycles of denaturation at 1 min, annealing at 59°C, extension at 72°C for 1 min and a final extension at 72°C for 5 min in thermal cycler (Minicycler, MJ Research). The PCR products were then subjected to electrophoresis on a 2.5% agarose gel (USB, Cleveland) prepared in TAE buffer along with DNA marker and electrophoresed at 60V. The presence of a 480 bp, and 215bp was indicative of the gstt1 and gstm1 genotypes. Albumin indicated by a 350 bp product was used as an internal control. Absence of 480 or 215 bp bands indicated the null genotypes for gstt1 and gstm1 genotypes. The internal control was always present irrespective of the gstm1 or gstt1 being of null type.
3. Detection of p53 gene mutations by PCR-SSCP

3.1 Genomic DNA isolation from bronchoscopic biopsies of lung cancer patients

The biopsies were washed with autoclaved Millipore filtered water to remove traces of blood. The biopsies were lysed by adding 500μl of lysis buffer containing 40mM Tris Cl (8.0), 1% SDS, 1mg/ml Pronase, 20mM EDTA and 10mM NaCl and kept for 12 hours at 42°C with constant shaking. DNA was isolated by the same procedure as mentioned above.

3.2 PCR for p53 gene

The genomic DNA from lung cancer patients was used for detecting mutations in p53 gene by PCR-SSCP method. Since the maximum mutations (more than 80%) have been reported in the exons 5-8 (the mutational hot spots), the present study was limited to these. The following primers were used:

<table>
<thead>
<tr>
<th>PRODUCT SIZE</th>
<th>EXON</th>
<th>SENSE PRIMER</th>
<th>ANTISENSE PRIMER</th>
<th>SIZE</th>
</tr>
</thead>
<tbody>
<tr>
<td>263 bases</td>
<td>Exon 5 sense: 5’ TGT GCC CTG GAC TTT CAA CTC-3’</td>
<td>Exon 5 antisense: 5’ AAC CAG CCC TGT CGT CTC TC-3’</td>
<td>263 bases</td>
<td></td>
</tr>
<tr>
<td>157 bases</td>
<td>Exon 6 sense: 5’ TGA TTC CTC ACT GAT TGC TC-3’</td>
<td>Exon 6 antisense: 5’ ACC CCA GTT GCA AAC CAG AC-3’</td>
<td>157 bases</td>
<td></td>
</tr>
<tr>
<td>181 bases</td>
<td>Exon 7 sense: 5’ AAG GCG CAC TGG CCT CAT CT-3’</td>
<td>Exon 7 antisense: 5’ CAG TGT GCA GGG TGG CAA GT-3’</td>
<td>181 bases</td>
<td></td>
</tr>
<tr>
<td>241 bases</td>
<td>Exon 8 sense: 5’ GGA CCT GAT TTC CTT ACT GC-3’</td>
<td>Exon 8 antisense: 5’ GAG GCA TAA CTG CAC CCT TG-3’</td>
<td>241 bases</td>
<td></td>
</tr>
</tbody>
</table>
The amplification for each exon was carried out in a 50μl reaction volume containing 1X reaction buffer (Sigma, St Louis, MI), 1.5mM MgCl₂, 100μg BSA, 5% DMSO, 200μm dNTPs, 1.5U Taq (MBI, Fermentas), 0.5μM of each sense and anti-sense primers for exons 5-8. Initial denaturation was performed at 95°C for 5 min, followed by 30 cycles of denaturation at 1 min, annealing at 55°C, extension at 72°C for 1 min and a final extension at 72°C for 5 min in thermal cycler (Minicycler, MJ Research). The annealing temperature for exons 5, 6 and 7 was 55°C and for exon 8, it was 58°C. 10μL of the PCR product was analyzed in 2% agarose gel (USB, Cleveland) prepared in TAE buffer along with DNA marker and electrophoresed at 60V. The amplified band size for the different exons was as follows: - Exon 5-263 bp, Exon 6 157 bp, Exon 7- 181 bp and Exon 8- 241 bp.

3.3 SSCP analysis

SSCP was performed according to the protocol of Vega et al. (1999). 8μl of the PCR product and equal amount of denaturing solution (95% formamide, 10mM EDTA, 5mM NaOH 0.01% xylene cyanol and 0.01% bromophenol blue) was boiled at 95°C for 5 min and snap chilled on ice. Then samples (16μl) were loaded on an 8-10% PAGE gel. To increase the sensitivity of the technique, all samples were run under three sets of gel conditions (firstly 8% acrylamide, 2% bis-acrylamide with 10% glycerol) run at 4°C and secondly 10% acrylamide, 1.3% bis-acrylamide with 10% glycerol run at room temperature for all four exons studied; finally 8% acrylamide, 2% bis-acrylamide with no glycerol and run at 4°C [exons 7 and 8] or 10% acrylamide, 1.3% bis-acrylamide with no glycerol and run at 4°C [exons 5 and 6]. The gel was stained with silver according to the protocol of Bassam et al. (1991).
3. 4 Silver staining

