IMMUNOHISTOCHEMICAL EVALUATION OF $_{P}$53 AND $_{P}$63 IN ORAL SQUAMOUS CELL CARCINOMA, ORAL LEUKOPLAKIA, ORAL SUBMUCOUS FIBROSIS AND NORMAL ORAL MUCOSA

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CERTIFICATE

This is to certify that this dissertation titled "**IMMUNOHISTOCHEMICAL EVALUATION OF p53 AND p63 IN ORAL SQUAMOUS CELL CARCINOMA, ORAL LEUKOPLAKIA, ORAL SUBMUCOUS FIBROSIS AND NORMAL ORAL MUCOSA**" is a bonafide dissertation performed by **VARUN.B.R** under our guidance during the postgraduate period 2007-2010.

This dissertation is submitted to THE TAMILNADU DR. M.G.R MEDICAL UNIVERSITY, in partial fulfillment for the degree of **MASTER OF DENTAL SURGERY** in **ORAL PATHOLOGY AND MICROBIOLOGY, BRANCH VI**. It has not been submitted (partial or full) for the award of any other degree or diploma.

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Oral cancer constitutes the sixth most common cancer worldwide and third most common cancer in South-East Asia[1]. Oral squamous cell carcinoma (OSCC), the most common type of oral cancer is often preceded by potentially malignant lesions or conditions such as leukoplakia and oral submucous fibrosis (OSF). On biopsy, most leukoplakias show histologic features of epithelial dysplasia. The standardization of histopathological diagnosis and grading of epithelial dysplasia remains subjective as there are many composite histologic criteria of cellular atypia and architectural disturbances[2]. OSF is a well recognized potentially malignant condition that is characterized by rigidity of oral mucosa and development of palpable fibrous bands, resulting from the deposition of collagen in juxtaepithelial and submucosal layers. The malignant transformation rate for leukoplakia ranges from 15-20%[3], while transformation rates as high as 7.6% over a 10-year period have been reported for OSF[4].

It has been generally considered that oral carcinogenesis develops through a multistep process of accumulation of genetic mutations related to cell proliferation and differentiation. The principal targets of genetic damage include growth-promoting protooncogenes, growth-inhibiting tumor suppressor genes and genes that regulate apoptosis. Mutations in the tumor suppressor p53 gene and resultant alteration in the protein are the most common abnormalities found in squamous cell carcinoma of the head and neck region[2,5].The p53 gene encodes a 53 kD nuclear phosphoprotein that is involved in DNA repair, programmed cell death and negative regulation of cell cycle. In normal cells, wild type p53 protein has a very short half life (6-20 minutes) and is present in such small quantities that it cannot be detected by immunohistochemical methods. However, mutations in p53 gene often result in a more stable product and

prolong the half life of p53 protein, causing it to accumulate within cell nuclei to the extent that it can be easily detected by immunohistochemistry[5].

The p63 gene mapped on chromosome 3q27-29 is a member of p53 gene family and is responsible for the transcription two groups of p63 protein (TAp63 and ΔNp63), both of which have α, β and γ isoforms. The TAp63 group contains an N-terminal transactivation domain and has functions similar to p53 such as, cell cycle arrest, apoptosis and cell differentiation. The ΔNp63 group lacks TA (Transactivation) domain and acts by inhibiting both p53 and TA p63 and thus favors cell proliferation[6]. It is suggested that p63, possibly in concert with p53 may play a role in the regulation of proliferation and differentiation in potentially malignant disorders and malignant lesions of the oral cavity[7].

This study is done to evaluate the expression of p53 and p63 proteins in OSCC, oral leukoplakia and OSF by immunohistochemistry.

AIMS AND OBJECTIVES:

1. To evaluate the expression of p53 and p63 proteins in formalin fixed, paraffin embedded sections of OSCC, oral leukoplakia and OSF specimens by immunohistochemistry.

2. To compare the expression of p53 and p63 proteins in formalin fixed, paraffin embedded sections of OSCC, oral leukoplakia and OSF with normal oral mucosa by immunohistochemical methods.

HYPOTHESIS:

There is altered expression of p53 and p63 proteins in

- Oral squamous cell carcinoma
- Oral leukoplakia
- Oral submucous fibrosis

when compared to normal oral mucosa.

Study setting:

The study was conducted in the Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital, Chennai.

Study subjects:

20 consecutive cases of oral squamous cell carcinoma (Group I), 20 consecutive cases of clinically diagnosed oral leukoplakia (Group II), 20 consecutive cases of OSF (Group III), and 10 cases of normal patients (Group IV), were collected over a period of 6 months. A preformatted clinical case sheet was used to record all the cases. Detailed case history including age, sex, and occupation, past medical and dental history along with the history of habits were recorded. This was followed by general examination and intra oral examination.

Selection criteria:

Group I: Clinically and histopathologically confirmed cases of OSCC **(Figure 3)**.

Group II: Clinically appearing white, non-scrapable patch associated with the history of tobacco habit, which was clinically diagnosed as leukoplakia and histopathologically graded as epithelial dysplasia (mild, moderate or severe) **(Figure 4)**.

Group III: The criteria for selection of OSF patients were difficulty in opening the mouth, burning sensation of the mouth, palpable vertical fibrous bands in the oral mucosa and history of areca nut chewing **(Figure 5)**.

Group IV: Ten patients who reported to the outpatient department of Oral and Maxillofacial Surgery for removal of impacted third molar constituted the normal control group.

Incisional biopsy of sufficient width and depth to ensure inclusion of connective tissue was taken from the buccal mucosa of the 60 study patients. Normal non-inflammed buccal mucosa adjacent to the site of surgery was biopsied for the control group. Informed consent was obtained from all the patients. The tissues taken were immediately transferred to 10% buffered formalin for further processing. After adequate fixation, paraffin blocks of the tissues were made.

IMMUNOHISTOCHEMISTRY PROCEDURE:

Armamentarium (Figure1):

- Microtome
- Autoclave
- Hot air oven
- Couplin jars
- Measuring jar
- Refrigerator
- Weighing machine DHONA 200D
- Cyclomixer
- APES (3-amino propyl triethoxy silane) coated slides
- Slide carrier
- Slide warmer
- Aluminum foil
- Micropipettes
- Tooth forceps
- Electronic timer
- Beakers

- Rectangular steel trays with glass rods
- Sterile gauze
- Cover-slips
- Light microscope

Reagents for IHC:

1. Conc. HCL

2. Laxbro solution

3. APES (3-Amino Propyl Triethoxy Silane)

4. Acetone

5. Citrate buffer (pH 6)

6. Phosphate buffer saline (pH 7.2-7.4)

7. 3% hydrogen peroxide (H_2O_2)

8. Deionized distilled water

9. Haematoxylin

10. Eosin

1 l. Alcohol 70%

12. Absolute alcohol

13. Xylene

Antibodies used (Figure 2):

1. Primary antibody
    a. Monoclonal mouse anti-human p53 (DO7, Biogenex)
    b. Monoclonal mouse anti-human p63 (4A4, Biogenex)
2. Secondary antibody
    a. Enhancer (Biogenex secondary kit)
    b. Streptavidin HRP (Biogenex secondary kit)
3. Chromogen DAB (3-Diaminobenzidine Tetrahydrochloride)

Procedure:

Pretreatment of the slides:

- The slides were first washed in tap water for few minutes.
- They were then soaked in detergent solution for 1 hour.
- After 1 hour, each slide was brushed individually using the detergent solution and were transferred to distilled water.
- Slides were washed in two changes of distilled water.
- The slides were then immersed in 1 N HCL (100 ml HCL in 900 ml distilled water) overnight.
- The following day, slides were taken out of acid and washed in two changes of autoclaved distilled water.
- All the slides were then transferred to slide trays, wrapped in aluminum foil and baked in hot air oven for 4 hours at 180 degrees centigrade.

APES coating:

Slides were first dipped in couplin jar containing acetone for 2 minutes

↓

Dipped in APES for 5 minutes

↓

Dipped in two changes of distilled water for 2 minutes each

↓

Slides were left to dry

Preparation of sections:

After the slides were dry, tissue sections of 0.5 micron thickness were made in a rotary manual microtome. The ribbons of tissue section were transferred onto the APES coated slide from the tissue float bath such that two tissue bits come on to the slide with a gap in between. One of the tissue sections was labeled positive (P) and the other negative (N).

Immunohistochemistry procedure:

The slides with tissue sections were treated with three changes of xylene to remove paraffin wax. They were put in descending grades of alcohol and then rehydrated with water. Then the slides were transferred to citrate buffer and autoclaved for antigen retrieval at 15 lbs pressure for 15 minutes. The slides were allowed to cool and then washed in cold phosphate buffer (PBS) solution for 5 minutes. Slides were treated with 3% hydrogen peroxide for 10 minutes to quench endogenous peroxidase activity of cells that would otherwise result in non-specific staining. After blotting the excess, the slides were treated with protein block reagent for another 10 minutes. The slides were then wiped carefully without touching the

tissue section with gauze to remove excess protein bock reagent. Circles were drawn around the tissues, so that the antibodies added later on do not spread and are restricted to the circle. The primary antibody (p53; DO7) (Biogenex) was added only to P tissue on the slide and PBS was added to the N tissue to prevent drying. The petridish containing the slides was incubated at room temperature for 1 hour. For p63, primary antibody (A4A, Biogenex) was added to P tissue and incubated for 1 hour. The sections taken out were washed in three changes of cold PBS for 5 minutes each to remove the excess antibody. Then the slides were wiped carefully without touching the tissue section to remove excess PBS. Then a drop of enhancer from the secondary antibody kit (Biogenex) was added on both the sections and the slides were incubated for 20 minutes. Later slides were washed in three changes of cold PBS for 5 minutes each. The slides were wiped carefully without touching the tissue section to remove excess PBS. Then a drop of Streptavidin from the secondary antibody kit (Biogenex) was added on both the sections and the slides were incubated for 30 minutes. The sections were washed in 3 changes of cold PBS for 5 minutes each. The slides were wiped carefully without touching the tissue section to remove excess PBS. Then a drop of freshly prepared DAB (3' Diaminobenzidine Tetrahydrochloride - a substrate chromogen) was added on both sections. Slides were washed in PBS to remove excess DAB and then counter stained with Hematoxylin. The slides were placed in a tray of tap water for 5 minutes for the process of blueing. Then the slides were transferred to 70% alcohol, 100% alcohol and xylene. The tissue sections were mounted with DPX. Slides were then observed under the microscope. Throughout the procedure care was taken not to dry the tissues.

Positive controls:

Oral squamous cell carcinoma (OSCC) tissue was used as a positive control. p53 and p63 staining in OSCC were used as a standard benchmark to evaluate the intensity of staining among the study groups.

Criteria for evaluation of p53 and p63 staining:

- Localization of stain – staining was limited only to basal layers of the epithelium or seen both in basal and supra basal layers.
- Labelling index (LI) was calculated by dividing the number of positive cells by the total number of cells counted in the slide and expressed as percentage. A minimum of thousand cells was counted for each slide.

$$LI = \frac{\text{Number of positive cells}}{\text{Total number of cells counted}} \times 100$$

IHC procedure flow chart:

APES coated slides with paraffin embedded tissue

↓

Placed in xylene I (5 min)

↓

Placed in xylene II (5 min)

↓

Placed in xylene III (5 min)

↓

Placed in 100% isopropanol (5min)

↓

Placed in 70% isopropanol (5min)

↓

Washed in distilled water thrice (5 min each)

↓

Kept in citrate buffer, autoclaved and allowed to cool

↓

Washed in PBS (5 min)

↓

Placed in 3% hydrogen peroxide (10 min)

↓

Blot excess

↓

Protein block (10 min)

Blot excess

Primary antibody added and incubated (1 hour)

Washed in PBS thrice (5 min each)

Secondary antibody (Enhancer) (20 min)

Washed in PBS thrice (5 min each)

Streptavidin HRP (30 min)

Washed in PBS thrice (5 min each)

DAB (chromogen)

Washed in PBS thrice (5 min each)

Stained with haematoxylin

Washed in tap water (5 min)

Placed in 70% isopropanol (1 min)

Placed in 100% isopropanol (1 min)

Placed in xylene (1 dip)

Slides were mounted using DPX

Slides were observed under the microscope

Statistical analysis was done using SPSS software (version 11.0.5). p value less than 0.05 was considered to be statistically significant.

- Comparison of the mean age among the groups was done using ANOVA
- Pearson's Chi-square test was done to compare the distribution of gender and habits among the study groups.
- Mean labeling index and localization of p53 and p63 was compared among the study groups using Kruskal-Wallis test
- To compare the staining characteristics between p53 and p63 in each group, Pearson's correlation test was used.

p53:

p53 is a tumor suppressor gene located on chromosome 17p13.1. The p53 gene consists of 11 exons, of which the first one is non-coding. This gene encodes a protein of molecular weight 53 KD. The p53 protein is a transcription factor that enhances the rate of transcription of six or seven known genes[8].

p53 domains: Structure-Function relationships

The human p53 protein contains 393 amino acids and has been divided structurally and functionally into four domains.

1. The first 42 amino acids at the N-terminus constitute a transcriptional activation domain. This domain interacts with the basal transcriptional machinery for specific activation of certain genes.
2. The central part of the protein consists of sequence-specific DNA-binding domain localized between amino acid residues 102 and 292. It is a protease-resistant and independently folded domain containing Zn^{2+} ion that is required for its DNA-binding activity.
3. The C-terminus consists of tetramerisation domain responsible for the formation of p53 tetramers, which is the most active form of p53 in transactivation.
4. The C-terminus also contains a regulatory domain, which can negatively regulate the central DNA-binding domain by binding to it and thereby inhibit specific binding of p53 protein to different promoters[9,10].

Activation of p53: Upstream events

p53 is involved in detection of DNA damage, after which it can stop the progression of cell cycle while the damage is repaired or can trigger apoptosis and death of the damaged cell. In this context, p53 acts as "molecular policeman" or "guardian of the genome". In the absence of functional wild-type p53, the 'guardian' function is lost; cells accumulate genetic damage and show marked genetic instability[11].

The p53 protein has the ability to sense different kinds of stress to which the cells are exposed such as, DNA damage caused by UV radiation, γ radiation and mutagenic chemicals. The cellular proteins like ATM (ataxia telengiectasia mutated) recognize damaged DNA and phosphorylate the p53 protein by interaction through their protein kinase domain. The phosphorylation results in a rapid increase in the levels of p53 in the cell and activation of p53 as a transcription factor. The p53 level increases because the half-life of protein is lengthened and also because the rate of translation of p53 mRNA is enhanced. To perform its function, the p53protein must, apart from being activated, be transported into the nucleus and also form tetrameric complexes with other p53 molecules. The activated p53 protein binds to specific DNA sequences and hence transactivates the downstream target genes[10].

Responses to activation of p53: Downstream events

The downstream events mediated by p53 take place by two major pathways:

1. **Cell cycle regulation:**

Activated p53 induce the transcription of p21 and GADD45 (Growth Arrest and DNA Damage) genes. p21 functions by inactivating the cyclin D-CDK4 complex, which is necessary for the progression of cell cycle through the S phase. p53-dependent transcription of p21 mediates cell cycle arrest in late G1 phase and allows enough time to repair the DNA damage. GADD45 gene encodes a protein involved in DNA repair. If the DNA damage is repaired successfully, p53 activates MDM2, whose product binds to and degrades p53, thus relieving the cell cycle block.

2. **Regulation of apoptosis:**

The p53 protein induces the activation of pro-apoptotic genes like Bax. Bax binds to and antagonizes the apoptosis-inhibiting protein Bcl-2; thus promotes cell death[9].

p53 mutations and cancer:

Mutation and subsequent inactivation of a tumor suppressor gene causes "loss of function". However, tumor suppressor genes are most commonly recessive to the normal allele, meaning that if one allele is mutated its phenotype is not expressed as long as the other allele's nature if of wild type. The inheritance of one mutant p53 allele predisposes individuals to develop malignant tumors because only one additional "hit" is needed to inactivate the second, normal allele. Such individuals, said to have "Li-Fraumeni syndrome", have a 25-fold greater chance of developing a malignant tumor by age 50 than the general population. The spectrum of tumors that develop in patients with Li-Fraumeni syndrome is quite varied; the most common types of tumors are sarcomas, breast cancer, leukemia, brain tumors and carcinomas of the adrenal cortex[12].

