Materials and Methods

The present human study was undertaken to investigate relevance of molecular genetic alterations involved in immortalization, angiogenesis, invasion and metastasis of oral cancer with their specific relevance to the most important tumor suppressor gene i.e. *p53*. The study was approved by Institutional Review Board (IRB) of the Gujarat Cancer & Research Institute (GCRI), Ahmedabad, Gujarat, India. Informed written consent was obtained from the study subjects.

1. Subjects

***Oral cancer patients*:** The study consisted of histopathologically confirmed and previously untreated 243 oral cancer patients from the out patients' department of the Institute.

Controls: A total of 216 controls were also enrolled in the study. The controls were volunteer blood donors, genetically unrelated to the patients.

Subjects enrolled in both the groups did not have any major illness in recent past. A detailed questionnaire was administered to all participants for gathering information on age, sex, occupation and tobacco habits. Clinical details of the subjects were obtained from patients' records.

Characteristics of the study subjects: Demographic details of study subjects are provided in table 3.1. In oral cancer cohort, there were 205 (84.4%) males with age range of 22 to 75 years (mean 44.9 years) and 38 (15.6%) females with age range of 22 to 65 years (mean 44.7 years). In control cohort, there were 195 (90.3%) males with age range of 19 to 56 years (mean 34.5 years) and 21 (9.7%) females with age range of 22 to 50 years (mean 34.4 years).

Tobacco habits were highly prevalent (89.8%) in oral cancer cases. The risk for oral cancer development was significantly higher in tobacco users as compared to tobacco non-users. (OR=9.8, 95%CI=5.96-15.95, $p<0.0001$). The habit of tobacco chewing was more common in all the groups. Chewers as well as smokers were significantly over-represented among cases as compared to the controls ($p<0.0001$). Exclusive chewers were also at increased risk of developing oral cancer (OR=7.1, 95%CI=4.26-11.9, $p<0.0001$). The number of smokers was comparatively less in both cases and controls. Still, exclusive smokers were at significantly higher risk for oral cancer development (OR=7.7, 95%CI=3.43 - 17.4, $p<0.0001$).

Table 3.1: Clinical details of controls and cases

Characteristics	Controls (216) No. (%)	Oral cancer cases (243) No. (%)
Sex: Male	195 (90.3)	205 (84.4)
Female	21 (9.7)	38 (15.6)
Age: Mean (Range)	35.4 (19 - 56)	44.8 (22 - 75)
Male: Mean (Range)	34.5 (19 - 56)	44.9 (22 - 75)
Female: Mean (Range)	34.4 (22 - 50)	44.7 (22 - 65)
Tobacco Habits: NHT	114 (52.8)	25 (10.3)
WHT	102 (47.2)	218 (89.8)
Types of tobacco habit:		
Exclusive Chewers	82 (80.4)	128 (58.7)
Exclusive Smokers	13 (12.7)	22 (10.1)
Others	0 (0)	7 (3.2)
Mixed	7 (6.9)	61 (28.0)

NHT: No habit of tobacco, WHT: With habit of tobacco

Clinico-pathological details of oral cancer patients are provided in table 3.2. Majority (46.9%) of patients had buccal mucosa involved as a primary site followed by tongue (20.2%). Histologically, majority (97.1%) of the oral carcinoma tissues was squamous cell carcinoma and moderately differentiated (53.9%). 65.0% of all the patients were in advanced stage at diagnosis.

Table 3.2: Clinico-pathological parameters of oral cancer patients

Clinico-pathological Characteristics (N=243)	N (%)
Histopathology: Oral squamous cell carcinoma	236 (97.1)
Verrucus carcinoma	7 (2.9)
Site: Buccal mucosa	114 (46.9)
Tongue	49 (20.2)
Alveolus	32 (13.1)
Lips	5 (2.1)
Others	18 (7.4)
Multiple sites	25 (10.3)
Tumor Differentiation: Well	84 (34.6)
Moderate	131 (53.9)
Poor	10 (4.1)
Undefined	18 (7.4)
Nuclear Grade: 1	41 (16.9)
2	113 (46.5)
3	6 (2.5)
Undefined	83 (34.2)
Tumor Size: < 4 cms	128 (52.7)
≥ 4 cms	109 (44.9)
Undefined	6 (2.5)

Stage: Early [Stage I + Stage II]	79 (32.5)
Advanced [Stage III + Stage IV]	158 (65.0)
Undefined	6 (2.5)
Lymph Node Metastasis: Non - Metastasis	133 (54.7)
Metastasis	99 (40.7)
Undefined	11 (4.5)
Node Status: N1	41 (41.4)
N2	56 (56.6)
N3	2 (2.0)
Mode of Invasion: Localized	84 (34.6)
Invasive	142(58.4)
Undefined	17 (7.0)

2. Reagent, kits and instruments

The fine chemicals were procured from Merck (Germany), Sigma Aldrich (USA), Amersco (USA), Bangalore Genei (India), Sisco Research Laboratory (India) and Qualigens (India). Kits, restriction enzymes and primers were procured from Qiagen (USA), Fermantas (Canada), R&D system (USA), New England Biolabs (MA) and IDT technologies (USA). Plastic ware was purchased from Tarsons (India) and Axygen (USA). Spectrophotometric analyses were performed on Systronic 220 (India) and Shimadzu UV-1800 (Japan). Eppendorf mastercycler gradient (Germany) was used to carry out PCR and reverse transcriptase-PCR (RT-PCR). Electrophoresis was done using vertical as well as horizontal electrophoresis system (GeNei, India). Gel Documentation System (Alpha Innotech, USA and BioRad GS 800, USA) was used for gel scanning and quantification. ELISA plates were read on ELISA reader (Labsystem Multiscan Spectrum, USA).

3. Sample collection and processing

3.1. Blood Samples:

Blood samples (5 ml) from 216 controls and 243 oral cancer patients were collected by venipuncture into plain as well as EDTA and heparin containing vacutainers. Plasma were separated from heparinized vacutainers by centrifugation at 5,500 rotation per minute (RPM) at 4^{o}C for 10 min and stored at -80^{0}C until analysis. WBCs were separated from whole blood collected in EDTA vacutainers. Whole blood was mixed with RBC lysis buffer and incubated at room temperature (RT) for 10 min to allow the lysis of RBC. After incubation, the samples were centrifuged at 11,000 RPM at 4^{o}C for 10 min. The supernatant so obtained was discarded and the WBC pellet was again washed with 3 ml of RBC lysis buffer, followed by vortex and

incubation at RT for 10 minutes. The cell suspension was again centrifuged to get clearer pellet of WBC (without the traces of RBC in it). These steps were repeated until the clear pellet of WBC was obtained. The pellet was dissolved in 200µl of chilled phosphate buffer saline (PBS, pH 7.4), transferred in 1.5 ml micro-centrifuge tube, and stored at -20^{o}C until analysis. Serum were separated from plain vacutainers by centrifuging at 2500 RPM at RT and stored at -80^{0}C until analysis.

