CHAPTER 3 MATERIALS AND METHODS

The present study was conducted in the Department of Biochemistry, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh (JNMC, AMU, Aligarh) from Nov. 2004 to March 2007. Cases for the present study comprised of patients of oral cancer attending the OPD of Department of Otorhinolaryngology (ENT) or admitted to their wards of JNMC, Aligarh. Age and sex matched controls were recruited from healthy individuals. Prior consent was taken from all the cases as well as controls.

The study comprised of total 250 human subjects with 100 patients and 150 controls. Biopsy specimens were taken from 60 cases & 10 controls. Blood samples were collected from all the cases & controls. A thorough proper history with special emphasis on the tobacco & betel quid intake was taken. Patients having habit of smoking and/or alcohol intake were excluded from the present study. Complete clinical evaluation and relevant investigations were done in all the patient samples. This all information was recorded in the following pro forma:

**PRO FORMA**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Name</th>
<th>Date</th>
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<td>Environment</td>
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<tr>
<td>Complaints</td>
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</table>
Past History:
Treatment History:
Personal History:
Tobacco and betel quid intake:
Duration: less than 6 months
: 6-12 months
: 12-18 months
: 18-24 months
Site of lesion:

INVESTIGATION
Routine:
Biopsy and histopathology:
Diagnosis:
Specific investigations:
Immunohistochemistry:
Agarose gel electrophoresis:
PCR-RFLP genotyping:

Histopathology

Post surgical specimens received included excisional and incisional oral biopsy specimens. They were processed by an automatic tissue processor (Histokinette).

Embedding of the block was done as follows:

a) Xylol with paraffin wax-one and half hour.
b) Paraffin wax-one and half hour.

Blocks were prepared in paraffin wax with the help of L-blocks. Sections were cut at 4-5 μm thickness with the help of rotatory microtome (SP-1120) and were subjected to following stains:

a) Haematoxylin and Eosin
b) Immunostaining
Staining Procedure

**Hematoxylin and Eosin methods:** The following protocol was observed:

1. Slides were dewaxed by application of heat and 1-2 min. in xylol.

2. Hydration of sections done by graded alcohol (absolute alcohol, 80%, 70%, 50%).

3. Slides were washed in running tap water for 1-2 min.

4. Dipped in filtered haematoxylin stain for 10 min.

5. Washed in running tap water for 5-19 min.

6. Decolonized using 1% acid alcohol to remove the excess stain.

7. Rinsed in running water.

8. Counter stained with eosin 1% for 2-4 min.

9. Washed in running water to wash off excess stain.

10. Dehydrated by dipping in 95% ethanol and absolute alcohol-15 dips in each.


**Result**

Cytoplasm of cells-pink

Nucleus- blue

Differentiation of tumors was analyzed according to the Broder's Classification as:

1. Well differentiated (Grade I) - < 25% undifferentiated cells

2. Moderately differentiated (Grade II) - <50% undifferentiated cells
3. Poorly differentiated (Grade III) - <75% undifferentiated cells

4. Anaplastic/pleomorphic (Grade IV) - >75% undifferentiated cells

Methodology:

**Immunohistochemistry Procedure:**

- **Positive Control:** - Breast carcinoma (known to express p53 /cyclin D1)

- **Preparation of sections:**
  1. Formalin fixed, paraffin embedded sections were mounted on slides coated with poly-L-lysine solution.
  2. Sections mounted on coated slides were incubated at 56°C overnight for better adhesion.
  3. Sections were deparaffinized in xylol and hydrated through graded alcohols to Tris buffered saline (0.005 M TRIS buffer, pH7.6).
  4. Antigen retrieval was done using citrate buffer (0.01 mol/L, pH 6.0) in a pressure cooker by heating up to one whistle and then allowing to cool to room temperature before opening the lid of cooker.
  5. Endogenous peroxidase activity was blocked by treating sections with 3% hydrogen peroxide in methanol (LSAB Kit) for 10-15 minutes.

- **IMMUNOSTAINING:**
  1. Primary antibody (FL-393 and H-295 antibodies, Santa Cruz Biotechnology, USA for p53 and cyclinD1 respectively) was added to the sections at room temperature and incubated overnight at 38°C in a moist chamber.
  2. Sections were then washed with three changes of TBS for 10 minutes each.
  3. Sections were incubated in biotinylated secondary (Link) antibody at room temperature for 30 minutes in a moist chamber and washed in TBS(x3) for 10 minutes duration each.
  4. Sections were incubated in streptavidin at room temperature for 45 minutes in a
moist chamber and washed in TBS.