In most cases, the inactivating mutations affect both p53 alleles and are acquired in somatic cells. The most common type of mutation is the mis-sense mutation (where a change in one or more bases of a codon changes the affected codon into specifying a different amino acid), constituting up to 79% of all mutations. More than 90% of these mis-sense mutations in p53 reside in the DNA-binding domain and they fall into two classes. Mutations in amino acid residues such as R248 and R273 results in defective contacts with DNA and loss of ability of p53 to act as a transcription factor. A second type of p53 mutation disrupts the structural basis of β sheet and loop-sheet helix motif, thereby altering the conformation of the protein. Since the mutations affect area of p53 protein outside the tetramerisation domain in the C-terminus, most p53 mutants are still able to form tetramers[9].

Mutations in p53 gene are found in up to 40-50% of squamous cell carcinomas of the head and neck region (SCCHN). Based on studies of SCCHN and cell lines, it has been suggested that after mutation of one p53 allele, the remaining wild type allele is deleted, and accordingly the mutant phenotype expressed. While the wild-type p53 protein has a short half life (about 20 minutes), the mutant protein has a greatly enhanced stability, allowing for the immunohistochemical detection. However, the protein can also remain in the tissue for longer duration for certain reasons such as a defect in degradation pathway or by binding to other proteins. For example, p53 is known to bind to certain virus-encoded proteins like SV40 large T, which blocks its DNA binding activity and adenovirus E1B, which blocks its transcriptional activity. p53 also binds to HPV E6, which targets it for accelerated degradation[10].

p53 expression in oral precancer and cancer:

Kaur J, Chakravarti N, Mathur M et al (2004)[13] studied the expression of p53 and retinoic acid receptor β (RAR β) proteins using IHC in 50 cases of OSF and 30 histologically normal oral tissues. p53 immunostaining was detected in 48% (24/50) of OSF cases. Increased nuclear expression of p53 was predominantly limited to the basal layer of the epithelium. A significantly increased p53 expression and decreased RAR β expression were observed in OSF lesions when compared to normal oral mucosa. 36% (18/50) of OSF lesions demonstrated concomitant alterations in both these proteins, suggesting that alteration in p53 an RAR β expression might not be mutually exclusive events in OSF. Further, altered expression of either p53 or RAR β in majority of OSF lesions suggested their association with disease

pathogenesis and warranted follow-up to determine whether OSF lesions harboring concomitant alterations are at a high risk for malignant transformation.

Yamazaki Y, Chiba R, Hirai A et al (2003)[14] analyzed p53 mutations in 121 primary OSCC sample using PCR-single strand conformation polymorphism or yeast functional assay. p53 mutations were detected in 42% (51/121) cases of OSCC, of which 45 were point mutation, 4 frameshift deletions and 2 splice junctional mutations. Twenty three mutations were within zinc-binding domain and 28 mutations were within DNA-binding surface regions. A significantly short survival was observed in patients whose tumors contained p53 mutations within the conserved regions compared to those with mutations outside these regions. The prognosis was significantly poor among the 28 patients with mutations affecting DNA-binding surface regions. The study strongly suggested that determining the specific mutational site by DNA sequencing was essential for evaluation of the nature of the mutated p53 gene and the tumor characteristics. Additionally, p53 mutations within DNA-binding surface regions or conserved regions could be useful markers for predicting prognosis among OSCC.

Cruz I, Snijders PJF, Houten VV et al (2002)[15] investigated for p53 protein expression by IHC in 55 samples of OSCC. Twenty two cases showed no p53 staining, 12 showed less than 25% positive cells, 6 cases showed 25-50% positive cells, 3 cases showed 50-75% positive cells and 12 cases showed staining in almost all of the tumor cells. Ten out of 55 cases, including five p53 immunopositive and five p53 immunonegative cases were subjected to microdissection followed by sequence analysis for the detection of TP53 mutations. Paired IHC and sequence

analysis revealed that p53 immunoexpression in more than 25% of tumor cells was indicative of TP53 mutations. p53 immunoexpression in more than 25% of the neoplastic cells was significantly associated with smoking. The authors concluded that 25% p53 immunopositive cells appeared to be a good cut off value to predict TP53 mutations and p53 immunonegativity was not informative for TP53 mutations.

Van Houten VMM, Tabor MP, van den Brekel MWM et al (2002)[16] analyzed 50 head and neck SCC samples for the presence of p53 mutations by direct sequencing. 60% (30/50) of the samples showed a p53 mutation in the primary tumor. Histopathologically tumor free surgical margins of these 30 patients were analyzed for p53 mutation using the p53 plaque hybridization assay, of which 19 patients showed p53 mutated DNA in one or more additional margin samples. IHC confirmed the presence of minimal residual cancer (MRC) in 12/19 mutated p53 positive cases. In 7/19 cases, p53 mutation was found in unresected dysplastic mucosal precursor lesions. The authors concluded that by using p53 mutations as a marker, both MRC and unresected mutated p53 positive mucosal precursor lesions were detected within surgical margins. Molecular assessment of surgical margins using p53 mutations enables the selection of head and neck SCC patients at high risk for tumor recurrence.

Shin DM, Charurul N, Lippman SM et al (2001)[17] analyzed p53 protein expression using IHC in 48 SCC of the head neck region, 31 sites of normal epithelium adjacent to tumors, 24 sites of hyperplasia and 26 sites of dysplastic lesions. p53 protein was detected in 58% (28/48) of carcinomas, 19% (6/31) of normal epithelium adjacent to tumors, 24% (7/24) of hyperplasia and 46% (12/26) of dysplasias, but none of the normal control epithelium expressed detectable levels of

p53. The frequency of p53 expressing cells gradually increased from normal to hyperplasia, dysplasia and SCC. The authors also performed *in situ* hybridization to detect polysomies of chromosomes 17 and 9. The lesions with dysregulated p53 expression showed nearly 2-3 fold increased levels of chromosomal polysomy. These findings suggested that altered p53 expression is associated with increased genetic instability in premalignant epithelium and acts as a driving force for increasing the rate of accumulation of genetic events in head and neck tumorigenesis.

Lingen MW, Chang KW, McMurray SJ et al (2000)[18] investigated the status of p53 in patients with SCC of tongue with no known risk factors. Immunohistochemistry was used for the detection of p53 protein and single strand conformation polymorphism was used to identify mutations of the p53 gene in 21 SCC specimens from patients less than 40 years of age. Nuclear staining for p53 was detected in 81% (17/21) of the SCC specimens. The pattern of overexpression of p53 was associated with the histologic grade of the tumor. All the 15 cases of grade I and grade II tumors were positive for p53, while only 2 of the 6 grade III tumors were positive for p53. None of the patients demonstrated mutations in p53 gene. This analysis included the evaluation of exons 5-9; therefore, it is possible that a mutation in one of the other exons had occurred. The results suggested that altered p53 in OSCC from young individuals with no risk factors is due to different molecular mechanisms when compared to the altered p53 in typical cases of OSCC.

Cruz IB, Meijer CJLM, Snijders PJF et al (2000)[19] used 42 samples of non-malignant mucosa adjacent to OSCC, the respective carcinomas and six lymph node metastases to investigate for p53 protein expression by IHC. In 17% (7/42) of

specimens from non-malignant areas adjacent to OSCC, p53 expression was noted above the basal cell layer. Six of these were adjacent to carcinomas showing p53 expression in more than 50% of the neoplastic cells. The majority of cases with supra basal p53 expression in the immediately adjacent non-malignant mucosa (86%) showed moderate or severe dysplasia in the same area. The lymph node metastases showed the same patterns of p53 expression as the carcinomas from which they were derived. Supra basal expression in the non-malignant mucosa was found only adjacent to p53 positive tumors, suggesting that p53 alterations occur early in carcinogenesis and that these alterations are maintained upon progression to overt malignancy. The authors concluded that p53 immunostaining in non-malignant mucosa of the resection margins of OSCC might be a valuable predictor for local recurrences and may therefore have implications for the management.

Shahnavaz SA, Regezi JA, Brdley G et al (2000)[20] analyzed 24 samples from 10 patients with two or more distinct lesions from the same site in the oral cavity with the diagnosis of hyperkeratosis, epithelial dysplasia or SCC. The samples comprised of one hyperkeratosis, 12 dysplasias, 3 carcinomas *in situ* and 8 SCC. Standard IHC with DO7 monoclonal antibody was used to detect p53 protein and mutational analysis was performed using direct sequencing technique. Mutations were identified in 1 of 12 dysplasias, 2 of 3 carcinomas *in situ* and 6 of 8 carcinomas. Eight of these mutations were mis-sense and one occurred at a splice site. The overexpression of p53 protein was identified in 12 lesions and did not correlate with the gene status. In six of the eight patients who progressed from epithelial dysplasia or carcinoma in situ to carcinoma, p53 mutation was found only in carcinoma and not in any of the non-invasive lesions. This suggested that during oral carcinogenesis, p53

mutations probably occurred relatively late and were associated with transformation to the invasive phenotype.

Partridge M, Kiguwa S, Emilion G et al (1999)[21] examined 45 cases of OSCC for the presence of p53 protein using IHC. These tumors were also screened by single strand conformation polymorphism for the presence of p53 mutations. High levels of p53 protein was detected in 25/45 cases and point mutations involving exons 4-9 were seen in 11 cases of OSCC. There was no relationship between tobacco use and detection of p53 by IHC, as 52% of non-smokers had p53 positive tumors compared with 50% of smokers. To examine loss of heterozygosity, PCR-RFLP analysis was performed and fraction allelic loss (FAL) score was calculated for each tumor. Tumors which were positive for p53 by IHC had a greater FAL score than cases which were negative by IHC. The authors also reported frequent detection of p53 by IHC in the absence of mutation, suggesting that in some cases, IHC might detect stabilized wild-type p53 as a consequence of ongoing DNA damage.

Chiang CP, Huang JS, Wang JT et al (1999)[5] used anti-p53 antibodies to examine the expression of p53 protein in 81 cases of OSCC from patients who were areca quid chewers and/or tobacco smokers. Positive p53 nuclear staining was observed in 58% (47/81) of cases. p53 positivity was detected predominantly in the peripheral cells of the tumor nests as well as in the basal and para basal layers of the epithelium. p53 overexpression was higher in patients without areca quid chewing and tobacco smoking than in those with these habits. Kaplan-Meier analysis showed that the prognosis for p53 negative tumors was significantly better than that for patients with p53 positive tumors. A significant correlation was also observed between

positive lymph node status and poor prognosis. The results suggested that areca nut chewing or tobacco smoking may not play a role in p53 overexpression of OSCC. Furthermore, p53 overexpression may serve as an adjuvant marker of poor survival in patients with OSCC in Taiwan.

Murti PR, **Warnakulasuriya KAAS, Johnson NW et al (1998)**[22] compared the p53 expression in archival tissues from 22 baseline biopsies of precancerous lesions that transformed to cancer in 4-25 years against that in 68 similar lesions that did not transform over the same time period. Twenty nine percent of precancerous lesions that transformed to cancer were p53 positive at baseline compared to 31% of the lesions that did not transform to malignancy. These results suggested that detection of p53 protein by IHC in biopsies of oral precancerous lesions had no significant relationship to their likely malignant transformation. Among 10 cases of malignant transformation that did not show p53 expression at baseline, nine developed p53 expression at the time of cancer formation. This suggests that p53 overexpression peaks close to the time of transition from precancer to cancer rather than early in the natural history of oral precancer.

Trivedy C, Warnakulasuriya KAAS, Tavassoli M et al (1998)[4] analyzed 21 cases of OSF and 27 cases OSCC, of which 6 had arisen from OSF, to study the aberrations in structure and expression of p53 gene. The expression of p53 protein was detected by IHC using monoclonal antibody DO7 and PCR-SSCP (Polymerase chain reaction-Single strand conformation polymorphism) method was used for mutation analysis. Positive immunostaining was noted in 75% (15/21) of OSF specimens, 50% (3/6) of OSCC arising from OSF and 67% (14/21) of OSCC not

arising from OSF. In OSF, p53 protein was observed mainly in the basal layer of the epithelium. In OSCC specimens, positively stained cells were distributed widely in some cases and limited to a few foci in other cases. Mutation in p53 or loss of heterozygosity was seen in 13/21 case of OSF and 15/27 cases of OSCC. An increased tendency for cases with immunopositivity to harbor a mutation in p53 gene was noted in both OSF and OSCC. The results suggested that p53 mutation/protein stabilization may a play a role in the pathogenesis of OSF and its progression to OSCC. The authors consider it prudent to recommend p53 immunostaining of OSF biopsy specimens as a biomarker of DNA damage.

Warnakulasuriya KAAS, Tavassoli M, Johnson NW et al (1998)[23] performed immunohistochemical analysis of p53, p21 and p27 proteins in 24 cases of OSCC. Positive nuclear staining was detected in 10/24 OSCC, but none of the normal mucosa specimens showed p53 positivity. Heterogeneous expression of p21 and p72 proteins were noted in 10/24 OSCC and 9/16 OSCC respectively. The expression of p21 and p27 did not correlate with p53 status. Eight OSCC specimens lacked the expression of both p21 and p27, but only two of these tumors over-expressed p53, suggesting that accumulation of p21/27 could be independent of the functional status of the p53 gene. The study supports the view that not all cell cycle regulators are affected in any one cancer and among other cellular factors, p27 may be reciprocally down-regulated when the p53 pathway is ineffective.

Kerdpon D, Rich AM & Reade PC (1997)[24] studied 8 cases of normal oral mucosa, 50 cases of oral mucosal hyperplasia, 41 cases of oral dysplasia and 40 cases of OSCC for the expression of p53 protein. All cases of normal oral mucosa were

negative for p53. In hyperplasia, p53 staining was noted mainly in the basal and supra basal layers, with none of the cases having more than 50% of positive cells. When compared to hyperplasia, significantly more cases of dysplasia expressed p53 staining. There was also a significant increase in the distribution pattern of positively stained cells from dysplasia to OSCC. When observed for periods of 21-80 months, none of the p53 negative cases progressed clinically, whereas for the 26 positive cases, 1/26 changed from mild to moderate dysplasia, 1/26 changed from moderate to severe dysplasia and 3/2 progressed from severe dysplasia to OSCC. Although the presence or absence of p53 staining could not be used to predict the outcome of potentially malignant oral mucosal lesions, the results indicated an involvement of p53 in neoplastic transformation.

p63:

A recently discovered gene, p63 is mapped on chromosome 3q27-29 and is a member of the p53 gene family. The p63 gene is approximately 65 kilobase and contains 15 exons. The p63 gene gives rise to an array of multiple protein isoforms due to differential mRNA splicing and alternative promoter usage. The p63 gene undergo multiple C-terminal splicing, but among the high number of spliced isoforms found at the RNA level, 3 of them are consistently found at the protein level. The 3 spliced forms of p63 produced are termed α (full structure), β (splicing of exon 13) and γ (splicing from exons 10 to 15).