3.2. Tissue samples:

A total of 194 tissue samples (97-paired tissues: malignant tumors and adjacent normal) were collected from oral cancer patients at the time of surgery from operation theatre. Tissue samples were defined by a pathologist as malignant and adjacent normal tissues (dissected from free margins at least 1 cm away from the tumor). The tissue samples were collected on ice, washed with chilled PBS (pH 7.4) and stored at -80^{o}C until analysis. For RT-PCR analysis, a fraction of these tissues were stored in RNA stabilizing reagent at -80^{0}C until processed.

Reagents:

- RBC lysis buffer: 8.29 g NH_4Cl; 0.79 g NH_4HCO_3; 0.037 g EDTA, in 1 litre Triple Distilled Water (TDW)
- PBS (pH 7.4): 8.01 g/l NaCl; 0.20 g/l KCl; 1.44 g/l $Na_2HPO_4 \cdot 2H_2O$; 0.24 g/l KH_2PO_4
- RNA stabilizing reagent

Methods:

Various methods were employed for analysis of proposed molecular markers (Table 3.3).

Table 3.3: Methods used to analyse proposed biomarkers

Parameter	Method	Source	Reference
p53 polymorphisms	PCR-RFLP	WBC	Mitra *et al.*, 2003
p53 mutations	PCR-SSCP-sequencing	Tissue	Saranath *et al.*, 1999
MDM2 polymorphism	PCR-RFLP	WBC	Yu *et al.*, 2011
HPV status	TS PCR	Tissue	Jain *et al.*, 2005
hTERT	RT- PCR	Tissue	Nakamura *et al.*, 1997
VEGFA VEGFC VEGFD	RT-PCR and ELISA	Tissue Serum	George *et al.*, 2001 Quantikine Human VEGF kit (R & D System, USA)
MMP-2/MMP-9	RT-PCR and Gelatin Zymography	Tissue Plasma	Edwards, 2000 Lorenzo *et al.*, 1992

4. DNA isolation from peripheral lymphocytes and tissues

DNA was isolated from peripheral lymphocytes and tissues (malignant and adjacent normal) using QIAamp DNA blood mini kit (Qiagen, USA) and QIAamp mini DNA

isolation kit (Qiagen, USA), respectively as per the manufacturer's instructions. The eluted DNA sample was stored at -20°C until analysis.

4.1. Spectrophotometric quantification of DNA

DNA concentration was determined by measuring absorbance at 260 nm (A260) in spectrophotometer. An absorbance of 1 unit at 260 nm corresponds to 50 µg of DNA per ml. Further, A260: A280 ratio was calculated to determine the purity of isolated DNA.

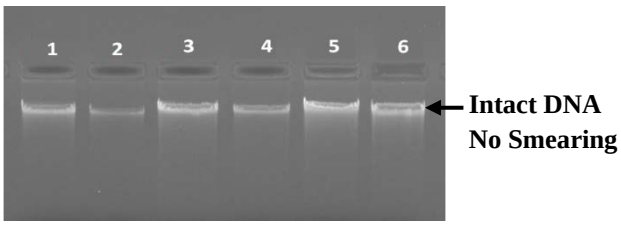
4.2. Agarose gel electrophoresis of DNA

High molecular weight DNA was checked for its integrity. DNA was run on 0.8% ethidium bromide (EtBr) stained agarose gel to check its integrity. Electrophoresis was done in midi submarine electrophoresis system. DNA samples (200 ng) were mixed with 3.0 µl of the DNA gel loading buffer. The gel was run under constant voltage (60 V), till the dye reached to the anode end. The bands were viewed under ultra violet (UV) light in gel documentation system (Figure 3.1).

Reagents:

- 0.8 % Agarose gel in Tris Borate EDTA (TBE) buffer (pH 8.0).
- TBE buffer (5X): 54.0 g Tris base; 27.5 g Boric acid; 20 ml 0.5M EDTA (pH 8.0) in one liter TDW
- EtBr: working concentration 0.5µg/ml in gel
- DNA gel loading buffer: 2.5 ml Glycerol; 1.2 ml Tris EDTA (TE) buffer (pH 8.0); Bromo phenol blue (BPB) (0.025%); TDW 1.3 ml

Figure 3.1: Agarose gel of genomic DNA extracted from WBCs to check integrity



Lanes 1- 6 showing yield of intact DNA

5. RNA isolation from tissues

Total cellular RNA was isolated from the frozen tissues using QIAamp RNA mini kit (Qiagen, USA) to manufacturer's instructions. RNA was suspended in RNAase free water.

5.1. Spectrophotometric quantification of RNA

RNA quantification was performed using spectrophotometer at 260/280 nm and stored at -80^{0}C until use. An absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA/ml.

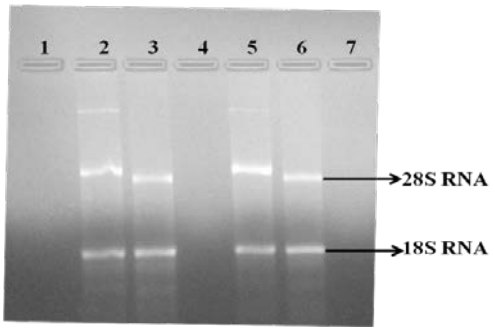
5.2. Agarose gel electrophoresis of RNA

RNA was run on 2.0% EtBr stained native agarose gel to check its integrity. Electrophoresis was done in midi submarine electrophoresis system. RNA samples (500 ng) were mixed with 3.0 µl of the RNA gel loading buffer. The gel was run under constant voltage (60 V), till the dye reached to the anode end. The bands were viewed under UV light in gel documentation system (Figure 3.2).

Reagents:

- 2 % Agarose gel in 1x Tris Acetate EDTA (TAE) buffer (pH 8.0).
- TAE Buffer (50X): 242.0 g Tris base; 57.1 ml glacial acetic acid; 100 ml 0.5M EDTA (pH 8.0) in 1 liter TDW
- EtBr: working concentration 0.5µg/ml gel
- RNA gel loading buffer: 95% v/v Deionized formamide; 0.025% w/v BPB; 0.025% Xylene cyanol; 5mM EDTA (pH 8.0); 0.025% w/v Sodium dodecyl sulfate (SDS)

Figure 3.2: Agarose Gel Electrophoresis (native) to check RNA integrity



Lanes 2,3,5,6: The 18S and 28S ribosomal RNA bands are clearly visible in the intact RNA sample

6. Analysis of *p53* and *MDM2* polymorphisms by PCR-RFLP method

Most frequently observed genetic variations in the genes are SNPs. The SNP genotyping method used for the present study was polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The first step in a PCR-RFLP analysis is amplification of a fragment containing the variation. This is followed by treatment of the amplified fragment with an appropriate restriction enzyme. Since the presence or absence of the restriction enzyme recognition site results in the formation of restriction fragments of different sizes, allele identification can be done by electrophoretic resolvement of the fragments.