5. Sections were incubated in freshly prepared 3, 3' diaminobenzidine tetrahydrochloride (DAB) solution. This was prepared by diluting DAB chromogen (1 drop) in 1 ml of DAB substrate.

6. Sections were washed in distilled water, counterstained in hemotoxylin (1-2 dips), dehydrated through graded alcohols, cleaned in xylol and mounted in DPX.

Results

Positive staining was identified in the form of strong dark brown nuclear staining of the epithelial cells.

Scoring of positive immunostaining

For protein expression, only nuclear positivity (strong brown staining) was assessed quantitatively. Cells with only cytoplasmic staining were not counted.

The quantification of protein positivity was done according to the method recommended by Hall and Lane (1994) and adopted by Chiang et al., (2000). Only the percentage was quantified and the percentage of positively stained cells in the whole layer of epithelium was determined by scanning the entire section and was recorded as follows:

For Squamous cell carcinomas:

- = No epithelial cells stained  
+ (+1) = up to 25% of cells positive  
++ (+2) = 26 to 50% of cells positive  
+++ (+3) = > 50% of cells positive
Genomic DNA Isolation from Whole Blood

For the present study, genomic DNA was isolated from 5ml of human whole blood samples, by Phenol-Chloroform method of DNA isolation.

1. Human blood (5ml) was collected in vial containing anti-coagulant EDTA (1mg/ml).
2. Ice cold red cell lysis buffer (20ml, PH=7.6) was added to the blood sample in a falcon tube and kept on ice for 15 minutes with intermittent mixing several times to lyse RBCs.
3. The lysate was centrifuged at 6000 rpm for 15 min at 4°C.
4. The supernatant was discarded.
5. Steps 2 to 4 were repeated until the white nuclear pellet was obtained.
6. The pellet was suspended in 5 ml of lysis buffer (pH 8.0) buffer, followed by 100ul of proteinase K solution and 500ul of 10% SDS solution and incubated at 37°C on a shaker, overnight to lyse the nuclei.
7. Equal volume of Tris equilibrated phenol (pH 7.5-8.0) was added, mixed well and centrifuged at 4000 rpm for 10 min at room temperature.
8. The aqueous layer was transferred to another tube and equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added, mixed and centrifuged at 4000 rpm for 10 min at room temperature.
9. The aqueous layer was transferred to a fresh tube and equal volume of chloroform: isoamylalcohol (24:1) was added, mixed and centrifuged at 4000 rpm for 10 min at room temperature.
10. DNA was precipitated by adding double the volume of ice cold absolute ethanol to the aqueous layer by gently mixing, spin and pellet was washed with 70% ethanol, air dried and dissolved in double distilled water.

Agarose Gel Electrophoresis

The isolated DNA samples were quantified by agarose gel electrophoresis using 0.8% concentration of agarose in 0.5x TBE (Tris borate EDTA). Agarose (3% in 0.5x TBE) was used for determination and identification of PCR products, after genomic DNA
samples were assayed by polymerase chain reaction. The location of DNA within the gel can be determined directly by staining with low concentrations of fluorescent intercalating dyes, such as Ethidium bromide. Agarose gels have a greater range of separation. DNA's from 50 bp to several megabases in length can be separated on agarose gels of various concentrations and configurations. Small DNA fragments (50-20,000 bp) are best resolved in agarose gels run in a horizontal configuration in an electric field of constant strength and direction. Under these conditions, the velocity of the DNA fragments decreases as their length increases and is proportional to electric field strength. The agarose gels casted with low concentration of agarose are capable of resolving extremely large DNA molecules.

**Preparation of Gel**

Prior to the preparation of agarose gel, appropriate combs were set into the gel plate according to the requirement of wells.

1. The solution was boiled, preferably in a microwave oven.
2. Then the solution was cooled down to 50°C.
3. Ethidium bromide (0.5 mg/ml) was added to the agarose solution, mixed and then poured into the gel plate.
4. When the gel was polymerized, combs were removed.
5. The gel was placed in the running apparatus filled with 0.5x TBE.

**Preparation of samples to be loaded**

The appropriate amount of each sample was transferred to a microfuge tube and mixed with loading buffer to give final 1x strength.
Loading of samples into the wells:

1. The DNA samples or the PCR products were loaded into the wells using micropipette.

2. The electrophoresis chamber was closed with a lid and current was applied (5V/cm).

3. The bromophenol blue dye in the DNA samples acts as a "front wave" that runs faster than the DNA itself. When the "front wave" approached the end of the gel (app 3/4th of the gel), electrophoresis was terminated.