The expression of p63 gene is regulated by the occurrence of two promoters that leads to the production of 2 different classes of proteins. The P1 promoter is located in the 5' untranslating region (UTR) of a non-coding exon 1 and leads to isoforms showing N-terminal transactivation domain (TAp63). The P2 promoter is located within the 23-kilobase-spanning intron 3 and encodes isoforms lacking the N-terminal transactivation domain (ΔNp63). The TA p63 group (comprising of α, β and γ isoforms) shows p53-like activities such as inducing cell-cycle arrest and apoptosis. The second group (ΔNp63 in α, β and γ isoforms) acts in a dominant negative fashion, inhibiting the transactivation activity of both TAp63 and p53. As a result, the TAp63 protein favors cell differentiation, while ΔNp63 favors cell proliferation[25,26].

p63 has an important role in the process of proliferation and/or differentiation of epithelial tissues including skin, breast and prostrate. The p63 gene also plays an essential role in the proliferation of limb and craniofacial structures during embryogenesis. Studies with murine model have revealed that p63-knockout mice are

born alive but they display severe deformation of limbs and profound defects in craniofacial development, as well as in differentiation of tissues with stratified epithelium including skin, oral cavity, esophagus, breast and prostrate. A similar pattern was observed in patients with ectrodactyly, ectodermal dysplasia and facial clefts (EEC) syndrome, an autosomal dominant disorder, where patients are found to have dominant heterozygous p63 mutations.

An experimental examination by the genetic elimination of p63 disclosed that it plays an essential role in epithelial development to maintain keratinocytes stem cells. This may be of practical importance for studies of epithelial tumorigenesis because it is thought that stem cells are involved in the formation of malignant tumors. Among the various isoforms, normal human keratinocytes express mainly the truncated dominant negative p63 isoforms during development. It has been reported that these ΔNp63-encoding transcripts are down-regulated during the irreversible growth arrest and differentiation of human keratinocytes. The overexpression of p63 has been noted in SCC of the head and neck region and this has been attributed to increased p63 gene copy number[27,28]**.**

p63 expression in oral precancer and cancer:

de Oliveira LR, Ribeiro-Silva A & Zucoloto S (2007)[29] studied paraffin-embedded sections from 106 OSCC patients for the expression of p63 and p53 using immunohistochemistry (IHC). p63 immunoreactivity was found in 87.8% (93/106) of the tumors, while p53 immunoreactivity was found in 52.8% (56/106) of the tumors. p63 expression was noted predominantly in well and moderately differentiated tumors, but absent in keratin pearl areas. The cases with lower intensity of p63

expression showed a higher number of metastasis than those with strong immunoexpression. A significant association was found between the intensity of p63 staining and increased odds of overall survival. The p53 positive cases showed a higher metastasis rate (78.6%) compared with p53 negative cases. The patients with negative p53 expression had a better prognosis than those with positive p53 expression and this was statistically significant. The authors concluded that p53 overexpression and decreased intensity of p63 immunostaining was associated with metastases and correlated with poor outcome.

Haniffa AM, Saitoh M, Abiko Y et al (2007)[30] investigated the expression pattern of p63 using IHC in 30 cases of SCC, 10 cases of epithelial dysplasia and 12 cases of oral submucous fibrosis (OSF). In normal oral epithelium, p63 positivity was noted in the nuclei of some of the basal cells and focally in para basal layer. In SCC, p63 positivity was observed mainly in the peripheral cells of tumor nests. In epithelial dysplasia, staining was predominantly seen in the upper spinous layers. The positivity for p63 staining was increased in epithelial dysplasia when compared to normal oral epithelium. In OSF, the p63 staining was wider and more intense than in normal and dysplastic epithelium. OSF showed a significantly higher percentage of positive cells than dysplastic epithelium or SCC. The authors suggested that p63 might play an oncogenic role in oral carcinogenesis through the expression of wild type forms rather than acting as a tumor suppressor via mutant forms. The disruption of the normal function of p53 by mutation might result in a compensatory up-regulation of p63. A higher frequency of p53 mutations in OSF might have led to the higher percentage of p63 positive cells noted in this study.

Takeda T, Sugihara K, Hirayama Y et al (2006)[2] studied the expression of p63 and ki-67 proteins using IHC in 10 normal oral mucosa, 10 hyperplasia, 10 mild dysplasia, 10 moderate dysplasia, 13 severe dysplasia, 10 carcinoma *in-situ* and 9 two-phase dysplasia. In the basal layer, p63 labelling index decreased from normal oral mucosa to carcinoma *in situ,* whereas in the supra basal layers, labelling index increased with the severity of dysplasia. ki-67 labelling index in the basal and supra basal layers increased according to the grade of dysplasia. The decrease of p63 labelling index in the basal layer of epithelial dysplasias suggested an alteration of stem cell function and the stem cells could be replaced by proliferating cells as demonstrated by positive ki-67. The authors concluded that the architectural disorganization of proliferating cells and stem cells in oral epithelium could be a useful index to estimate the grading of epithelial dysplasias if added to histomorphological examinations in H & E sections.

Kovesi G & Szende B (2006)[31] determined the expression of p63, cyclin D and p27 using IHC in 18 samples taken from homogenous leukoplakia, nodular leukoplakia and erythroleukoplakia. Immunoexpression of p63 appeared predominantly in the para basal and middle spinous layers. The p63 index was 10% in homogenous leukoplakia, 5% in nodular leukoplakia and 20% in erythroleukoplakia. The authors suggested that decrease in p63 expression was found in nodular leukoplakia as a part of a defense mechanism against malignant transformation. In erythroleukoplakia this mechanism appears to fail because the expression of p63 was increased significantly. The increase in p63 as well as cyclin D and decrease in p27 expression correlated with the severity of leukoplakia and hence the authors concluded by pointing out the possibility that immunohistochemical demonstration of

these gene products might be a useful tool for a more precise prognosis of oral leukoplakia.

Chen Y-K, Hsue S-S & Lin L-M (2005)[7] evaluated the expression of p63 protein using immunohistochemistry in 90 samples of epithelial dysplasia (30 cases of mild, 30 moderate and 30 severe dysplasia), 15 samples of hyperplastic oral mucosa and 15 cases of normal oral mucosa. In normal buccal mucosa specimens, p63 nuclear staining was predominantly seen in the basal layer of epithelium and focally in para basal layers. In hyperplastic oral epithelium, staining was detected chiefly in basal cells and occasionally in cells above the basal layer. In mild epithelial dysplasia, p63 staining was seen up to the middle spinous layer, whereas in moderate and severe dysplasias, the full thickness of the epithelium showed positive immunostaining. Furthermore, in 5 years follow-up, 16.7% (5/30) of moderate dysplasia and 30% (9/30) of severe dysplasia with positive p63 staining have undergone malignant transformation to squamous cell carcinoma.

RT-PCR was also done to detect p63 mRNA in 4 samples of mild and moderate dysplasia, 5 samples of severe dysplasia, 5 samples of hyperplastic epithelium and 5 normal mucosa. ΔNp63 mRNA was detected as a band corresponding to a 681-bp PCR product for all the specimens, whereas expression of TA (Transactivation) isotype was not detected in any of the specimen.

The authors concluded by suggesting that the overexpressed p63 proteins might exert an alterative mechanism to overcome p53 tumor suppressor function and hence induce clonal expansion of the dysplastic keratinocytes. Therefore, p63-stained

keratinocytes could be important in neoplastic transformation of the squamous cell, favoring neoplastic proliferation and anti-differentiation effect.

Muzio LL, Santarelli A, Caltabiano et al (2005)[32] studied p63 expression in 94 samples of OSCC and 10 cases of normal oral mucosa using IHC. Normal oral mucosa showed 10% of stained cells, with the immunostaining localized to basal and para basal layers. 5.3% of OSCC showed less than 10% of positive tumor cells, 35% had 10-30% of positive tumor cells, 38% of the cases had 30-50% of positive tumor cells, while 21% of OSCC had more than 50% positive tumor cells. There was a statistically significant increase in the expression of p63 staining in poorly differentiated OSCC when compared to well and moderately differentiated OSCC. Survival analysis revealed that the patients with increased p63 expression had poor survival rates than those with reduced p63 expression. The authors suggested that p63 expression may be useful to identify cases of OSCC with more aggressive and invasive phenotype providing novel prognostic information on individual patient survival.

Mognetti B, Trione E, Corvetti G et al (2005)[33] evaluated ΔNp63 mRNA expression using RT-PCR in the surgical margins of a case of SCC of the floor of the mouth in a 68 year old male patient. A sample of healthy contralateral mucosa served as a baseline control. Routine histopathological evaluation revealed clear margins, while ΔNp63 mRNA expression in one of the margins was 6.6 folds higher than the baseline level, and comparable to that measured in the tumor. In the same margin, a more accurate and time consuming histological analysis on serial sections revealed the presence of neoplastic cells. The authors therefore considered this technique as a

good method to screen the clear surgical margins to support the classic histologic analysis.

Bortoluzzi MC, Yurgel LS, Dekker N et al (2004)[34] assessed the expression of p63 protein in 10 cases of hyperkeratosis, 9 cases of mild dysplasia, 11 moderate dysplasia, 10 severe dysplasia/*in situ* carcinoma, 22 SCC and 5 normal mucosa using IHC. The normal mucosa showed intense p63 staining of basal, para basal and supra basal cells that gradually decreased at the mid-level of the epithelium. p63 staining was also noted in the basal and supra basal keratinocytes of hyperkeratosis and epithelial dysplasia specimens. The number of p63 positive cells was significantly greater in epithelial dysplasias when compared to hyperkeratosis. The majority of tumor cells stained positive for p63 and a significantly greater number of positive cells was found in SCC than that observed in epithelial dysplasias. Moderately differentiated SCC had significantly greater number of positive cells than well differentiated SCC. As p63 was expressed in nondiagnostic patterns in a variety of oral lesions, the authors concluded that p63 expression in oral keratinocytes might be more closely related to stem cell maintenance and cell differentiation than tumorigenesis.

Chen Y-K, Huse S-S & Lin L-M (2004)[35] investigated the differential expression of p63, p53 and p73 protein and mRNA for DMBA-induced hamster buccal pouch squamous cell carcinomas (SCC) using IHC and RT-PCR respectively. Using IHC, nuclear staining of p63 protein was noted for all of the 20 hamster buccal-pouch tissue specimens treated with DMBA, as well as for all the untreated and mineral oil treated buccal pouch tissue specimens. p53 and p73 proteins were detected

for a subset of hamster buccal pouch tissue specimens treated with DMBA and were not noted in the untreated and mineral oil treated pouch mucosa. For the carcinoma samples, both p63 and p73 immunoreactivity was chiefly observed in the less differentiated cells located at the periphery of carcinomatous clusters.

Using RT-PCR, ΔNp63 mRNA was detected for all the 20 hamster buccal pouch tissue specimens treated with DMBA, all mineral oil treated and untreated buccal pouch specimens, whereas expression of Tap63 was not detected in any of the specimen. Differential expression of p53, p63 and p73 protein in experimental group was as follows: p63+/p73+/p53+ (70%), p63+/p73+/p53- (10%) and p63+/p73-/p53- (20%). Additionally, significant correlation between p63, p73 and p53 expression was demonstrated for the hamster buccal pouch carcinoma samples. These results indicated that both p63 and p73 may be involved in the development of chemically induced hamster buccal pouch carcinomas, in concert with p53.

Foschini MP, Gaiba A, Cocchi R et al (2004)[6] investigated 39 samples of OSCC for the presence of p63 protein and mRNA using immunohistochemistry and RT-PCR and compared it with the non-neoplastic mucosa adjacent to the tumor. IHC revealed that percentage of positive cells increased from normal to neoplastic mucosa and p63 positive cells were predominantly seen in the basaloid cells present at the periphery of the neoplastic nests. The percentage of p63 positive cells was higher in poorly differentiated OSCC than in well differentiated OSCC. In the non-neoplastic mucosa, p63 positivity was confined to the basal and para basal layers of keratinocytes, with positive cells never exceeding 10% of the keratinocytes. RT-PCR revealed that ΔNp63 was the most frequently expressed isoform, being present in

97.4% (38/39) of the cases. The truncated isoforms ΔNp73L and Δ4TAp63 were more frequently expressed in patients presenting with lymph node metastases. The authors concluded that an impaired expression of the p63 isoforms might favor cell proliferation and indirectly enhance the metastasizing capacity of OSCC.

Thurfjell N, Coates PJ, Uusitalo T et al (2004)[36] used quantitative real-time RT-PCR to study p63 isoforms in 13 cases of SCC of the head and neck. Both the ΔNp63 and p63β isoforms were significantly more expressed in tumors compared to normal tissue from the dame patient. Eleven of the 13 tumors had diagnostic biopsies available for immunohistochemical analysis. All 11 were positive for p63 antibody and the staining was found throughout the tumors, with the exception of foci of more differentiated islands of keratinizing cells.

Matched pairs of samples from smokers and non-smokers were analyzed for expression of p63 mRNA and protein by RT-PCR and IHC respectively. As with the tumor samples, ΔN isoform predominated, although low levels of TA isoform could also be detected. Although the highest levels of p63 were seen in individuals with a history of smoking, no significant difference in p63 expression was seen between smokers and non-smokers, indicating that p63 might not be involved in the response to tobacco-derived genotoxic agents. The results suggested that SCC of head and neck maintain expression of high levels of ΔNp63 in combination with varying levels of other isoforms, and this expression significantly influences the differentiation status of the neoplastic cells.

Chen YK, Hsue SS & Lin LM (2003)[37] performed immunohistochemical analysis to study the differential expression of p63, p53 and p73 proteins in 40 samples of well differentiated buccal SCC and compared it with 10 specimens of normal buccal mucosa. Nuclear staining of p63 and p73 were noted in the basal layers of the normal buccal mucosa, while p53 expression was not noted in the normal epithelium. In the carcinoma samples, both p63 and p73 immunoreactivity were chiefly observed for the less-differentiated cells located at the periphery of carcinomatous clusters. For p53 protein, positive staining was demonstrated for some cells in the upper layers of tumor islands. Differential expression of p63, p73 and p53 proteins in carcinomas were: p63+/p73+/p53+ (70%; n=28/40), p63+/p73+/p53- (10%; n=4/40) and p63+/p73-/p53- (20%; n=8/40). In this study, p63 and p73 were frequently expressed simultaneously and were positively correlated with each other, suggesting a synergistic effect with respect to tumor development in the oral cavity.

Chen YK, Hsue SS & Lin LM (2003)[38] analyzed p63 expression in DMBA induced hamster buccal pouch SCC using immunohistochemical studies. For all the untreated and mineral oil treated pouch mucosa, nuclear positivity for p63 was mainly observed in basal/para basal cell layers, whereas, positive staining was observed throughout the whole epithelial layer in DMBA treated pouch mucosa specimens. For carcinoma specimens, p63 staining was more uniform and homogenous in less differentiated tumor areas, while expression was noted mainly in the peripheral cells of tumor nests in well differentiated tumors. The authors suggested that p63 expression in DMBA induced pouch carcinogenesis will block the growth inhibition and apoptosis-indication activities of p53, and thus, may help maintain the proliferative capacity of progenitor cells in hamster buccal pouch mucosa.

Choi HR, Batsakis JG, Zhan F et al (2002)[39] performed immunohistochemical analysis of p63, p73 and p53 proteins in 38 samples of head and neck carcinoma, 16 dysplastic lesions and 25 samples of histologically normal squamous epithelium. In normal epithelia, nuclear p63 staining was noted in the basal and para basal layers of the epithelium in all the specimens. p63 positivity increased with histologic progression in all 16 dysplasias. In carcinomas, p63 staining was more uniform and intense than p53 and p73 in 94.7% (n=36/38) of the samples. In well-differentiated tumor areas, p63 was noted mainly in the peripheral cells of tumor nests and generally absent in terminally differentiated cells. In normal mucosa, p53 expression was focal to patchy and was confined wo the basal/parabasal layers. In dysplastic lesions, p53 positive cells increased in number and intensity with progression of dysplasia in 68.7% (n=11/16) of cases. Of the 38 carcinomas, p53 staining was noted in 52.6% of the samples. A significant association between p63 and p73 expression was observed, but no correlation between p63 or p73 with p53 was noted. The study suggested a synergistic cooperation of p63 and p73 in the early development of dysplasias and/or a compensatory up-regulation in response to p53 alteration.

p63 expression in other oral lesions:

Ebrahimi M, Wahlin Y-B, Coates PJ et al (2007)[40] analyzed the sera from 20 consecutive patients diagnosed with oral lichen planus (OLP) for the presence of antibodies against p63 and p73 using western blotting. The sera from two OLP patients reacted with p63 proteins. Both of these patients reacted with all six p63 isoforms with varying intensity. Sera from one of these two OLP patients also reacted with the four p73 isoforms. The strong reaction seen against p63 isoforms could be an

indication that the epithelial cells in these lesions could not differentiate normally and thus are considered foreign to the body and evoke an immune response. Further, patients with a milder disease or disease of shorter duration could have antibodies against p63 at levels too low to be detected by Western blotting.