6.1. Genotyping of p53 intron 3 (rs17878362) and exon 4 (rs1042522) polymorphism

Intron 3 and exon 4 were amplified together within which the polymorphisms fall, using primers as mentioned previously (Mitra *et al.*, 2003). The PCR was carried out

in a 25 µl reaction mixture containing 100 ng genomic DNA, 12.5 µl of PCR mastermix, 0.2 µM of each forward and reverse primer and nuclease free water. The primers used were 5'-CCT GAA AAC AAC GTT CTG GTA A-3' (forward) and 5'-GCA TTG AAG TCT CAT GGA AG-3' (reverse). The amplification was performed by initial denaturation at 95^0C for 3 minutes, followed by 30 cycles of denaturation at 94^0C for 1 minute, annealing at 47.4^0 C for 1 minute, extension at 72^0C for 1 minute and a final extension at 72^0C for 10 minutes. The PCR products were resolved by 6% native polyacrylamide gel electrophoresis (PAGE) and visualized after staining with Etbr under UV light in gel documentation system. 16 bp duplication in intron 3 was directly interpreted from 6% PAGE analysis of the PCR products. Resulting PCR products were either 432 or 448 bp DNA fragments depending on the absence or presence of 16 bp duplication in intron 3 in template genomic DNA. Homozygotes for the absence of duplication (A1/A1) produced band of 432 bp DNA fragment; heterozygotes produced both the bands (A1/A2); homozygotes for the presence of duplication (A2/A2) produced band of 448 bp DNA fragment (Figure 3.3).

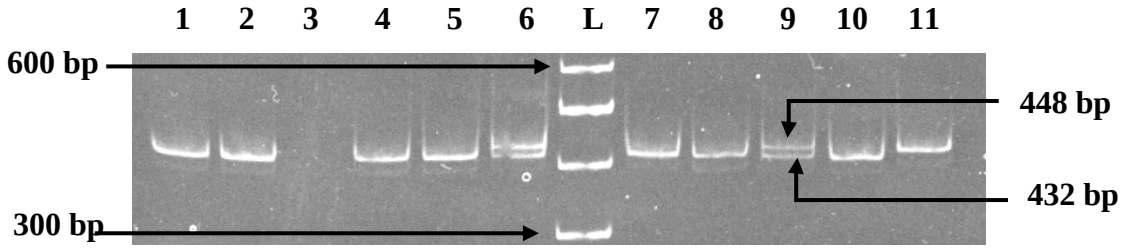
Further, aliquots of the same PCR products were subjected to restriction digestion with *BstUI*. The products were separated by 6% PAGE and visualized after staining with Etbr under UV light in gel documentation system. There were four DNA fragments of different sizes. Pro/Pro homozygotes produced band of 448 bp DNA fragment with the intron 3 duplication or produced band of 432 bp DNA fragment without the intron 3 duplication; Pro/Arg heterozygotes produced bands of 448 bp/432 bp, 246 bp/230 bp (depending upon the presence or absence of intron 3 duplication) and 202 bp DNA fragments. Arg/Arg homozygotes produced two bands of 246 bp and 202 bp DNA fragments with the intron 3 duplication or produced two bands of 230 bp and 202 bp DNA fragments without intron 3 duplication (Figure 3.4).

Reagents:

- PCR Mastermix (2X): 0.4 mM of each dNTPs, 4.0 mM $MgCl_2$ and 0.05 units/µl Taq DNA polymerase
- Primers
- Nuclease free water
- Gene ruler: 100-1000 bp DNA ladder and PUC19/MspI digest
- Restriction Enzyme: *BstUI*
- Polyacrylamide gel solution: 30.0% Acrylamide:bisacrylamide (6.0 ml), 5X TBE (6.0 ml), TDW water (17.65 ml), 10% Ammonium persulfate (APS) (210 µl), N,N,N',N'-tetramethylethane-1,2-diamine (TEMED) (21 µl)
- DNA gel loading buffer: 2.5 ml Glycerol; 1.2 ml TE buffer (pH 8.0); BPB (0.025%); TDW 1.3 ml

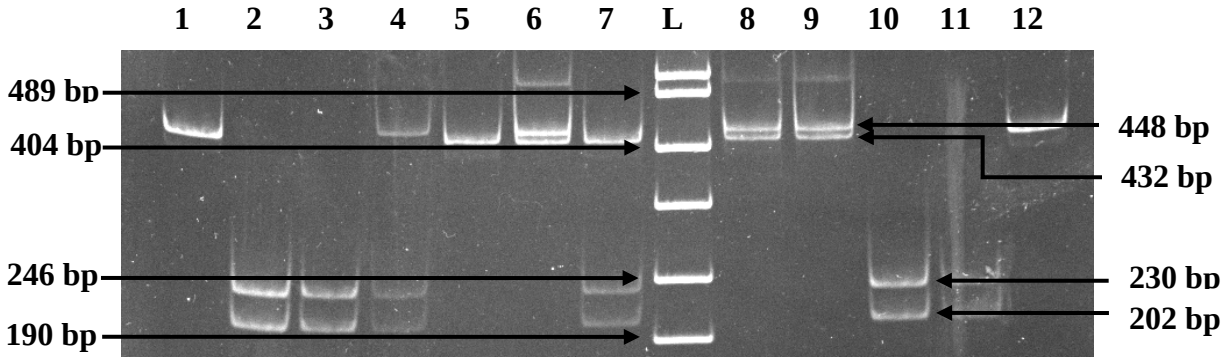
- 1X TBE buffer
- Staining solution: 0.5 µg EtBr/ml TDW

Figure 3.3: Representative pattern for three genotypes of *p53* intron 3 (16 bp duplication) polymorphism



Lanes 1, 2, 4, 5, 7, 8, 10 represent homozygous for absence of 16 bp duplication (A1/A1)
Lanes 6,9 represent heterozygous for 16 bp duplication 9 (A1/A2)
Lane 11represents homozygous for presence of 16 bp duplication (A2/A2)
Lane 3 represents Negative control
Lane L= 100 bp ladder

Figure 3.4: Representative pattern for three genotypes of *p53* exon 4 (Arg72Pro) polymorphism after *Bst*UI digestion



Lanes 1, 5, 6, 8, 9, 12 represent Pro/Pro homozygous
Lanes 4,7 represent Arg/Pro heterozygous
Lanes 2, 3, 10, 11 represent Arg/Arg homozygous
Lane L = PUC19/Msp1 digest DNA ladder