4. The DNA or PCR product was visualized in a UV transilluminator.

PCR-RFLP Genotyping

Polymerase chain reaction is a molecular biology technique which allows a small amount of the DNA molecule to be amplified exponentially. PCR is an iterative process consisting of three elements: denaturation of the template by heat (temperature, 94-96°C), annealing of the oligonucleotide primers to the single stranded target sequence(s), the temperature of this stage depends on the primers and is usually 5°C below their melting temperature (45-60°C), and extension of the annealed primers by a thermostable DNA polymerase, (Taq polymerase), this step takes 1 minute per thousand base pairs. In PCR-RFLP, PCR products are subjected to restriction digestion overnight at 37°C, the digestion products are resolved on agarose gel and bands are visualized.

hOGGI Genotyping

hOGGI genotyping was performed using a PCR-RFLP technique. The primers used to identify the polymorphism at codon 326 of hOGGI were adopted from those published in literature (Table 3.1). A 40-μl reaction mixture containing 29.71μl of double-distilled...
water, 4 μl of 10xPCR buffer, 1 μl of each primer (5 mM/μl), 1 μl of the mixture of deoxynucleoside triphosphates (2.5 mM/μl), 1.2 μl of MgCl₂ (50 mM/μl), and 0.45 unit of (5 unit/μl) Taq DNA polymerase (Amersham Pharmacia Biotech) was used. The PCR condition was initiated by a 4 min denaturation step at 94°C, followed by 35 cycles at 94°C for 40 s, 55°C for 30 s, 72°C for 2 min, and a final step at 72°C for 10 minutes. The PCR products were subjected to restriction digestion overnight at 37°C by Fnu4HI. The digestion products were resolved on 3% agarose gel.

**XRCCI Genotyping**

XRCCI genotyping was performed using a PCR-RFLP technique. The primers used to identify the polymorphism at codon 280 of XRCCI were adopted from those published in literature (Table 3.1). A 40-μl reaction mixture containing 29.71 μl of double-distilled water, 10xPCR buffer (4 μl), 1 μl of each primer (5 mM/μl), 1 μl of the mixture of deoxynucleoside triphosphates (2.5 mM/μl), 1.2 μl of MgCl₂ (50 mM/μl), and 0.45 unit of (5 unit/μl) Taq DNA polymerase (Amersham Pharmacia Biotech) was used. The PCR condition was initiated by a 4-min denaturation step at 94°C, followed by 35 cycles at 94°C for 40 s, 55°C for 30 s, 72°C for 2 min, and a final step at 72°C for 10 min. The PCR products were subjected to restriction digestion overnight at 37°C by Rsal. The digestion products were resolved on 3.0% agarose gel.

**CYP2E1 Genotyping**

The RFLPs in the 5' flanking region and in intron 6 of the CYP2E1 gene were determined by PCR amplification followed by digestion with Rsal and Dral restriction enzymes.
respectively. The primers used to identify the polymorphism at Rsal and Dral sites of CYP2E1 were adopted from those published in literature (Table 3.1). Briefly, a 40-μl reaction mixture containing 29.71 μl of double-distilled water, 10x PCR buffer (4μl), 1 μl of each primer (5 mM/μl), 1 μl of the mixture of deoxynucleoside triphosphates (2.5 mM/μl), 1.2 μl of MgCl₂ (50 mM/μl), and 0.45 unit of (5 unit/μl), TaqDNA polymerase (Amersham Pharmacia Biotech) was used. The PCR condition was initiated by a 4-min denaturation step at 94°C, followed by 35 cycles at 94°C for 40 s, 55°C for 30 s, 72°C for 2 min. and final step at 72°C for 10 min. The PCR products were subjected to restriction digestion overnight at 37°C by Rsal and Dral (New England Biolabs, Inc., Beveraly, MA) respectively. The digestion products were resolved on 3.0% agarose gels.

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<th>Target</th>
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<td>F-Rsa ccagtcagctacattgca</td>
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<td>R-Rsa Ttcattctgtcttcaactgg</td>
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<td>CYP2E1</td>
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<td>GRXRCC1</td>
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<td>GRhOGG1</td>
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Statistical Analysis

An SPSS for windows computer programme (SPSS Inc. Chicago 11, USA, version 13) was used for statistical analysis. The association between protein expression and tumor location was analyzed by the Chi-square test. The relationship between protein expression and histopathological grade was analyzed by Kruskal-Wallis analysis of variance (ANOVA). Wilcoxon paired sample test was used to analyze the differences within the three categories of histopathological grade and protein expression.

The chi-square test for heterogeneity was used for categorical variables to test the hypothesis that the distribution of allele prevalence was same for the cases and controls. Conditional logistic regression technique was used to examine association of polymorphisms with risk of oral cancer.