Ebrahimi M, Wahlin Y-B, Coates PJ et al (2006)[41] studied the expression of p63 and p53 proteins using IHC in 46 samples of OLP and 8 samples of graft vs host disease (GVHD) and compared it with 16 normal oral mucosa and 12 samples of SCC of the head and neck region. In normal oral mucosa only a few cells in the basal cell layer expressed p53, whereas OLP and GVHD samples showed increased expression of p53. A decreased expression of all p63 isoforms were seen in OLP and GVHD when compared to normal oral mucosa. In SCC of the head and neck region, ΔNp63 proteins were detected at a higher level compared with normal mucosa. It is likely that decreased p63 an increased p53 proteins inn OLP and GVHD might represent a protective response to increased levels of DNA damage resulting from chronic inflammation. The coordinated stabilization of p53 and decreased expression of p63 enables apoptosis of epithelial cells to remove damaged cells with the potential for malignancy. In SCC, increased p63 expression might provide an advantage to the initiated cells exposed to further damage by allowing their continued survival and thereby increasing the likelihood of accumulating the successive oncogenic alterations.

Kumamoto H, Ohki K & Ooya K (2005)[42] evaluated tissue specimens of 9 tooth germs and 48 benign and 5 malignant ameloblastomas for the immunohistochemical expression of p63 protein. In tooth germs, expression of p63 was found in most cells of the cells of inner and outer enamel epithelium and in

fewer cells of stratum intermedium and stellate reticulum. Ameloblastomas showed p63 reactivity predominantly in the peripheral columnar or cuboidal cells and only in fewer central polyhedral cells. Desmoplastic ameloblastomas demonstrated significantly higher p63 expression when compared to acanthomatous and granular cell ameloblastomas. P63 expression in ameloblastic carcinomas was significantly higher than in metastasizing ameloblastomas. RT-PCR done for identification of p63 mRNA revealed that mRNA transcripts for ΔNp63 were detected in all ameloblastomas and inn tooth germs. The results suggested that p63 expression is associated with proliferation of neoplastic odontogenic epithelial cells and also might be involved in malignant transformation of odontogenic epithelium.

Edwards PC, Bhuiya T, Kelsch RD et al (2004)[43] determined the expression of p63 immunoreactivity in 17 samples of polymorphous low grade adenocarcinoma (PLGA), 15 adenoid cystic carcinomas (ACC), 6 canalicular adenoma and 11 basal cell adenoma. Nuclear p63 reactivity was uniformly positive in PLGA, suggesting that the neoplastic cells in PLGA might represent a population either of p63 positive epithelial stem/reserve cells similar to the basal cells of stratified epithelium, or of modified myoepithelial cells. Positive reactivity was also identified in 87% of ACC, primarily in the nonluminal myoepithelial-like cells surrounding luminal cells. No p63 staining was seen in canalicular adenoma, whereas variable staining was noted in basal cell adenoma. All basal adenoma of parotid gland origin stained strongly positive for p63, but none of the basal cell adenoma of the upper lip stained for p63. The authors concluded that p63 was neither an ideal marker for distinguishing between PLGA and ACC, nor useful in separating canalicular adenoma from basal cell adenoma in the upper lip.

Patient characteristics:

Twenty cases of OSCC (Group I), 20 cases of leukoplakia (Group II), 20 cases of OSF (Group III) and 10 histologically normal oral mucosal samples (Group IV) were assayed for p53 and p63 proteins using immunohistochemistry.

The patients' ages ranged from 38 to 85 years (mean of 55.2 years) in group I, from 30 to 73 years (mean of 43.6 years) in group II, from 23 to 51 years (mean of 36.4 years) in group III and from 19 to 42 years (mean of 29.5 years) in group IV **(Table 1, Graph 1).** Of the 20 patients in group I, 16 (80%) were men and 4 (20%) were women. In group II, all 20 (100%) were men. In group III, 19 (95%) were men and only one (5%) was a woman, whereas in group IV, 4 (40%) were men and 6 (60%) were women **(Table 2, Graph 2).** All the samples in group I, II, III and IV were taken from the buccal mucosa.

The oral habits of the patients in the study group were categorized as smoking or chewing. In group I, 4 (20%) patients had smoking habit, 11 (55%) had chewing habit, while 5 (25%) had both smoking and chewing habits. In group II, 9 (45%) patients had smoking habit, 5 (25%) had chewing and 6 (30%) had both smoking and chewing habits. In group III, 17 (85%) had chewing habit, whereas 3 (15%) had both chewing and smoking habits. All the patients in group III had the habit of chewing pan masala. In group IV, none of the patients had any oral habits **(Table 3, Graph 3).**

Of the 20 cases in group I with features of squamous cell carcinoma histologically, 14 (70%) were well differentiated, 4 (20%) moderately differentiated and 2 (10%) poorly differentiated **(Table 4, Graph 4).**

All the cases in group II showed features of epithelial dysplasia histologically, with 9 (45%) mild dysplasia, 7 (35%) moderate dysplasia and 4 (20%) severe dysplasia **(Table 5, Graph 5).**

Distribution of p53 staining among study groups:

p53 immunostaining revealed positivity in all the cases of group I, II and III (**Group I**: **Figures 8,9**; **Group II**: **Figures 16,17**; **Group III**: **Figures 24,25**). In group IV, only 3 (30%) of the cases showed positive staining for p53, while the remaining 7 (70%) cases were negative for p53. **Figures 32 and 33** are representative pictures of p53 staining in group IV.

Mean labeling index (LI) was calculated for all the four study groups and was expressed as percentage of positive cells. The mean LI for group I, II, III and IV were 56.9 ± 21.3, 37.6 ± 12.6, 34.6 ± 8.7 and 15.1 ± 9 respectively; the difference in mean LI among the groups was statistically significant (p=0.00) **(Table 6, Graph 6).** There was statistically significant difference in the mean LI of p53 between group I and group II (p=0.00), between group I and group III (p=0.00) and between group I and group IV (p=0.00). Mean LI of p53 was found to be significantly different between group II and group IV (p=0.00) and between group III and IV (p=0.00).

p53 immunostaining was observed only in the basal layer of the epithelium or both in the basal and supra basal layers of the epithelium in group II, III and IV. Four (20%) cases in group II had basal staining and 16 (80%) had both basal and supra basal staining. In group III, 8 (40%) of the cases showed basal stain, while 12 (60%) showed both basal and supra basal staining. Of the 3 positive cases in group IV, 2

(20%) had basal stain, whereas one (10%) case had both basal and supra basal stain. The tissue localization of p53 staining was significantly different among the 3 study groups (p=0.00) **(Table 7, Graph 7)**. There was statistically significant difference in the localization of p53 stain between group II and group IV (p=0.01) and between group III and IV (p=0.00).

Distribution of p63 staining among study groups:

p63 immunostaining revealed positivity in all the cases of group I, II, III and IV. (**Group I**: **Figures 12,13**; **Group II**: **Figures 20,21**; **Group III**: **Figures 28,29; Group IV: Figures 36,37**). The mean LI for group I, II, III and IV were 56.8 ± 19.6, 42.3 ± 10.5, 32.8 ± 12.1 and 26.4 ± 9.4 respectively and the difference was statistically significant (p=0.00) **(Table 8, Graph 8).** There was statistically significant difference in the mean LI between group I and group II (p=0.02), between group I and group III (p=0.00) and also between group I and group IV (p=0.00). Mean LI of p63 was found to be significantly different between group II and III (p=0.01) and between group II and group IV (p=0.00).

Two (10%) cases in group II had only basal staining and 18 (90%) had both basal and supra basal staining. In group III, 6 (30%) cases showed basal stain, while 14 (70%) showed both basal and supra basal staining. In group IV, 5 (50%) cases had basal stain, whereas the remaining 5 (50%) had both basal and supra basal stain. The tissue localization of p63 staining was not statistically significant among the 3 study groups (p=0.05) **(Table 9, Graph 9)**.

Correlation between p53 and p63:

Pearson's correlation analysis of p53 and p63 mean LI showed statistically significant positive correlation in OSCC (r=0.72) (p=0.00) and OSF (r=0.60) (p=0.00). Analysis also showed positive correlation between p53 and p63 in leukoplakia (r=0.28) and normal samples (r=0.45), but the positive correlation did not reach statistical significance (p=0.2; p=0.1) **(Graphs 10, 11, 12, 13)**.

OSCC is considered to develop through a multistep process of accumulations of genetic mutations related to cell proliferation, differentiation and apoptosis. Mutations in the p53 gene are the most common genetic abnormality found in 40-50% of SCC of head and neck[9.] p63 gene, a member of p53 gene family, has a remarkable structural similarity to p53 and is responsible for the transactivation of 6 isoforms. Three isoforms (TAp63α, TAp63β, TAp63η) contain an N-terminal transactivation domain (TA) and can induce apoptosis. The other 3 isoforms (ΔNp63α, ΔNp63β, ΔNp63η), lack the TA domain and may function by inhibiting the transactivation functions of p53 and TAp63 proteins, and thus act as oncoproteins[34].

p63 protein is known to play an essential role in epithelial development and proliferation of limb and craniofacial structures. p63 expression has been described in head and neck tumor of squamous lineage, suggesting that p63 overexpression may play a role in oncogenesis of these tumors[34].

Strikingly, squamous cell carcinoma of skin and oral cavity as well as the oral epithelial dysplastic lesions produce high levels of ΔNp63mRNA[7,35]. The most commonly used clone of p63, the 4A4 antibody does not discriminate the different isoforms of p63[6]. However, the presence of TAp63 mRNA in skeletal muscle, in the absence of staining with 4A4 antibody, has been reported, indicating that this antibody may not identify all p63 isoforms by IHC[27].

In the present study we have examined the expression of p53 and p63 proteins in OSCC, leukoplakia and OSF samples and compared their expression with that of normal oral mucosa using immunohistochemistry.

Patient characteristics:

OSCC is a malignancy commonly encountered in the older age group between 50 - 70 years of age and rarely in patients younger than 40 years[3]. In a study by **Mehrothra et al (2003),** 7.6% of the patients with OSCC were between 30 to 40 years[44]. In our study the age of the patients with OSCC ranged from 38 to 85 years with a mean age of 55.2 years.

Lumerman et al had examined 308 cases of leukoplakia and had shown that the average age of presentation was 59.3 years[45]. Studies by **Saito et al** have shown that the mean age for patients with leukoplakia was 54 years[46]. In our study, the minimum age of presentation for leukoplakia was 30 years and the maximum age was 73 years with a mean of 43.6 years.

Ranganathan et al reported that the youngest and oldest age of occurrence of OSF was 16 years and 57 years respectively, with a mean age of 32.4 years[47]. In our study, the age of presentation for OSF ranged from 23 to 51 years, the mean age being 36.4 years.

OSCC has been shown to exhibit gender predilection, with males being more commonly affected[3]. The male to female ratio recorded by **Mehrothra et al (2003)** was 3.27:1[44]. In our study 80% of the OSCC patients were males. This may be due to the reason that more number of males have the habit of smoking tobacco than females[3].

Neville et al had reported a strong male predilection of 70% for oral leukoplakia, while **Lumerman et al** had shown that 52.8% of the patients in their study were males[3,45]. In our study all the patients with leukoplakia were males.

Our study showed a high preponderance of OSF in males (95%), similar to the study done by **Ranganathan et al** who had examined 185 patients with OSF and had given a male to female ratio of 9.9:1[47].

The use of tobacco in either smoked (cigarettes, beedi, cigar, pipes) or chewed form is considered to be the most common factor associated with the etiology of OSCC[48]. In the study conducted by **Mehrothra et al**, 78.6% of OSCC patients were tobacco users in chewing or smoking form[44]. In the present study, 20% of OSCC patients were smokers, 55% chewers and 25% had both smoking and chewing habit.

Out of 20 patients with leukoplakia in our study, 45% of them had the habit of smoking tobacco, 25% were chewers and 30% had both chewing and smoking habit. This is in agreement with the report by **Bouquot et al** who related tobacco smoking with leukoplakia and reported that 66% of the patients with leukoplakia were smokers[49].

Areca nut is considered to be the main etiological factor for the development of OSF. In vitro studies on human fibroblasts have shown that arecoline and arecaidine, the alkaloids present in areca nut, causes fibroblast proliferation and stimulation of collagen synthesis[50]. In our study, all the 20 patients with OSF had the habit of chewing pan masala (powdered areca nut with additives, flavoring agents and tobacco). These findings were consistent with that of **Ranganathan et al**, who reported that the prevalence of chewing pan masala was more in the males than in females[47].

p53 staining characteristics:

Our study showed p53 immunoexpression in 100% of OSCC, leukoplakia and OSF samples. p53 immunoexpression in OSCC as reported by **Cruz et al (2000),**

Trivedy et al (1998) and in the study done by **Partrige et al (1999)** ranged from 52-62%[19,4,21]. **Kerdpon et al (1997)** reported positive p53 staining in 94% of OSCC and 85% of oral epithelial dysplasias[24]. Expression of p53 protein has been reported in 10-50% of oral leukoplakia cases showing features of epithelial dysplasia histologically[14,20]. **Kaur et al (2004)** observed p53 expression in 48% of OSF samples[13]. **Trivedy et al** (1998) reported p53 immunoexpression in 75% (15/20) of OSF samples[4].

70% of normal oral mucosa samples in the present study were negative for p53 staining, while 30% showed positive expression. This is in agreement with studies by **Yanamoto et al (2002)** and **Kaur et al (2004),** who reported 20% and 10% positivity for p53 in normal mucosa respectively[51,13]. The detection of p53 in normal epithelium has been attributed to the physiological stabilization of the wild type p53 due to genotoxic stress caused by UV radiation, hypoxia and viral proteins, which might lead to increased half life of p53 protein and therefore detection by IHC[11,13,19]. In contrast, **Kerdpon et al (1997)** in their study found that all cases of normal oral mucosa were p53 negative[24].

The mean LI was significantly higher in OSCC (56.9 ± 21.3), leukoplakia (37.6 ± 12.6) and OSF (34.6 ± 8.7) when compared to normal mucosa (15.1 ± 9) (p=0.00; p=0.00; p=0.00). These results are similar to the study by **Kerdpon et al (1997)** who showed that p53 expression increased from normal to hyperplasia to dysplasia to OSCC[24].

In our study, p53 immunostaining was localized to both basal and supra basal layers of the epithelium in 80% of leukoplakia and 60% of OSF samples. **Nylander et al** stated that the expression of p53 stain in the supra basal layers was an indication of malignant development in oral premalignant lesions[10]. **Cruz et al (1998)** observed

p53 expression in supra basal cell layer of 25% of premalignant lesions. They demonstrated that p53 supra basal expression was significantly associated with development of carcinoma. They also stated that this pattern of p53 expression explained the presence of proliferating cells with DNA damage in the superficial compartments of the epithelium, especially in dysplasia. These authors suggested that p53 protein detected in the supra basal layer indicate an irreversible process during carcinogenesis[52].

p63 staining characteristics:

p63 immunostaining was positive in all the cases of OSCC, leukoplakia, OSF and normal oral mucosal samples in the present study. This is in accordance with previous reports on p63 expression in OSCC by **Choi et al (2002), Bortoluzzi et al (2004**) and **Foschini et al (2004)[39,34,6].** Positive p63 staining was noted in 100% of oral leukoplakia and 100% of OSF samples by **Kovesi et al (2006)** and **Haniffa et al (2007)** respectively[31,30]. **Bortoluzzi et al (2004), Foschini et al (2004), Chen et al (2005)** & **Haniffa et al (2007)** have demonstrated p63 expression in 100% of normal oral mucosa samples[34,6,,7,30].