6.2. Genotyping of p53 intron 6 (rs1625895) polymorphism

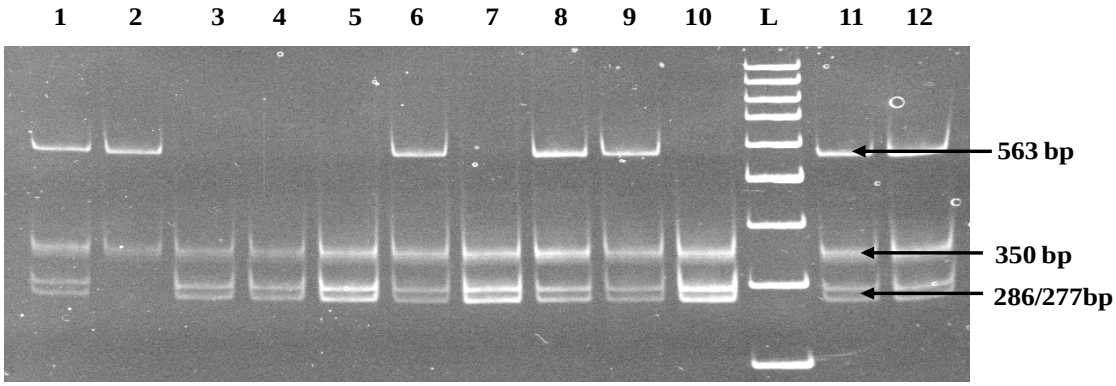
Intron 6 was amplified separately using primers as described previously (Mitra *et al.*, 2003). The PCR was carried out in a 25 µl reaction mixture containing 100 ng genomic DNA, 12.5 µl of PCR mastermix, 0.2 µM of each forward and reverse primers and nuclease free water. The primers used were 5'-GTA AAG CTT GAG CGC TGC TCA GAT AGC GAT-3' (forward) and 5'-ACA AAG CTT CCT GGA GTC TTC CAG TGT GAT-3' (reverse). The amplification was performed by initial denaturation at 95^0C for 3 minutes, followed by 30 cycles of denaturation at 94^0C for 1 minute, annealing at 58.0^0 C for 1 minute, extension at 72^0C for 1 minute and a final extension at 72^0C for 10 minutes. After PCR, amplification was checked on 1.5%

agarose gel. The 913 bp PCR products were digested with *NciI*, separated by 6% PAGE and visualized after staining with Etbr under UV light in gel documentation system. Homozygotes for the absence of *NciI* restriction site produced band of 563 bp DNA fragment (A/A); heterozygotes for the *NciI* restriction site produced bands of 563 bp, 286 bp and 277 bp DNA fragments (G/A); homozygotes for the presense of *NciI* restriction site produced two bands of 286 bp and 277 bp DNA fragments (G/G). All the three types of individuals contained band of 350 bp DNA fragment due to presence of a nonpolymorphic NciI site in the amplicon (Figure 3.5).

Reagents:

- Gene ruler: 100-1000 bp DNA ladder
- Restriction Enzyme: *NciI*
- 1.5% Agarose Gel
- All other reagents are same as mentioned in *p53* exon 4 and intron 3 genotyping (section 6.1)

Figure 3.5: Representative pattern for three genotypes of *p53* intron 6 (G>A) polymorphism after *Nci*I digestion



Lane 2 represents homozygous for absence of *Nci*I restriction site (A/A)
Lanes 1,6,8,9,11,12 represent heterozygous for *Nci*I restriction site (A/G)
Lanes 3,4,5,7,10 represent homozygous for presense of *Nci*I restriction site (G/G)
Lane L = 100 bp ladder

6.3. Genotyping of MDM2 (SNP 309 T>G, rs rs2279744) polymorphism

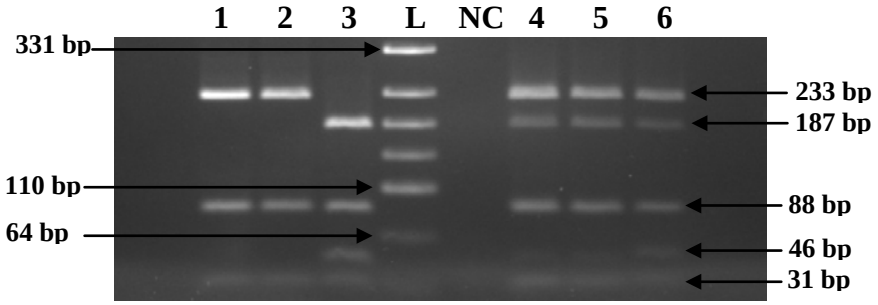
MDM2 SNP309 was genotyped by the PCR-RFLP assay. The primers used in the PCR-RFLP for *MDM2* SNP309 were: 5'-CGGGAGTTCAGGGTAAAGT-3' (forward) and 5'-AGCAAGTCGGTGCTTACCTG-3' (reverse) (Hu *et al.*, 2011). PCR was carried out in a 25 µl reaction mixture containing 300 ng genomic DNA, 12.5 µl of PCR mastermix, 0.1 µM of each forward and reverse primer and nuclease free water. The amplification was performed by initial denaturation at 95^0C for 5 minutes, followed by 38 cycles of denaturation at 94^0C for 30 seconds, annealing at 60.0^0 C for 45 seconds, extension at 72^0C for 45 seconds and a final extension at 72^0C for 5 minutes. After PCR, amplification was checked on 1.5% agarose gel. The 352

bp PCR products were digested with *MSPA1I*, separated on 2.5% agarose gel stained with Etbr and visualized under UV light in gel documentation system. The T/T genotype produced three bands of 233, 88, and 31 bp; G/T genotype produced 233, 187, 88, 46, and 31 bp and G/G genotype produced 187, 88, 46, and 31 bp (Figure 3.6).

Reagents:

- PCR Mastermix (2X): 0.4 mM of each dNTPs, 4.0 mM MgCl2 and 0.05 units/µl Taq DNA polymerase
- Primers
- Nuclease free water
- Gene ruler: 100-1000 bp DNA ladder
- Restriction Enzyme : *MspA1I* (New England BioLabs, MA)
- 1.5% and 2.5% Agarose Gel
- DNA gel loading buffer: 2.5 ml Glycerol; 1.2 ml TE buffer (pH 8.0); BPB (0.025%); TDW 1.3 ml
- 1X TBE buffer
- Staining solution: 0.5 µg EtBr/ml TDW

Figure 3.6: Representative pattern for three genotypes of *MDM2* (SNP309 T>G) polymorphism after *MspA1I* digestion



Lanes 1, 2 represent T/T homozygous
Lanes 4, 5, 6 represent G/T heterozygous
Lane 3 represents G/G homozygous
Lane L = PUC19/Msp1 digest DNA ladder
Lane NC= Negative control

7. *p53* mutation analysis by Polymerase Chain Reaction-Single Strand Conformational Polymorphism (PCR-SSCP) followed by DNA sequencing

7.1. PCR-SSCP

Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis is one of the simplest and most sensitive methods for detection of mutations (Hayashi, 1991). PCR is used to amplify the region of interest and the resultant DNA is denatured and separated as single-stranded molecules by electrophoresis in a non-denaturing polyacrylamide gel. A strand of single-stranded DNA folds differently

from another if it differs by a single base, resulting in different mobilities for the two strands (mutation-induced changes of tertiary structure of the DNA).

Steps involved in SSCP:

1. *Amplification of the target region of the gene by PCR and agarose electrophoresis of amplified PCR products*

PCR was performed for exons 4-9. The PCR reaction was carried out in a total volume of 25 µl, containing 100 ng genomic DNA, 12.5 µl PCR mastermix, 0.2 µM of each forward and reverse primers and nuclease free water as described by Saranath *et al.* (1999) (Table 3.4).