Silver staining was done by applying the protocol of Bassam et al. (1991).

a) The gel was fixed in fixative (300ml) containing 50% distilled water, 40% methanol and 10% glacial acetic acid and was kept in the fixative for 1 hour.

b) It was washed thrice with deionized water for 2 minutes each.

c) The gel was then stained with silver nitrate solution (200ml) containing 200mg silver nitrate and 37% formaldehyde and kept in the dark for 30 minutes.

d) It was washed for 1 minute in deionized water and then developed in solution containing 3% sodium carbonate, 37% formaldehyde and 60μl of (10mg/ml) sodium thiosulphate solution. The gel was then kept on a gel rocker or shaker for band development.

e) As soon as the bands developed, stop solution containing 10% glacial acetic acid was added and kept for 5 minutes on a shaker.

f) The gel was then washed twice with deionized water for 5 minutes each.

g) Finally the gel was stored in 1% glycerol.

4. Telomerase activity by TRAP method

To assess the telomerase activity, the bronchial washings from the lung cancer patients were used. Briefly, 5ml of bronchial washings were collected in a centrifuge tube and immediately cooled on ice and then centrifuged at 2000 R.P.M for 5 minutes at 4°C. To avoid contamination with red blood cells that could potentially interfere with PCR, the pelleted cells were resuspended in 5 ml of filtrated autoclaved hypotonic solution (1.2114g Tris, 0.7456g KCL and 0.2033g MgCl₂ to 1L of distilled water) and then the
mixture was pipetted for 15 times to disperse the pellet and immediately kept on ice. It was followed by the addition of 2ml of filtrated ice-cold hypertonic solution (0.4g KCl, 5.795g Na₂HPO₄·12H₂O and 0.4g KH₂PO₄ to 1L water. The suspended cells were then centrifuged at 2000 R.P.M. for 5 minutes at 4°C and the cell pellet thus obtained was stored at –85°C.

4.1 Preparation of extracts from bronchial washings

The frozen cell pellets were thawed and centrifuged at 3000 R.P.M. for 5 minutes. The pellet was washed with 3 ml of ice-cold washing buffer [10mM Hepes-potassium hydroxide (pH 7.5), 1.5mM magnesium chloride, 10mM potassium chloride and 1mM dithiothreitol], pelleted again and then dissolved in 20μl of ice-cold CHAPS lysis buffer containing 10mM Tris-HCl (pH 7.5), 1mM Magnesium chloride, 1mM EGTA (Sigma), 0.1mM PMSF (Sigma), 5mM β-mercaptoethanol (Merck), 10% glycerol and 0.5% CHAPS buffer (Sisco). The pellet was then incubated on ice for 30 minutes and the lysate was centrifuged at 16000 R.P.M. for 40 minutes at 4°C. The supernatant (20μl) was carefully removed so that no debris of the cells was transferred. The protein concentration of the extracts was calculated by Bradfords method and the supernatant was then immediately stored at –85°C.

4.2 TRAP (Telomeric Repeat Amplification Protocol) Reaction.

Telomerase activity was assayed by the TRAP method. Briefly 2-4μl of the cell extract (2-3μg protein) was incubated in a 25 μl of reaction mixture containing 20mM Tris-HCl (pH 8.3), 1.5mM magnesium chloride, 1mM EGTA, 63mM KCl, 0.5% Tween-20, 50mM dNTPs, 100μg BSA and 0.5μM TS primer (AAT CCG TCG AGC AGA GTT) at 23°C for 1 hour and then heated at 90°C for 3 minutes. After the hot start, 0.5μM CX primer (CCC TTA CCC TTA CCC TTA CCC TAA) and 2 units of Taq polymerase was added, and then subjected to 34 cycles of initial denaturation of 94°C for 45secs, annealing of 50°C for 45 secs, and extension at 72°C for 90 secs and final extension at 72°C for 5 min. For assessment of the sensitivity of
the telomerase assay, extracts containing 3μg, 0.3μg and 0.03μg of protein were used in the TRAP reaction. Confirmations of the RNAase A sensitivity of positive telomerase assays were performed as follows: 10μl of cell extract was digested with 0.5μg of RNase for 20 min at 37°C, and 4μl was used in the TRAP reaction. The TRAP assay results were visualized by electrophoresis of 20μl of the PCR products in 1X Tris-borate EDTA buffer in 12% polyacrylamide non-denaturing gels. Gels were visualized by silver staining method for the presence of a 6 base pair ladder that was indicative of telomerase activity.

STATISTICAL ANALYSIS

Odds ratio were calculated to assess the relative risk with respect to genotype and were expressed together with 95% CI. p-values were also calculated. All statistical analysis was performed on Epical 2000 (Version 1.02)