According to **Chen et al (2005),** p63 protein is expressed in the basal layers of the normal oral mucosa, where it likely serves to maintain the proliferative potential of the basal keratinocytes. They authors also stated that upon dysplastic change, the dysplastic keratinocytes above the basal layers also shows p63 expression, indicating an antidifferentiation effect and proliferative capacity of the dysplastic cells[7].

The mean LI was significantly higher in OSCC (56.8 ± 19.6) and leukoplakia (42.3 ± 10.5) when compared to normal mucosa (26.4 ± 9.4) (p=0.00; p=0.00). Similar findings have been reported by **Foschini et al**, who showed that the

percentage of positive cells increased from normal to neoplastic mucosa[6]. In the present study, the mean LI was significantly higher in OSCC when compared to oral leukoplakia. These findings are in contrast to the studies done by **Bortoluzzi et al (2004)** and **Haniffa et al (2007)** who did not find significant difference in the percentage of p63 positive cells between dysplasia and OSCC[34,30].

A significantly higher mean LI was noted in OSCC and leukoplakia when compared to OSF samples in our study. Studies by **Haniffa et al** have shown that OSF samples showed significantly higher percentage of p63 positive cells than in OSCC or leukoplakia[30]. These authors suggest that the disruption of normal function of p53 by mutation may result in compensatory up-regulation of p63. They also state that a higher frequency of p53 mutations in OSF might lead to the higher percentage of p63 positive cells than that seen in epithelial dysplasia[30].

In the present study, 90% of leukoplakia, 70% of OSF and 50% of normal samples exhibited both basal and supra basal staining. **Bortoluzzi et al** and **Foschini et al** have demonstrated p63 staining in both basal and supra basal layers of normal oral epithelium[34,6]. Extension of the p63 expression to the superficial layers of the normal oral epithelium has been noted in areas of chronic inflammation[6]. Supra basal expression of p63 protein, extending up to the upper spinous layers of the epithelium has been reported in oral epithelial dysplasias by **Kovesi et al, Haniffa et al** and **Bortoluzzi et al[31,30,34].**

Correlation between p53 and p63 staining:

We observed a significant positive correlation between the mean LI of p53 and p63 in OSCC and OSF. Association between p53 and p63 immunoexpression in the buccal squamous cell carcinoma was shown by **Chen et al**[38]. Another study done by **Chen et al** also showed significant correlation between p53 and p63 expression in DMBA-treated hamster buccal pouch carcinomas[35.] In our study, correlation was not significant in oral leukoplakia and normal mucosa. In leukoplakia, this could be probably due to variation in grades of dysplasia seen in our cases.

To conclude, our study showed that there is increased expression of p53 and p63 proteins in OSCC, oral leukoplakia and OSF when compared to normal oral mucosa using IHC. These results suggest that alteration of p53 and p63 protein expression occur during oral oncogenesis. Further, correlation analysis shows that there is a direct correlation between p53 and p63 expression in OSCC and OSF. Thus, based on our study findings, we state that both p53 and p63 proteins are increased in potentially malignant lesions and cancer when compared to normal.

- A total of 70 patients were included in the study, comprising of 20 cases of OSCC (group I), 20 cases of leukoplakia (group II), 20 cases of OSF (Group III) and 10 patients with normal oral mucosa (group IV), showing mean ages of 55.2, 43.6, 36.4 and 29.5 years respectively.

- In group I, 80% were males and 20% were females. In group II, 100% were males.

  In group III, 95% were males and 5% were females. In group IV, 40% were males and 60% were females.

- The p53 mean LI for group I, II, III and IV were 56.9 ± 21.3, 37.6 ± 12.6, 34.6 ± 8.7 and 15.1 ± 9 respectively; the difference in mean LI among the groups was statistically significant (p=0.00). The p63 mean LI for group I, II, III and IV were 56.8 ± 19.6, 42.3 ± 10.5, 32.8 ± 12.1 and 26.4 ± 9.4 respectively and the difference was statistically significant (p=0.00).

- In group II, p53 staining was seen in the basal layer in 20% and both basal and supra basal layers in 80% of the cases. In group III, 40% exhibited basal staining, while 60% had both basal and supra basal p53 staining. In group IV, 20% of the samples had basal p53 staining and 10% had both basal and supra basal staining. The tissue localization of p53 staining was significantly different among the 3 study groups (p=0.00).

- In group II, 10% of the cases exhibited p63 staining in the basal layer, while 90% had both basal and supra basal staining. In group III, 30% had basal stain and 70% showed basal and supra basal staining. In group IV, basal staining was seen in 50% of the samples while both basal and supra basal staining was observed in the remaining 50% of the samples. The tissue localization of p63 staining was not statistically significant among the 3 study groups (p=0.05).

- p53 and p63 staining showed a significant positive correlation in group I and III (p=0.00; p=0.00).

In conclusion, the results of the current study show that there is significantly higher expression of p53 and p63 proteins in OSCC, oral leukoplakia and OSF samples when compared to normal oral mucosa. Over-expression of p63 protein in OSCC, leukoplakia and OSF samples might be due to

- Compensatory up-regulation of p63, resulting from mutation of p53 gene
- Transcriptional dysregulation of p63 gene

However, the 4A4 antibody against p63 protein, which was used in our study does not discriminate TA or ΔN type of p63, both of which has opposite functions. Further mRNA studies by RT-PCR using isoform-specific primers, will ascertain the exact role of p63 in oral carcinogenesis and also its relationship to p53 protein.

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Anti-P63 [4A4]

AM418-5M
AM418-10M
MU418-UC
AZ418-10X

BioGenex

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Doc. No. 932-418M-4 Rev. E
Release Date: June 12, 2007

ENGLISH

Anti-Human P63	Immunogen	Clone	Species	Immunoglobulin Class	Protein Conc
	Amino terminal fragment of the deltaNp63 isoform expressed in E. coli as a GST fusion protein	4A4	Mouse	IgG2a	10 – 15 mg/ml*

*Lot specific Ig concentration available upon request

Catalog No.	Description	Recommended Detection System:
AM418-5M	6 ml of Ready-to-Use Antibody for Use with BioGenex Super Sensitive Detection Systems.	LINK-LABEL
AM418-10M	10 ml of Ready-to-Use Antibody for Use with BioGenex Super Sensitive Detection Systems and BioGenex Automated Staining Systems.	LINK-LABEL
MU418-UC	1 ml of Concentrated Antibody for Use with BioGenex Super Sensitive Detection Systems or Other Equivalent Detection Systems.	LINK-LABEL
AZ418-10X	10 ml of EZ Ready-to-Use Antibody for Use with BioGenex Super Sensitive Detection Systems and BioGenex Automated Staining Systems.	LINK-LABEL

Intended Use
This antibody is currently available for in vitro diagnostic use. This antibody is designed for the specific localization of p63 antigen in formalin-fixed, paraffin-embedded tissues.

Summary and Explanation
This antibody will detect all isoforms of p63 since the epitope is within the DNA binding domain. The p63 protein is a member of the p53 family, which also includes p73. Gene encoding p63 expresses at least 8 isoforms ranging in size from 40-80 kDA, with all isoforms containing common central domains but variable amino and carboxyl terminal extensions. p63 protein is detected in proliferating cells of epithelium, cervix, urothelium and prostate. It is also expressed in most poorly differentiated squamous cell carcinomas. The delta Np63 isoform is also abundantly expressed in nasopharyngeal carcinomas. Anti-p63 antibody enables the differentiation of benign/malignant prostatic lesions. The predominant localization of p63 protein is in the basal layer of stratified squamous and transitional epithelia. These basal cells act as the progenitors for the suprabasal cells, which undergo differentiation and cell death in regenerative epithelia. p63 is also an essential gene that is critical for regenerative proliferation of cells involved in limb, craniofacial and epidermal morphogenesis.

Principles of the Procedure
The demonstration of antigens by immunohistochemistry is a two-step process involving first, the binding of a primary antibody to the antigen of interest, and second, the detection of bound antibody by a chromogen. The primary antibody may be used in immunohistochemistry using manual techniques or using BioGenex Automated Staining System. BioGenex offers a variety of Super Sensitive detection systems including link-label and polymer based technologies to detect the chromogenic signal from the stained tissues and cells.

Reagents Provided
Purified mouse IgG diluted in PBS, pH 7.6, containing 1% BSA and 0.09% sodium azide.

Dilution of Primary Antibody
This Ready-to-Use antibody has been optimized for use with detection system as indicated above and should not require further dilution. Further dilution may result in loss of sensitivity. The user must validate any such change. BioGenex Concentrated antibodies must be diluted in accordance with the staining procedure when used with BioGenex Super Sensitive Detection Systems. Use of non-BioGenex systems other than recommended systems and protocols require validation by the user. Antibody dilutions should be appropriately adjusted and verified according to the detection system used.

Materials Required But Not Provided
All the reagents and materials required for immunohistochemistry are not provided. Pre-treatment reagents, Super Sensitive detection systems, control slides, control reagents and other ancillary reagents are available from BioGenex. Please refer to the product insert(s) of the BioGenex Super Sensitive Immunohistochemistry detection systems for detailed protocols and instructions. The immunohistochemistry procedure may need other lab equipment that are not provided including oven or incubator (capable of maintaining 56-60°C), BioGenex Automated Staining System, Humidity Chamber, Microwave oven, Staining Jars or baths, Timer (capable of 3-20 minute intervals), Wash Bottles, Absorbent Wipes, Microscope slides (pre-treated with poly-L-Lysine), Coverslips, Lens paper and Light microscope with magnification of 200X.

Storage and Handling
Antibodies should be stored at 2-8°C without further dilution. Fresh dilutions, if required, should be made prior to use and are stable for up to one day at room temperature (20-26°C). Unused portions of antibody preparations should be discarded after one day.
This antibody is suitable for use until expiry date when stored at 2-8°C. Do not use product after the expiration date printed on vial. If reagents are stored under a condition other than those specified in the package insert, they must be verified by the user (U.S. Congress, 1992).
The presence of precipitate or an unusual odor indicates that the antibody is deteriorating and should not be used. Positive and negative controls should be run simultaneously with all patient specimens. If unexpected staining is observed which cannot be explained by variations in laboratory procedures and a problem with the antibody is suspected, contact BioGenex Technical Support at 925-275-0550 or your local distributor.

Specimen Collection and Preparation
Tissues fixed in 10% (v/v) formalin are suitable for use prior to paraffin embedding. Consult references (Kiernan, 1981: Sheehan & Hrapchak, 1980) for further details on specimen preparation.

Treatment of Tissues Prior to Staining
Pretreatment of tissues if any, should be done as suggested in the staining procedure section.

Precautions
This antibody contains no hazardous material at a reportable concentration according to U.S. 29 CFR 1910.1200, OSHA Hazard Communication Standard and EC Directive 91/155/EC. However, this product contains sodium azide, at concentrations of less than 0.1%. Sodium azide is not classified as a hazardous chemical at the product concentrations. However, toxicity information regarding sodium azide at product concentrations has not been thoroughly investigated. Sodium azide may react with lead or copper plumbing to form highly explosive metal azides. Upon disposal, flush with large volumes of water to prevent azide build-up in plumbing (Center for Disease Control, 1976, National Institute for Occupational Safety and Health, 1976). For more information, a Material Safety Data Sheet for sodium azide in pure form is available upon request. Do not pipette reagents by mouth, and avoid contact of reagents and specimens with skin and mucous membranes. If reagents or specimens come in contact with sensitive area, wash with copious amounts of water. Minimize microbial contamination of reagents or increase in nonspecific staining may occur. Refer to appropriate product inserts for instructions of use and safety information on detection reagents and other materials, which may be used with the antibody.

Staining Procedure
Refer to the following table for conditions specifically recommended for this antibody. Refer to the detection system package insert for guidance on specific staining protocols or other requirements.

Parameter	BioGenex Recommendations
Control Tissue	SKIN
Tissue Type	Formalin-fixed, Paraffin-embedded
Concentrated Dilution	5-20
Pretreatment	AR Citra Plus
Pretreatment (EZ Antibody)	EZ-AR Common + EZ-AR3
Incubation Time and Temperature	30 min. @ RT
Incubation Time and Temperature (EZ)	30 min. @ RT

Quality Control
The recommended positive control tissue for this antibody is SKIN. FB-418M/ FG-418M tissues are available from BioGenex for QC. Refer to the appropriate detection system package inserts for guidance on general quality control procedures.

Troubleshooting
Refer to the troubleshooting section in the package inserts of BioGenex Super Sensitive Detection Systems (or other equivalent detection systems) for remedial actions on detection system related issues, or contact BioGenex Technical Support Department at 925-275-0550 to report unusual staining.

Expected Results
This Antibody stains the nuclei of basal or progenitor cells in a variety of epithelia. Interpretation of the staining result is solely the responsibility of the user. Any experimental result should be confirmed by a medically established diagnostic product or procedure.

Limitations of the Procedure
Immunohistochemistry (IHC) is a complex technique involving both histological and immunological detection methods. Tissue processing and handling prior to immunostaining can also cause inconsistent results. Variations in fixation and embedding or the inherent nature of the tissue may cause variations in results (Nadji and Morales, 1983). Endogenous peroxidase activity or pseudoperoxidase activity in erythrocytes and endogenous biotin may cause non-specific staining depending on detection system used. Tissues containing Hepatitis B Surface Antigen (HBsAg) may give false positive with horseradish peroxidase systems (Omata et al, 1980). Improper counterstaining and mounting may compromise the interpretation of results.

Performance Characteristics
BioGenex has conducted studies to evaluate the performance of the antibody with BioGenex detection systems and accessories. The antibodies have been found to be sensitive and show specific binding to the antigen of interest with minimal to no binding to non-specific tissues or cells. BioGenex antibodies have shown reproducible and consistent results when used within a single run, between runs, between lots and wherever applicable between manual and automated runs. The products have been determined to be stable for the periods specified on the labels either by standard real time or accelerated methods. BioGenex ensures product quality through 100% quality control for all products released and through surveillance programs.

Anti-p53 Protein [D07]

AM239-5M
AM239-10M
MU239-UC
AZ239-10X

BioGenex

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Doc. No. 932-239M-4 Rev. F
Release Date: November 10, 2005

ENGLISH

Anti-Human p53 Protein	Immunogen	Clone	Species	Immunoglobulin Class	Protein Conc
	Recombinant wild-type p53 protein	D07	Mouse	IgG2b	10 – 15 mg/ml*
*Lot specific Ig concentration available upon request					

Catalog No.	Description	Recommended Detection System:
AM239-5M	6 ml of Ready-to-Use Antibody for Use with BioGenex Super Sensitive Detection Systems.	LINK-LABEL
AM239-10M	10 ml of Ready-to-Use Antibody for Use with BioGenex Super Sensitive Detection Systems and BioGenex Automated Staining Systems.	LINK-LABEL
MU239-UC	1 ml of Concentrated Antibody for Use with BioGenex Super Sensitive Detection Systems or Other Equivalent Detection Systems.	LINK-LABEL
AZ239-10X	10 ml of EZ Ready-to-Use Antibody for Use with BioGenex Super Sensitive Detection Systems and BioGenex Automated Staining Systems.	LINK-LABEL

Intended Use
This antibody is currently available for in vitro diagnostic use. This monoclonal antibody is designed for the specific localization of p53 protein in formalin-fixed, paraffin-embedded tissue sections.

Summary and Explanation
p53 is a tumor suppressor gene product identified in a wide variety of tumors. Point mutations result in a mutant p53 protein with an altered conformational structure and an increased half life (T 1/2) and hence accumulation in nucleus of malignant cells. p53 protein is present in low concentration in normal cells, but elevated levels of mutant p53 have been found in many common cancers. Accumulation of mutant p53 detected by immunohistochemical staining has been reported in breast, lung, colon, stomach, bladder, and testis carcinomas, soft-tissue sarcomas, and melanomas. Bártek et al reported that mutation of the p53 gene with over-expression of the mutant protein is a frequent specific genetic change in malignant breast cancer. Immunohistological staining of colorectal carcinomas with antibodies specific to p53 demonstrated over-expression of the protein in about 50% of the malignant tumors.