Table 3.4: Primers used for amplification of exon 4 to 9 of *p53* gene

Exon 4	F	5'-TGG ATG ATT TGA TGC TGT CCC-3'	256 bp
	R	5'- CGT GCA AGT CAC AGA CTT GGC-3'	
Exon 5	F	5'-TAC TCC CCT GCC CTC AAC AA-3'	186 bp
	R	5'-CAT CGC TAT CTG AGC AGC GC-3'	
Exon 6	F	5'-GTC TGG CCC CTC CTC AGC AT-3'	111 bp
	R	5'-CTC AGG CGG CTC ATA GGG CA-3'	
Exon 7	F	5'-TCT CCT AGG TTG GTC CTG AC-3'	133 bp
	R	5'-CAA GTG GCT CCT GAC CTG GA-3'	
Exon 8-9	F	5'-CCT ATC CTG AGT AGT GGT AAT C-3'	332 bp
	R	5'-CCC AAG ACT TAG TAC CTG AAG-3'	

The reaction mixture was subjected to cycles of denaturation, annealing, extension as mentioned in table 3.5. The PCR products after amplification were checked on 1.5% EtBr stained agarose gel under UV light in gel documentation system.

Table 3.5: PCR condition for amplification of exon 4 to 9

Steps	Initial Denaturation	Denaturation	Annealing	Extension	No. of Cycles	Final Extension
Exon 4	94^{o}C/3 mins	94^{o}C/1 min	67^{o}C/2 min	72^{o}C/1 min	30	10 mins
Exon 5	94^{o}C/3 mins	94^{o}C/1 min	53.4^{o}C/1 min	72^{o}C/1 min	30	10 mins
Exon 6	94^{o}C/3 mins	94^{o}C/1 min	57^{o}C/1 min	72^{o}C/1 min	30	10 mins
Exon 7	94^{o}C/3 mins	94^{o}C/1 min	52.3^{o}C/1 min	72^{o}C/1 min	30	10 mins
Exon 8-9	94^{o}C/3 mins	94^{o}C/1 min	46.7^{o}C/1 min	72^{o}C/1 min	30	10 mins

Reagents:

- PCR Mastermix (2X): 0.4 mM of each dNTPs, 4.0 mM MgCl2 and 0.05 units/µl Taq DNA polymerase
- Primers
- Nuclease free water
- Gene ruler: 100-1000 bp DNA ladder
- 1.5% Agarose Gel
- DNA gel loading buffer: 2.5 ml Glycerol; 1.2 ml TE buffer (pH 8.0); BPB (0.025%); TDW 1.3 ml

- 1X TBE buffer
- Staining solution: 0.5 µg EtBr/ml TDW

2. *Denaturation of the PCR product, electrophoresis of the single-sanded DNA through the non-denaturing gel and detection of bands by silver staining*

2.0 µl of amplified samples each for exon 4, 5, 6, 7, 8 and 9 was denatured by mixing the samples in 9.0 µl of denaturing dye, 2.0 µl 100mM NaOH and heating it to 95ºC. Denatured samples were immediately kept on ice and then loaded onto 6.0% of non-denaturing polyacrylamide gel. After electrophoresis at low temperatures (4ºC and 20^0C) at constant 160V for each plate, the gels were silver stained to detect the mobility shift and scanned in gel documentation system.

Reagents:

- Formamide -loading Buffer (Denaturing dye): Formamide (95%), EDTA (20mM) (pH 8.0), BPB (0.05%), Xylene cynol (0.05%)
- NaOH (100 mM)
- Polyacrylamide gel solution: 30.0% Acrylamide:bisacrylamide (6.0 ml), 50% Glycerol (6.0 ml), 1X TBE (7.5 ml), Deionised water (9.17 ml), 10% APS (300 µl), TEMED (30 µl)
- Gene ruler: 100-1000 bp DNA ladder
- DNA gel loading buffer: 2.5 ml Glycerol; 1.2 ml TE buffer (pH 8.0); BPB (0.025%); TDW 1.3 ml
- 1X TBE buffer
- Fixative: 9.5% ethanol; 0.5% glacial acetic acid
- Staining solution : 0.1% AgNO3
- Developing solution: 1.5% NaOH; 2 ml 37% formaldehyde

7.2. DNA Sequencing

Sample showing mobility shift in SSCP gels were further subjected to DNA sequencing (Chromos Biotech Private limited, Bangalore). Sequencing data were analyzed using mutation surveyor software (version 4.0.9) as well as manually by considering NM_000546 as a reference sequence.

8. Detection of HPV 16 and 18 by Type specific PCR

Type specific PCR (TS-PCR) was carried out using HPV 16 and 18 specific primers and *β-globin* as internal control (Jain *et al.*, 2005, table 3.6).

Table 3.6: Primer sequence used for amplification of *HPV16* and *HPV18* gene

HPV16	F	5’-TGG ATG ATT TGA TGC TGT CCC-3’	217 bp
	R	5’- CGT GCA AGT CAC AGA CTT GGC-3’	
HPV18	F	5’-TAC TCC CCT GCC CTC AAC AA-3’	100 bp
	R	5’-CAT CGC TAT CTG AGC AGC GC-3’	
β-globin	F	5’-GTC TGG CCC CTC CTC AGC AT-3’	268 bp
	R	5’-CTC AGG CGG CTC ATA GGG CA-3’	

25 µl PCR reaction mixture was prepared by mixing 12.5 µl of PCR master mix, 100 ng DNA, 0.2µM of each forward and reverse primers and nuclease free water. The PCR conditions for HPV 16 were as follows: initial denaturation at 94 ^{o}C for 3 minutes, followed by 35 cycles of denaturation at 94 ^{o}C for 1 minute, annealing at 52.1 ^{o}C for 30 seconds, extension at 72 ^{o}C for 30 seconds which was extended for 5 minutes at the final cycle. For HPV 18, the PCR conditions were: initial denaturation at 94 ^{o}C for 3 minutes, followed by 30 cycles of denaturation at 94 ^{o}C for 1 minute, annealing at 47.5 ^{o}C for 1 minute, extension at 72 ^{o}C for 1 minute which was extended for 10 minutes at the final cycle. HPV 16 and 18 positive cervical tissues were used as positive controls. After PCR, PCR products were confirmed for their respective amplicon size on 1.5% EtBr stained agarose gel with known size DNA ladders under UV light in gel documentation system.

Reagents:

- Gene ruler: 100-1000 bp DNA ladder (Fermentas, canada) and PUC19/MspI digest (Genei, India)
- All other reagents are same as mentioned in *p53* exon 4-9 amplification method (section 7.1)

9. RT-PCR for *hTERT*, *VEGFA* isoforms, *VEGFC, VEGFD, MMP2* and *MMP9*

One step RT-PCR was carried out using one step RT-PCR kit and primers mentioned earlier (Nakamura *et al.*, 1997, George *et al.*, 2001, Edwards, 2000) listed in table 3.7. The amplifications were performed on a thermal cycler. The reaction mixture was prepared by adding 1X of RT-PCR buffer, 400µM dNTPs, One step RT-PCR Enzyme mix, 500 ng RNA, 0.6µM of each forward and reverse primers and nuclease free water. The RT-PCR reaction conditions are mentioned in table 3.8.

β-actin was used as an internal control. PCR products were run on a 1.5% agarose gel for *hTERT*, *VEGFC*, *VEGFD* and *MMP2* and *MMP9*. For *VEGFA* isoforms, PCR products were run on a 6% PAGE. Image was captured and analyzed by gel documentation system. The figure 3.7, 3.8, 3.9, 3.10, 3.11 and 3.12 show representative patterns for *hTERT*, *VEGFA* isoforms, *VEGFC*, *VEGFD*, *MMP2* and *MMP9* mRNA expression from malignant as well as adjacent normal tissues, respectively. The band intensity of target gene as well as *β-actin* gene was quantified. Ratio of target gene to *β-actin* was calculated to find out the expression index of target gene.

Table 3.7: Primers used for RT-PCR analysis of *hTERT, VEGFA, VEGFC, VEGFD, MMP2* and *MMP9*

hTERT	F 5'-CGG AAG AGT GTC TGG AGC AA-3'	150 bp
	R 5'-GGA TGA AGC GGA GTC TGG A-3'	
VEGFA	F 5'-CTC ACC AAG GCC AGC ACA TAG G-3'	159,291, 363,345, 414 bp
	R 5'-ATC TGG TTC CGA AAA CCC TGA G-3'	
VEGFC	F 5'-GTC TGT GTC CAG TGT AGA TG-3'	360 bp
	R 5'-AGG TAG CTC GTG CTG GTG TT-3'	
VEGFD	F 5'-CAG TGA AGC GAT CAT CTC AGT C-3'	393 bp
	R 5'-TAC GAG GTG CTG GTG TTC ATA C-3'	
MMP2	F 5'-GGC CCT GTC ACT CCT GAG AT-3'	474 bp
	R 5'-GGC ATC CAG GTT ATC GGG GA-3'	
MMP9	F 5'-TGG ACG ATG CCT GCA ACG TG-3'	455 bp
	R 5'-GTC GTG CGT GTC CAA AGG CA-3'	
β-actin	F 5'-GAG AAG ATG ACC CAG ATC ATG T-3'	463 bp
	R 5'-ACT CCA TGC CCA GGA AGG AAG G-3'	
	F 5'-GGT CAC CCA CAC TGT GCC CAT-3'	350 bp
	R 5'-GGA TGC CAC AGG ACT CCA TGC-3'	

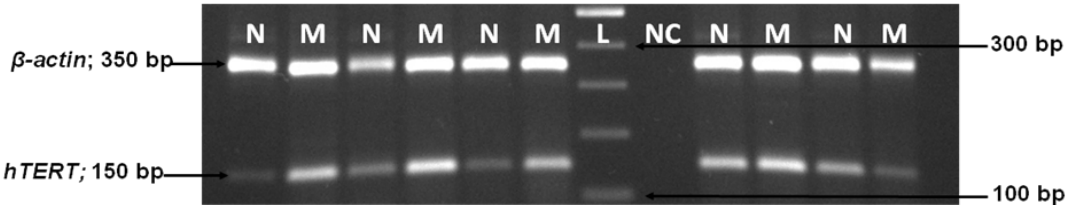
Table 3.8: RT-PCR conditions for *hTERT, VEGFA, VEGFC, VEGFD, MMP2* and *MMP9*

Genes	***hTERT***	***VEGFA***	***VEGFC***	***VEGFD***	***MMP2***	***MMP9***
Reverse Transcription- cDNA synthesis - 30 minutes at 50^0C						
Initial PCR activation 15 minutes at 95^0C						
Denaturation	94^0C; 1min	94^0C; 1min	94^0C; 1min	94^0C; 1min	94^0C; 1min	94^0C; 1min
Annealing	52.4^0C; 1min	53.6^0C; 30 Sec	53.6^0C; 1min	53.6^0C; 1min	63^0C; 1min	69^0C; 30 Sec
Extension	72^0C; 30 Sec	72^0C; 30 Sec	72^0C; 30 Sec	72^0C; 30 Sec	72^0C; 30 Sec	72^0C; 30 Sec
No. of cycles	40	35	35	35	30	34
Final extention	72^0C; 5 mins	72^0C; 5 mins	72^0C; 5 mins	72^0C; 5 mins	72^0C; 5 mins	72^0C; 5 mins

Reagents:

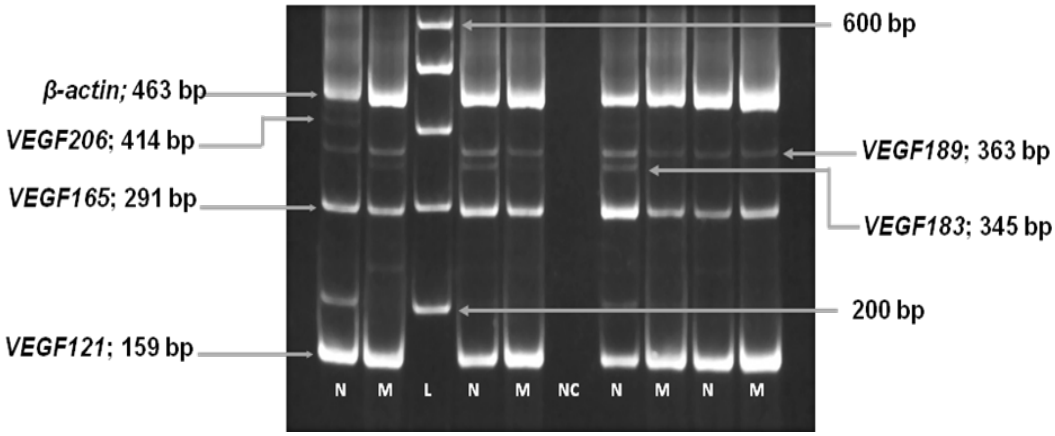
- One Step RT-PCR Kit
- Primers
- Nuclease free water
- Gene ruler: 100-1000 bp DNA ladder
- 1.5% Agarose Gel
- Polyacrylamide gel solution: 30.0% acrylamide:bisacrylamide (6.0 ml), 5X TBE (6.0 ml), TDW water (17.65 ml), 10% APS (210 µl), TEMED (21 µl)
- DNA gel loading buffer: 2.5 ml Glycerol; 1.2 ml TE buffer (pH 8.0); BPB (0.025%); TDW 1.3 ml
- 1X TBE buffer
- Staining solution: 0.5 µg EtBr/ml TDW

Figure 3.7: Representative pattern for *hETRT* mRNA expression using RT-PCR



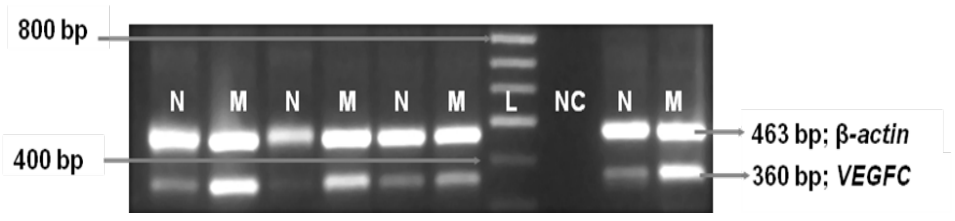
Lanes N represents bands of *hTERT* (150 bp) and housekeeping gene as internal control i.e. *β-actin* (350 bp) in adjacent normal tissues
Lanes M represents *hTERT* and *β-actin* in malignant tissues
Lane L is 100 bp ladder
Lane NC represents negative control