Principles of the Procedure
The demonstration of antigens by immunohistochemistry is a two-step process involving first, the binding of a primary antibody to the antigen of interest, and second, the detection of bound antibody by a chromogen. The primary antibody may be used in immunohistochemistry using manual techniques or using BioGenex Automated Staining System. BioGenex offers a variety of Super Sensitive detection systems including link-label and polymer based technologies to detect the chromogenic signal from the stained tissues and cells.

Reagents Provided
Mouse monoclonal antibody from tissue culture supernatant diluted in PBS, pH 7.6, containing 1% BSA and 0.09% sodium azide.

Dilution of Primary Antibody
This Ready-to-Use antibody has been optimized for use with detection system as indicated above and should not require further dilution. Further dilution may result in loss of sensitivity. The user must validate any such change. BioGenex Concentrated antibodies must be diluted in accordance with the staining procedure when used with BioGenex Super Sensitive Detection Systems. Use of non-BioGenex systems other than recommended systems and protocols require validation by the user. Antibody dilutions should be appropriately adjusted and verified according to the detection system used.

Materials Required But Not Provided
All the reagents and materials required for immunohistochemistry are not provided. Pre-treatment reagents, Super Sensitive detection systems, control slides, control reagents and other ancillary reagents are available from BioGenex. Please refer to the product insert(s) of the BioGenex Super Sensitive Immunohistochemistry detection systems for detailed protocols and instructions. The immunohistochemistry procedure may need other lab equipment that are not provided including oven or incubator (capable of maintaining 56-60°C), BioGenex Automated Staining System, Humidity Chamber, Microwave oven, Staining Jars or baths, Timer (capable of 3-20 minute intervals), Wash Bottles, Absorbent Wipes, Microscope slides (pre-treated with poly-L-Lysine), Coverslips, Lens paper and Light microscope with magnification of 200X.

Storage and Handling
Antibodies should be stored at 2-8°C without further dilution. Fresh dilutions, if required, should be made prior to use and are stable for up to one day at room temperature (20-26°C). Unused portions of antibody preparations should be discarded after one day.
This antibody is suitable for use until expiry date when stored at 2-8°C. Do not use product after the expiration date printed on vial. If reagents are stored under a condition other than those specified in the package insert, they must be verified by the user (U.S. Congress, 1992).
The presence of precipitate or an unusual odor indicates that the antibody is deteriorating and should not be used. Positive and negative controls should be run simultaneously with all patient specimens. If unexpected staining is observed which cannot be explained by variations in laboratory procedures and a problem with the antibody is suspected, contact BioGenex Technical Support at 925-275-0550 or your local distributor.

Specimen Collection and Preparation
Tissues fixed in 10% (v/v) formalin are suitable for use prior to paraffin embedding. Consult references (Kiernan, 1981: Sheehan & Hrapchak, 1980) for further details on specimen preparation.

Treatment of Tissues Prior to Staining
Pretreatment of tissues if any, should be done as suggested in the staining procedure section.

Precautions
This antibody contains no hazardous material at a reportable concentration according to U.S. 29 CFR 1910.1200, OSHA Hazard Communication Standard and EC Directive 91/155/EC. However, this product contains sodium azide, at concentrations of less than 0.1%. Sodium azide is not classified as a hazardous chemical at the product concentrations. However, toxicity information regarding sodium azide at product concentrations has not been thoroughly investigated. Sodium azide may react with lead or copper plumbing to form highly explosive metal azides. Upon disposal, flush with large volumes of water to prevent azide build-up in plumbing (Center for Disease Control, 1976, National Institute for Occupational Safety and Health, 1976). For more information, a Material Safety Data Sheet for sodium azide in pure form is available upon request. Do not pipette reagents by mouth, and avoid contact of reagents and specimens with skin and mucous membranes. If reagents or specimens come in contact with sensitive area, wash with copious amounts of water. Minimize microbial contamination of reagents or increase in nonspecific staining may occur. Refer to appropriate product inserts for instructions of use and safety information on detection reagents and other materials, which may be used with the antibody.

Staining Procedure
Refer to the following table for conditions specifically recommended for this antibody. Refer to the detection system package insert for guidance on specific staining protocols or other requirements.

Parameter	BioGenex Recommendations
Control Tissue	BREAST CARCINOMA.
Tissue Type	Formalin-fixed, Paraffin-embedded
Concentrated Dilution	100-200
Pretreatment	AR Citra
Pretreatment (EZ Antibody)	EZ-AR Common + EZ-AR3
Incubation Time and Temperature	2 hrs. @ RT
Incubation Time and Temperature (EZ)	30 min. @ RT

Quality Control
The recommended positive control tissue for this antibody is BREAST CARCINOMA.. FB-239M/ FG-239M tissues are available from BioGenex for QC. Refer to the appropriate detection system package inserts for guidance on general quality control procedures.

Troubleshooting
Refer to the troubleshooting section in the package inserts of BioGenex Super Sensitive Detection Systems (or other equivalent detection systems) for remedial actions on detection system related issues, or contact BioGenex Technical Support Department at 925-275-0550 to report unusual staining.

Expected Results
This antibody stains positive in nucleus of a variety of tumor cells in formalin fixed paraffin embedded tissues. Interpretation of the staining result is solely the responsibility of the user. Any experimental result should be confirmed by a medically established diagnostic product or procedure.

Limitations of the Procedure
Immunohistochemistry (IHC) is a complex technique involving both histological and immunological detection methods. Tissue processing and handling prior to immunostaining can also cause inconsistent results. Variations in fixation and embedding or the inherent nature of the tissue may cause variations in results (Nadji and Morales, 1983). Endogenous peroxidase activity or pseudoperoxidase activity in erythrocytes and endogenous biotin may cause non-specific staining depending on detection system used. Tissues containing Hepatitis B Surface Antigen (HBsAg) may give false positive with horseradish peroxidase systems (Omata et al, 1980). Improper counterstaining and mounting may compromise the interpretation of results.

Performance Characteristics
BioGenex has conducted studies to evaluate the performance of the antibody with BioGenex detection systems and accessories. The antibodies have been found to be sensitive and show specific binding to the antigen of interest with minimal to no binding to non-specific tissues or cells. BioGenex antibodies have shown reproducible and consistent results when used within a single run, between runs, between lots and wherever applicable between manual and automated runs. The products have been determined to be stable for the periods specified on the labels either by standard real time or accelerated methods. BioGenex ensures product quality through 100% quality control for all products released and through surveillance programs.

Figure 1: Armamentarium

Figure 2: Antibody Kit

CLINICAL PHOTOGRAPHS

Figure 3: Oral squamous cell carcinoma

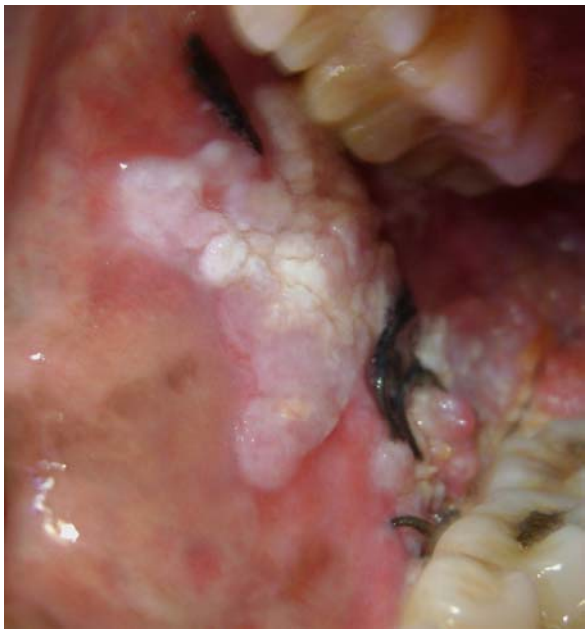


Figure 4: Leukoplakia

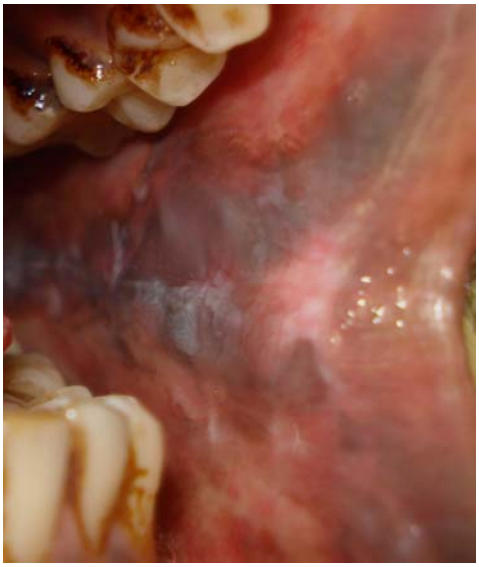
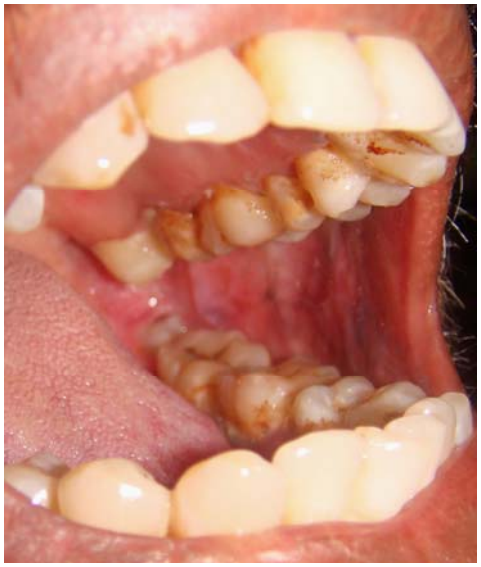


Figure 5: Oral submucous fibrosis



p53 STAINING IN ORAL SQUAMOUS CELL CARCINOMA

Figure 6: H & E; 10 x

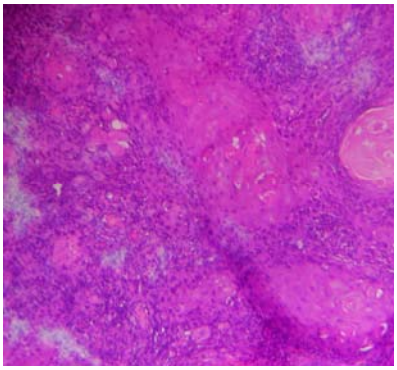


Figure 7: Negative control; 10 x

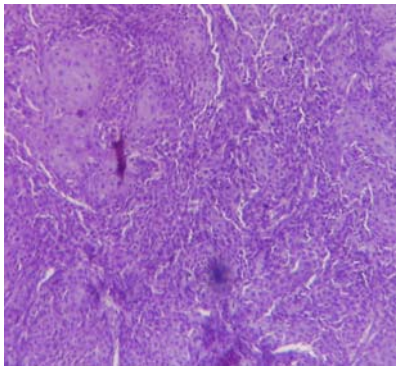


Figure 8: p53 stain; 10 x

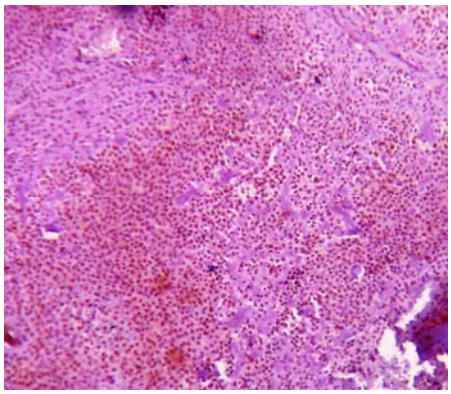
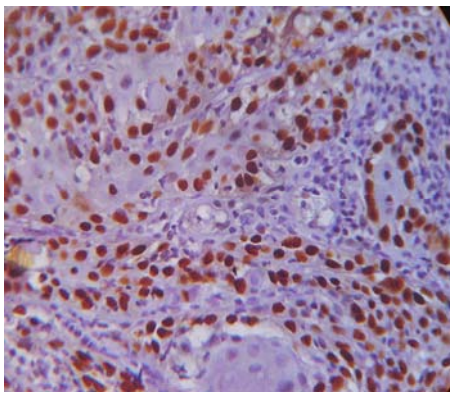


Figure 9: p53 stain; 40 x



p63 STAINING IN ORAL SQUAMOUS CELL CARCINOMA

Figure 10: H & E; 10 x

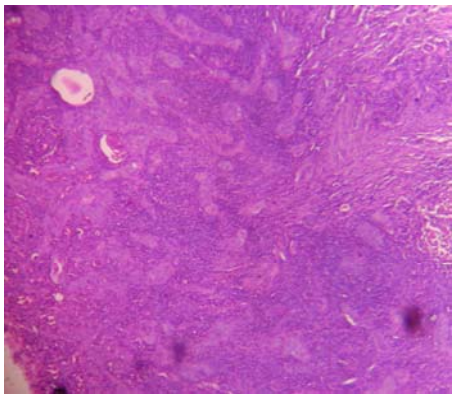


Figure 11: Negative control; 10 x

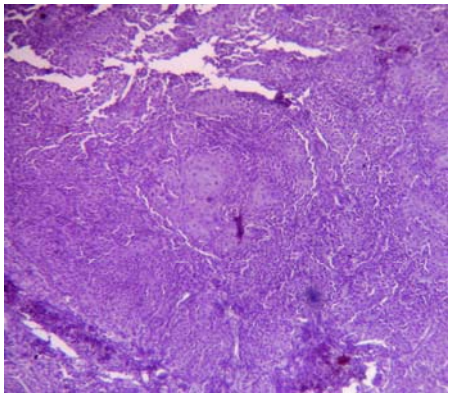


Figure 12: p63 stain; 10 x

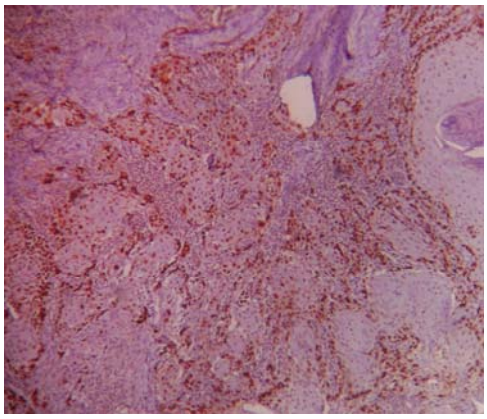
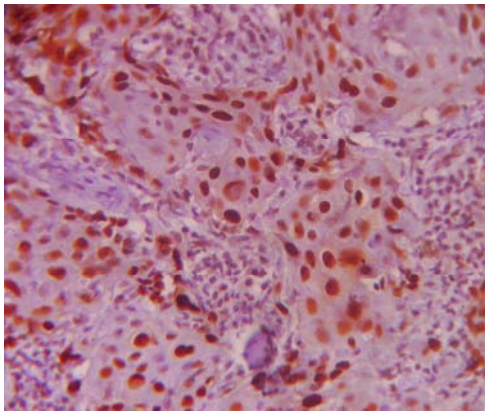