Figure 3.8: Representative pattern for mRNA expression of *VEGFA* isoforms using RT-PCR



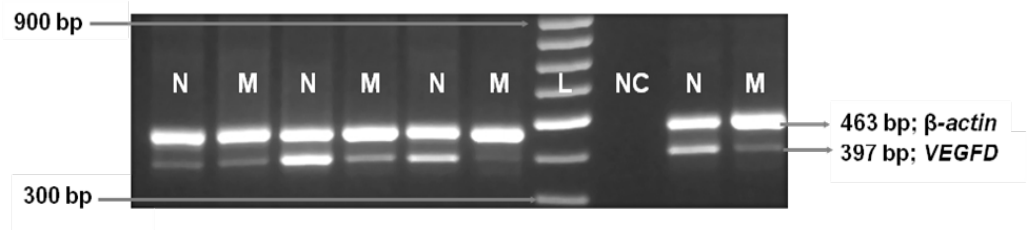
Lanes N represents bands of *VEGFA* isoforms, i.e. *VEGF206* (414 bp), *VEGF189* (363 bp), *VEGF183* (345 bp), *VEGF165* (291 bp), *VEGF121* (159 bp) and housekeeping gene as internal control i.e. *β-actin* (463 bp) in adjacent normal tissues
Lanes M represents *VEGFA* isoforms and *β-actin* in malignant tissues
Lane L is 100 bp ladder
Lane NC represents negative control

Figure 3.9: Representative pattern for *VEGFC* mRNA expression using RT-PCR



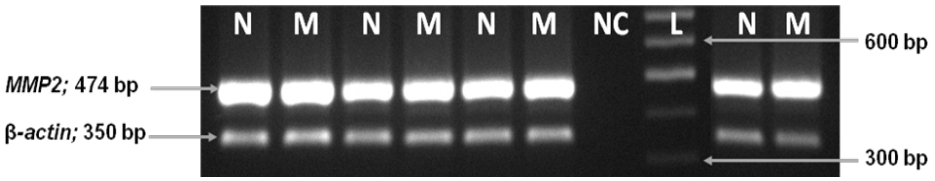
Lanes N represents bands of *VEGFC* (360 bp) and housekeeping gene as internal control i.e. *β-actin* (463 bp) in adjacent normal tissues
Lanes M represents *VEGFC* and *β-actin* in malignant tissues
Lane L is 100 bp ladder
Lane NC represents negative control

Figure 3.10: Representative pattern for *VEGFD* mRNA expression using RT-PCR



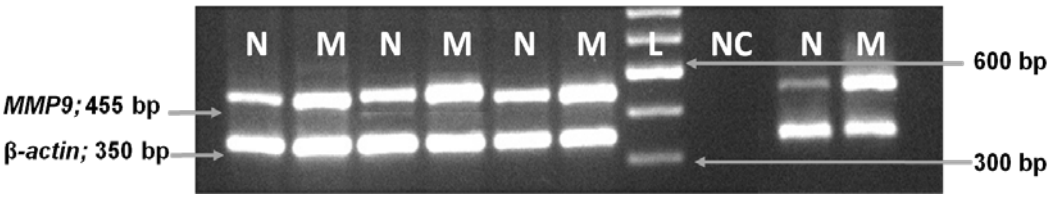
Lanes N represents bands of *VEGFD* (397 bp) and housekeeping gene as internal control i.e. *β-actin* (463 bp) in adjacent normal tissues
Lanes M represents *VEGFC* and *β-actin* in malignant tissues
Lane L is 100 bp ladder
Lane NC represents negative control

Figure 3.11: Representative pattern for *MMP2* mRNA expression using RT-PCR



Lanes N represents bands of *MMP2* (474 bp) and housekeeping gene as internal control i.e. *β-actin* (350 bp) in adjacent normal tissues
Lanes M represents *MMP2* and *β-actin* in malignant tissues
Lane L is 100 bp ladder
Lane NC represents negative control

Figure 3.12: Representative pattern for *MMP9* mRNA expression using RT-PCR



Lanes N represents bands of *MMP9* (455 bp) and housekeeping gene as internal control i.e. *β-actin* (350 bp) in adjacent normal tissues
Lanes M represents *MMP9* and *β-actin* in malignant tissues
Lane L is 100 bp ladder
Lane NC represents negative control

10. Enzyme-Linked Immunosorbent assay for estimation of VEGF-A, VEGF-C and VEGF-D

For the quantitation of serum VEGF-A, VEGF-C and VEGF-D, ELISA was performed using commercially available ELISA kits (R&D system, USA).

Principle: This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for VEGF-A, VEGF-C and VEGF-D has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any VEGF-A, VEGF-C and VEGF-D present is bound by the immobilized

antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for VEGF-A, VEGF-C and VEGF-D is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of VEGF-A, VEGF-C and VEGF-D bound in the initial step. The color development is stopped and the intensity of the color is measured at 450nm.

Procedure: The ELISA assays for VEGF-A, VEGF-C and VEGF-D was carried out from serum samples on a 96-well microtitre plate as per manufacturer's instructions. 100 µl serum samples for VEGF-A, 50 µl 5 fold diluted serum for VEGF-C and 50 µl serum samples for VEGF-D were added to the assay. Standards were diluted as per manufacturer's instructions**.** The immobilized antibody binds to VEGF-A, VEGF-C and VEGF-D present in the standards and samples, which were pipetted onto the wells. In all analyses, the wells were thoroughly washed with the wash buffer between each step of the procedure. After washing away unbound substances, an enzyme-linked polyclonal antibody specific for VEGF-A, VEGF-C and VEGF-D were added to the wells. It was followed by a wash to remove unbound antibody-enzyme reagent. Then, a substrate solution was added to the wells which resulted in to color development in proportion to the amount of total serum VEGF-A, VEGF-C and VEGF-D bound in the initial step. The reaction was stopped and the intensity of the color was measured on ELISA reader 450 nm and wavelength correction was done at 540 nm. Protein levels were measured in duplicate for each sample in order to minimize intra-assay variation. The absorbance values for standards and the standard curves constructed for each assay were compared and used to calculate inter-assay variations. Serum VEGF-A, VEGF-C and VEGF-D concentrations in the samples was determined by comparison of the optical density of the samples to the standard curves.

11. Expression of MMP-2 and MMP-9 by gelatin zymography

Activity of gelatinases (MMP-2 and MMP-9) was analyzed from the euglobulin fractions obtained from heparinized plasma samples of the subjects using gelatin zymography following procedure by Lorenzo *et al.* (1992) with minor modifications.

Zymography principle:

In zymography, the proteins are separated by electrophoresis under denaturing, nonreducing conditions. The separation occurs in a polyacrylamide gel containing a

specific substrate (gelatin) that is co-polymerized with the acrylamide. During electrophoresis, the SDS causes the MMPs to denature and become inactive. After electrophoresis, the gel is washed, which causes the exchange of the SDS with Triton® X-100, after which the enzymes partially renature and recover their activity. Additionally, the latent MMPs are autoactivated without cleavage. The activation of latent MMPs during zymography is believed to involve the "cysteine switch" because the dissociation of Cys73 from the zinc molecule is caused by SDS. Subsequently, the gel is incubated in an appropriate activation buffer. During this incubation, the concentrated renatured MMPs in the gel will digest the substrate (gelatin). After incubation, the gel is stained with Coomassie Brilliant Blue (CBB) and the MMPs are detected as clear bands against a blue background of undegraded substrate. The clear bands in the gel can be quantified by densitometer.

Procedure:

Zymography was performed using SDS-PAGE containing 0.5 mg/ml gelatin.

Electrophoresis of samples:

- Prepare 7.5% running gel with 0.5mg/ml gelatin and 5% staking gel
- 1:1 diluted samples (euglobulin fractions) with 2x sample buffer were subjected to 7.5% SDS- PAGE under non-reducing conditions.
- The gels were run at constant voltage (60V) for 1hr (till the tracking dye pass through the staking gel). Then it was increased to 120V till tracking dye reaches to the bottom.

Activation of gelatinases:

- After electrophoresis the gels were taken out gently from the glass plates and kept in washing buffer (0.25% Triton x-100) for 15 min to remove the SDS. Repeat this step.
- This was followed by overnight incubation of gels at RT in activation buffer.

Staining of gels and detection of gelatinases

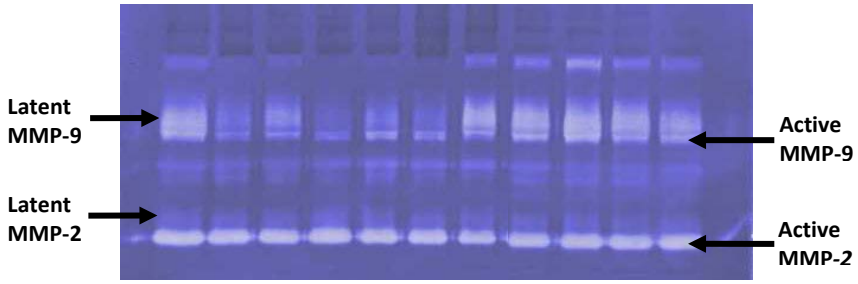
- Next day, the incubation buffer was decanted and gels were stained with 0.1% (w/v) CBB-R250 for 1 hour.
- After staining, gels were distained with 7% acetic acid.

- After distaining, the gelatinase activity was detected as unstained white bands on a blue stained background of gels, indicating the proteolysis of the gelatin substrate (Figure 3.13).
- These zymograms were quatitated using gel documentation system.

Reagents:

1. Stock Acrylamide: 30.0% acrylamide:bisacrylamide
2. 0.5% gelatin solution
3. 10% APS
4. TEMED
5. Running gel buffer: 1.5 M Tris HCl (pH 8.8) and 0.4% SDS
6. Stacking gel buffer: 1 M Tris HCl (pH 6.8) and 0.8% SDS
7. 2x Sample Buffer: 0.125M Tris HCl (pH 6.8); 0.004% BPB; 20% (v/v) Glycerol; 4% SDS
8. 1X Electrode buffer: 0.025 M Tris base; 0.192 M of Glycine and 0.2% SDS (pH 8.3)
9. Activation Buffer: 50 mM of Tris HCl (pH 7.5) containing 10 mM $CaCl_2$; 1μM $ZnCl_2$; 1% v/v Triton X-100 and 0.02% NaN_3
10. Washing solution: 0.25% (v/v) Triton X-100
13. Staining Solution: 0.1% CBB R-250 (w/v) in 40 % propanol
14. Destaining solution: 7% acetic acid

Figure 3.13: Representative gelatin zymogram for MMP-2 and MMP-9 protein expression



(C= Healthy individuals, S = Patients sample)

Statistical Analysis:

Statistical analysis was performed using statistical software (SPSS; version 15.0).

- Reproducibility of the assays for *p53* and *MDM2* genotyping, *p53* mutations and HPV infection were analyzed by repeating the samples.
- Power analysis shows that using 200 cases and controls each, this study has power of 90% of detecting association to risk allele with odds ratio as low as 2.0 and with minor allele frequency >0.1 when using QUANTO software (Version 1.2) (Gauderman and Morrison, 2006).
- The cases as well as controls were compared for genotype frequencies with the expected frequencies by the Hardy–Weinberg equilibrium (HWE) by

goodness-of-fit $\chi 2$ test. Pearson's $\chi 2$ test was performed to compare genotypic distributions between cases and controls. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated for the risk estimation of genotypes for oral cancer development. The highest proportion of a homozygous genotype at any particular locus in the control group was considered as referent genotype to estimate risk associated with these polymorphisms (Mitra *et al.*, 2005). Frequencies of the pair-wise and extended haplotypes resulting from 3 polymorphisms in the *p53* gene were estimated using PHASE software (Stephens *et al.*, 2001). ORs and 95% CIs were also calculated to assess the association of genotypes with age, habit, clinico-pathological parameters and recurrence of the disease.

- Pearson's $\chi 2$ test was performed to analyze association of *p53* mutations and age, habit, clinico-pathological parameters as well as recurrence of the disease. ORs and 95%CIs were also calculated to study the association of *p53* mutations with tobacco habit, clinico-pathological parameters and recurrence of the disease.
- mRNA as well as protein levels were expressed as Mean ± SEM. The samples were analyzed in duplicates. The inter assay and intra assay co-efficient of variations were less than 10%.
- Paired "t" test was used to compare mRNA levels between malignant and adjacent normal tissues. Independent "t" test was used to compare protein levels between controls and oral cancer cases. Independent "t" test as well as Multivariate analysis were performed to analyze association of biomarkers with different clinico-pathological parameters. Independent "t" test as well as Multivariate analysis were performed to analyze association of biomarkers with recurrence of the disease according to various clinico-pathological parameters. Logistic regression analysis was performed as well as ORs and 95%CI were calculated to predict recurrence of the disease according to levels of biomarkers. Independent "t" test was carried out to compare mRNA and protein levels with *p53* genotypes and *p53* mutation status. Correlation between all the parameters was analyzed by Pearson correlation coefficient.
- Receiver's operating characteristic (ROC) curves were plotted and area under curves (AUCs) was estimated to calculate sensitivity and specificity of the biomarkers.

- Disease free survival (DFS) and overall survival (OS) were estimated by Kaplan Meier Survival curve analysis. Comparison of DFS and OS between various groups was performed using log-rank test. DFS was estimated from date of treatment initiation to occurrence of loco-regional recurrence or last follow-up date. OS was estimated from date of diagnosis to date of death or last follow-up date. Mean transcript levels of primary tumors were set as cut-off and tumors were categorized into having low and high transcript levels. Patients were also categorized into having low and high protein levels. Mean protein levels of controls were set as cut-off. Univariate and multivariate cox proportional hazard regression analysis performed to estimate additional prognostic values of the biomarkers.
- $p<0.05$ was considered as statistically significant for all the assays.