Figure 13: p63 stain; 40 x



p53 STAINING IN EPITHELIAL DYSPLASIA

Figure 14: H & E; 10 x

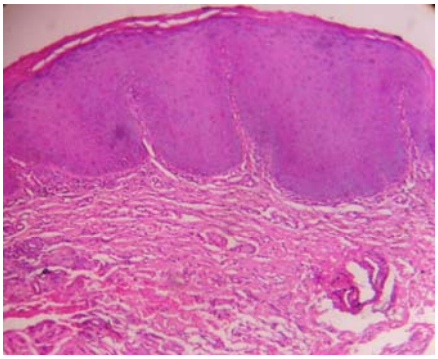


Figure 15: Negative control; 10 x

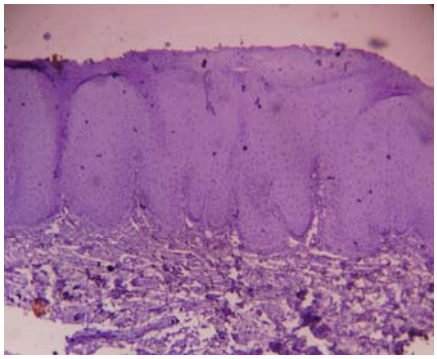


Figure 16: p53 stain; 10 x

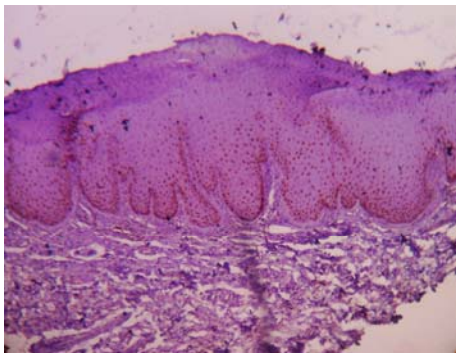
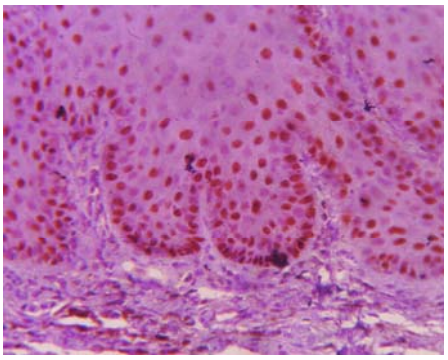


Figure 17: p53 stain; 40 x



p63 STAINING IN EPITHELIAL DYSPLASIA

Figure 18: H & E; 10 x

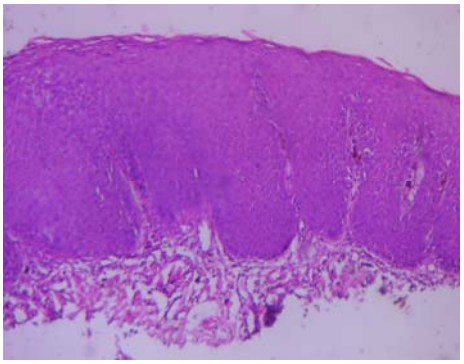


Figure 19: Negative control; 10 x

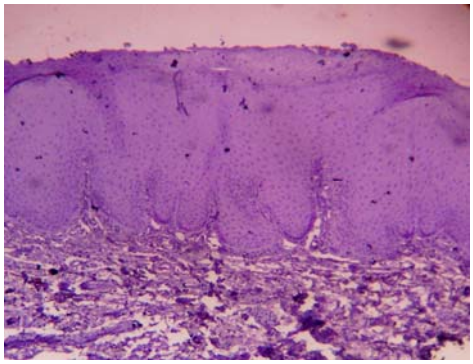


Figure 20: p63 stain; 10 x

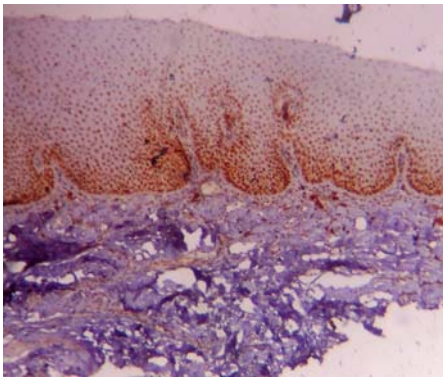
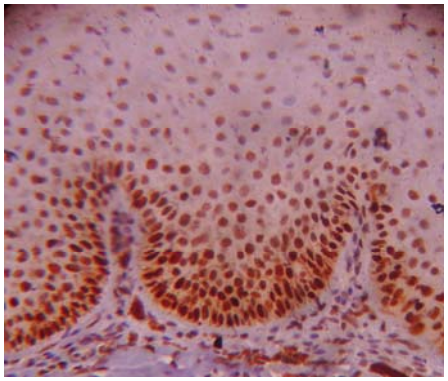


Figure 21: p63 stain; 40 x



p53 STAINING IN ORAL SUBMUCOUS FIBROSIS

Figure 22: H & E; 10 x

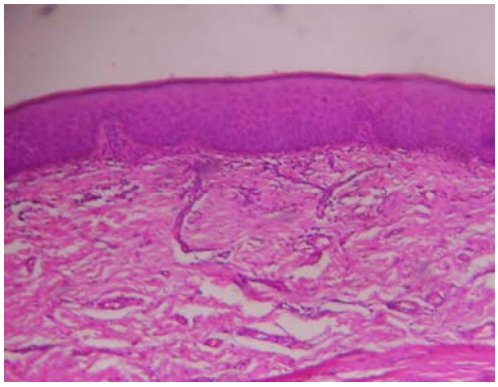


Figure 23: Negative control; 10 x

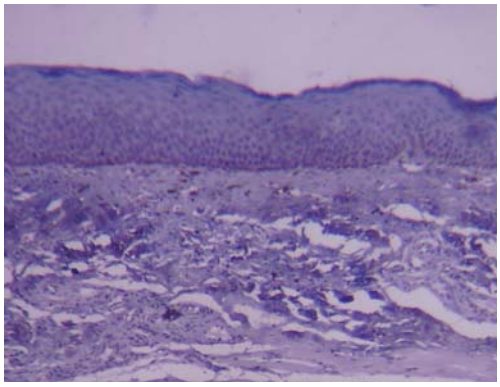


Figure 24: p53 stain; 10 x

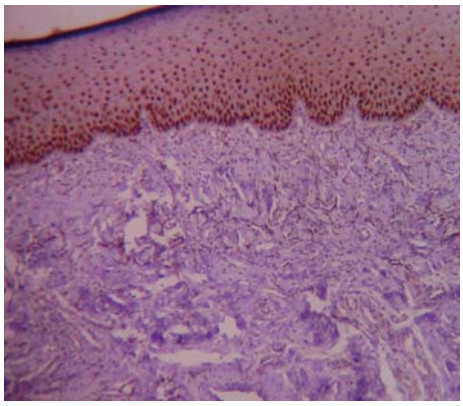
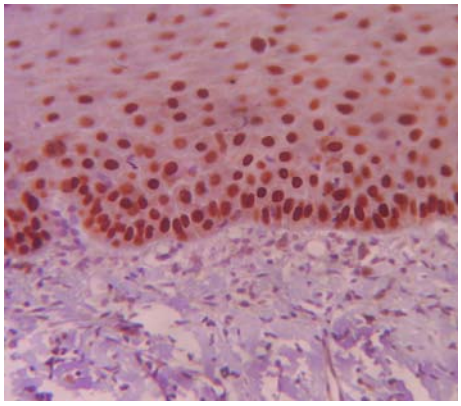


Figure 25: p53 stain; 40



p63 STAINING IN ORAL SUBMUCOUS FIBROSIS

Figure 26: H & E; 10 x

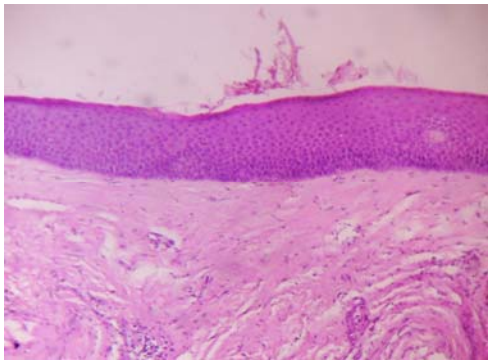


Figure 27: Negative control; 10

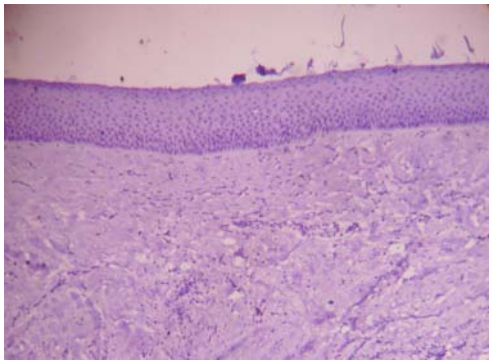


Figure 28: p63 stain; 10 x

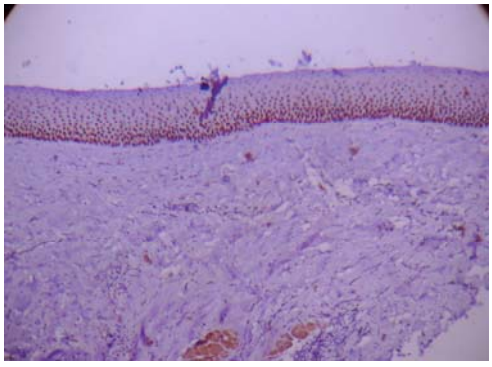
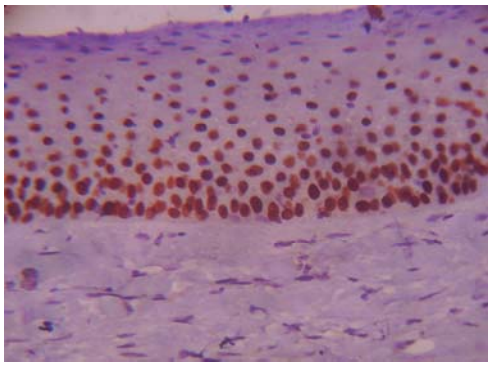


Figure 29: p63 stain; 40 x



p53 STAINING IN NORMAL ORAL MUCOSA

Figure 30: H & E; 10

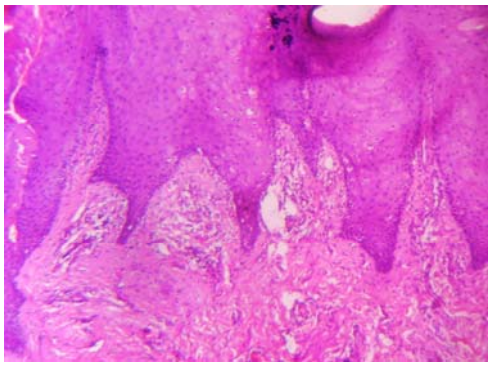


Figure 31: Negative control; 10 x

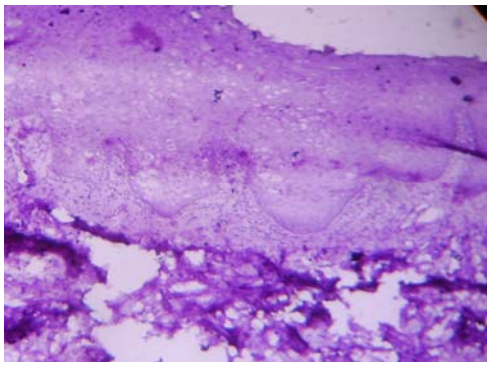


Figure 32: p53 stain; 10 x

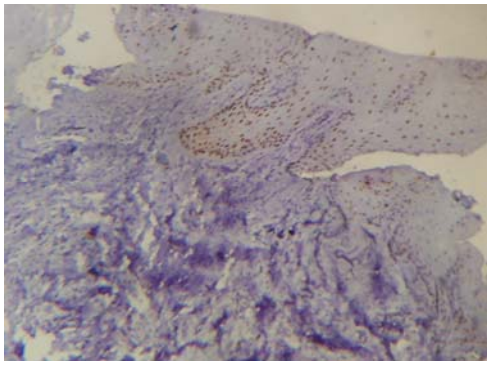
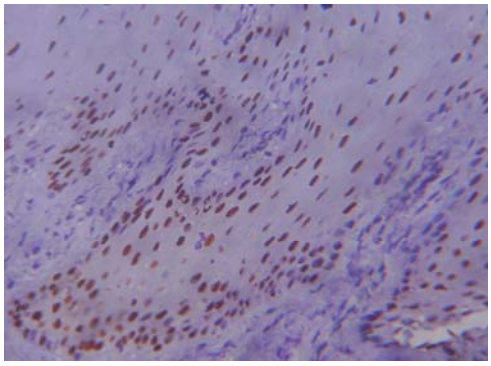


Figure 33: p53 stain; 40 x



p63 STAINING IN NORMAL ORAL MUCOSA

Figure 34: H & E; 10 x

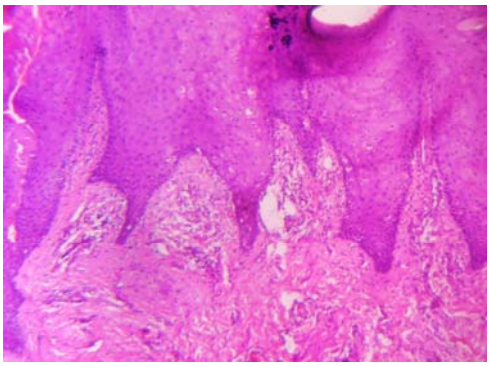


Figure 35: Negative control; 10 x

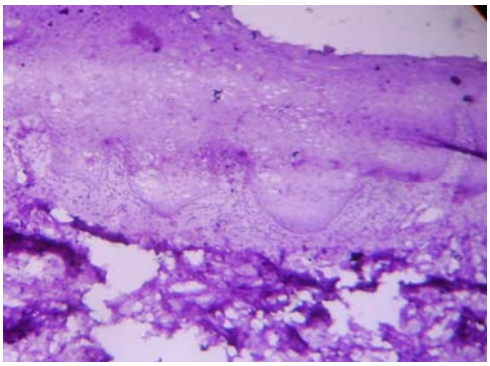


Figure 36: p63 stain; 10 x

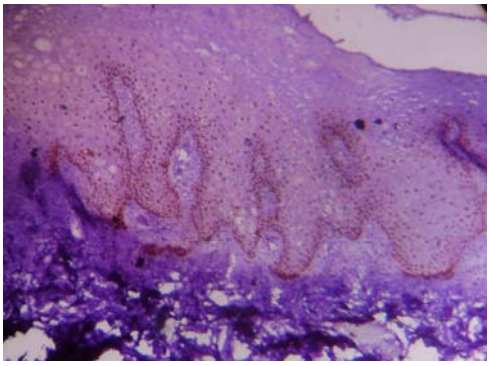


Figure 37: p63 stain; 40 x

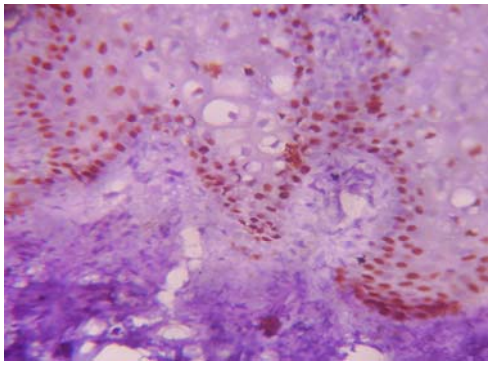


Table 1: Mean age distribution among the study groups

Groups	Age in years		p-value
	Mean	SD	
OSCC (Group I) (n=20)	55.2	9.9	0.000*
Leukoplakia (Group II) (n=20)	43.6	10.6	
OSF (Groups III) (n=20)	36.4	7.3	
Normal (Group IV) (n=10)	29.5	7.9	

*p<0.05 is statistically significant at 5% level

GRAPH 1: MEAN AGE DISTRIBUTION AMONG STUDY GROUPS

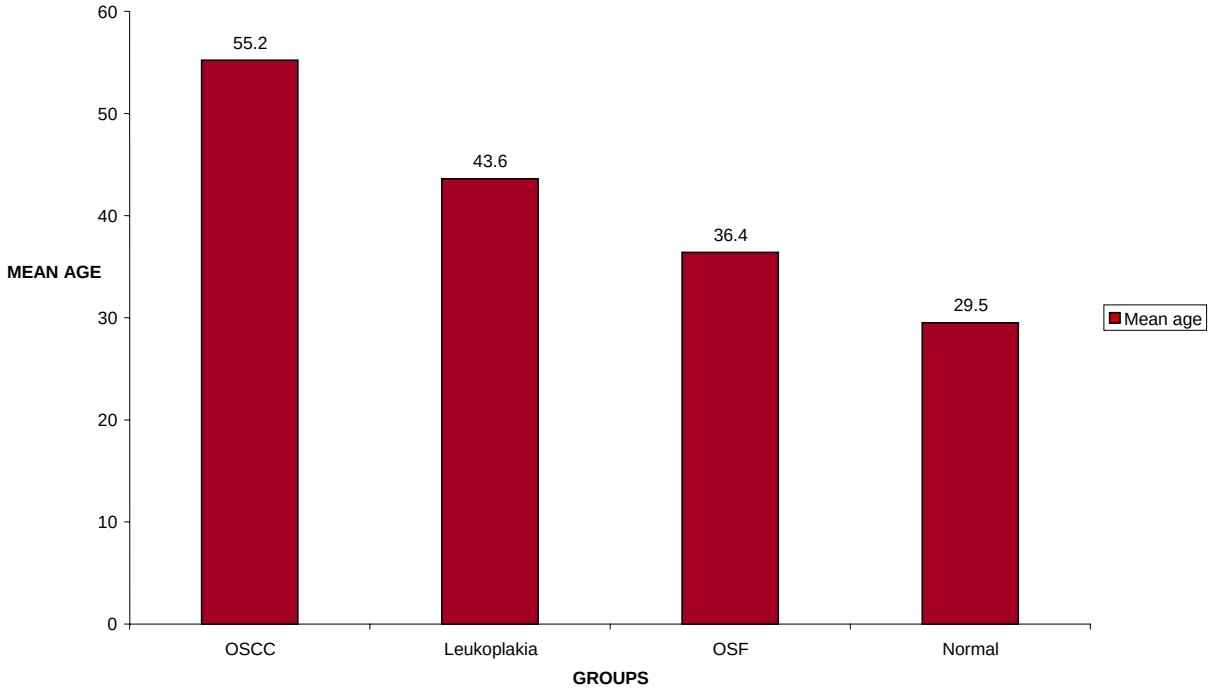


Table 2: Comparison of sex distribution among study groups

Groups	Males		Females		p-value
	n	%	n	%	
OSCC (Group I) (n=20)	16	80	4	20	0.000*
Leukoplakia (Group II) (n=20)	20	100	0	0	
OSF (Group III) (n=20)	19	95	1	5	
Normal (Group IV) (n=10)	4	40	6	60	

*p<0.05 is statistically significant at 5% level

GRAPH 2: COMPARISON OF SEX DISTRIBUTION AMONG STUDY GROUPS

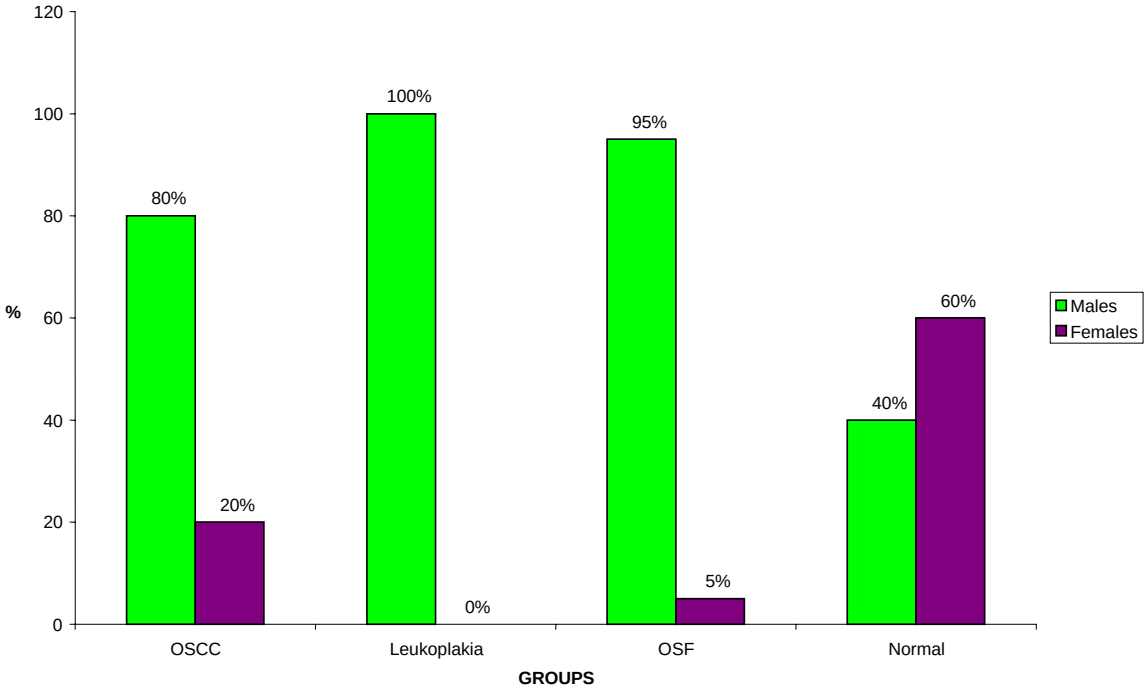


Table 3: Comparison of habits among study groups

Groups	Habits								p-value
	Smoking		Chewing**		Smoking + Chewing		No habits		
	n	%	n	%	n	%	n	%	
OSCC (Group I) (n=20)	4	20	11	55	5	25	0	0	0.000*
Leukoplakia (Group II) (n=20)	9	45	5	25	6	30	0	0	
OSF (Group III) (n=20)	0	0	17	85	3	15	0	0	
Normal (Group IV) (n=10)	0	0	0	0	0	0	10	100	

*p<0.05 is statistically significant at 5% level

**Tobacco or Pan masala

GRAPH 3: COMPARISON OF HABITS AMONG STUDY GROUPS

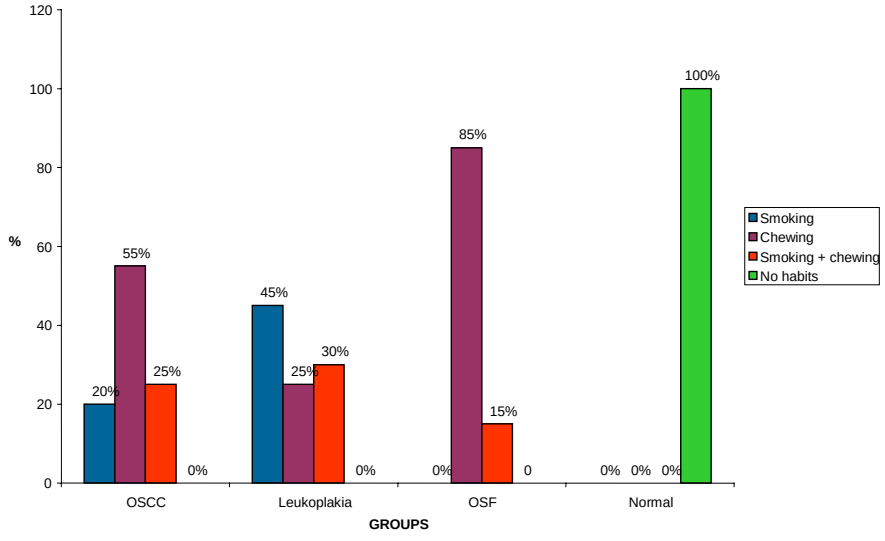


Table 4: Distribution of histopathological grading in OSCC

Grade	OSCC	
	n	%
Well differentiated	14	70
Moderately differentiated	4	20
Poorly differentiated	2	10

GRAPH 4: DISTRIBUTION OF HISTOPATHOLOGICAL GRADING IN OSCC

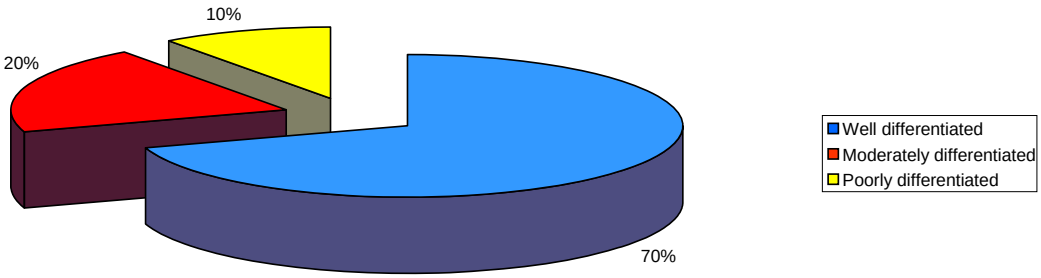


Table 5: Distribution of histopathological grading in Leukoplakia

Grade	Leukoplakia	
	n	%
Mild dysplasia	9	45
Moderate dysplasia	7	35
Severe dysplasia	4	20

GRAPH 5: DISTRIBUTION OF HISTOPATHOLOGICAL GRADING IN LEUKOPLAKIA

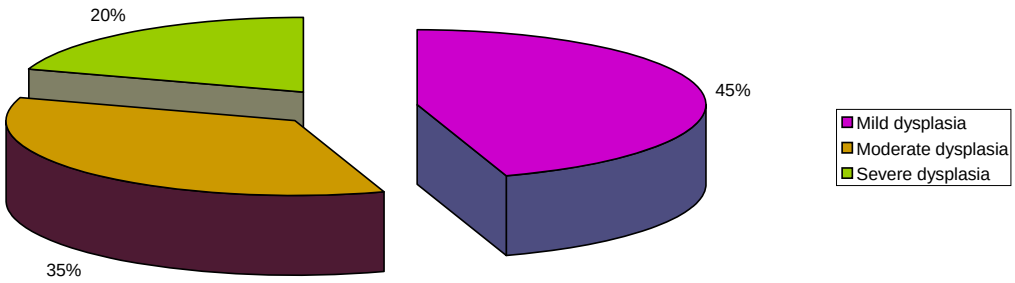


Table 6: Comparison of p53 labelling index among study groups

Groups	Labelling index		P- value
	Mean	SD	
OSCC(Group I) (n=20)	56.9	21.3	0.000*
Leukoplakia (Group II) (n=20)	37.6	12.6	
OSF(Group III) (n=20)	34.6	8.7	
Normal(Group IV) (n=10)	15.1	9	

*p<0.05 is statistically significant at 5% level

GRAPH 6: COMPARISON OF p53 MEAN LABELLING INDEX AMONG STUDY GROUPS

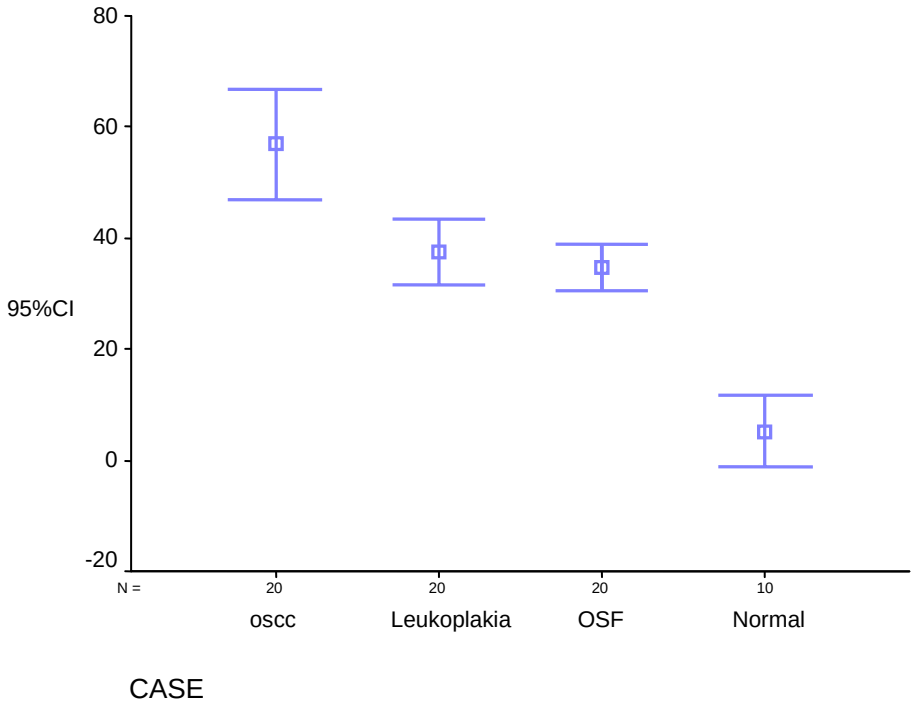


Table 7: **Comparison of p53 localization among study groups**

Groups	Localization						p- value
	Only Basal		Basal + Suprabasal		No stain		
	n	%	N	%	n	%	
Leukoplakia (Group II) (n=20)	4	20	16	80	0	0	0.00*
OSF (Group III) (n=20)	8	40	12	60	0	0	
Normal (Group IV) (n=10)	2	20	1	10	7	70	

*p<0.05 is statistically significant at 5% level

GRAPH 7: COMPARISON OF p53 LOCALIZATION AMONG STUDY GROUPS

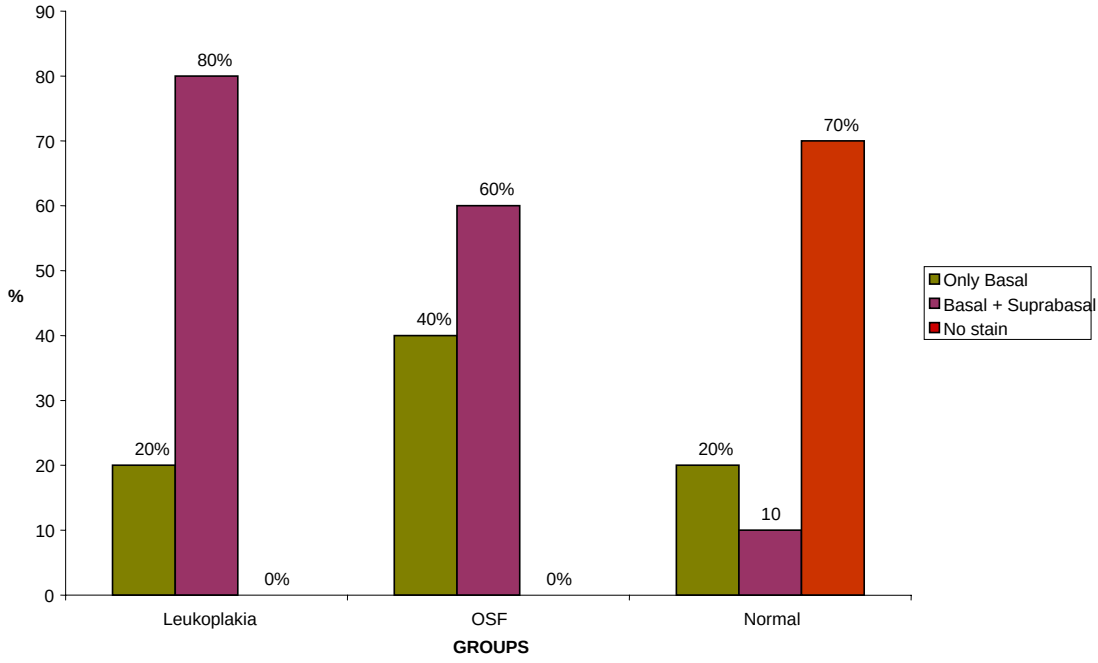


Table 8: Comparison of p63 labelling index among study groups

Groups	Labelling index		p- value
	Mean	SD	
OSCC (Group I) (n=20)	56.8	19.6	0.00*
Leukoplakia (Group II) (n=20)	42.3	10.5	
OSF (Group III) (n=20)	32.8	12.1	
Normal (Group IV) (n=10)	26.4	9.4	

*p<0.05 is statistically significant at 5% level

GRAPH 8: COMPARISON OF p63 MEAN LABELLING INDEX AMONG STUDY GROUPS

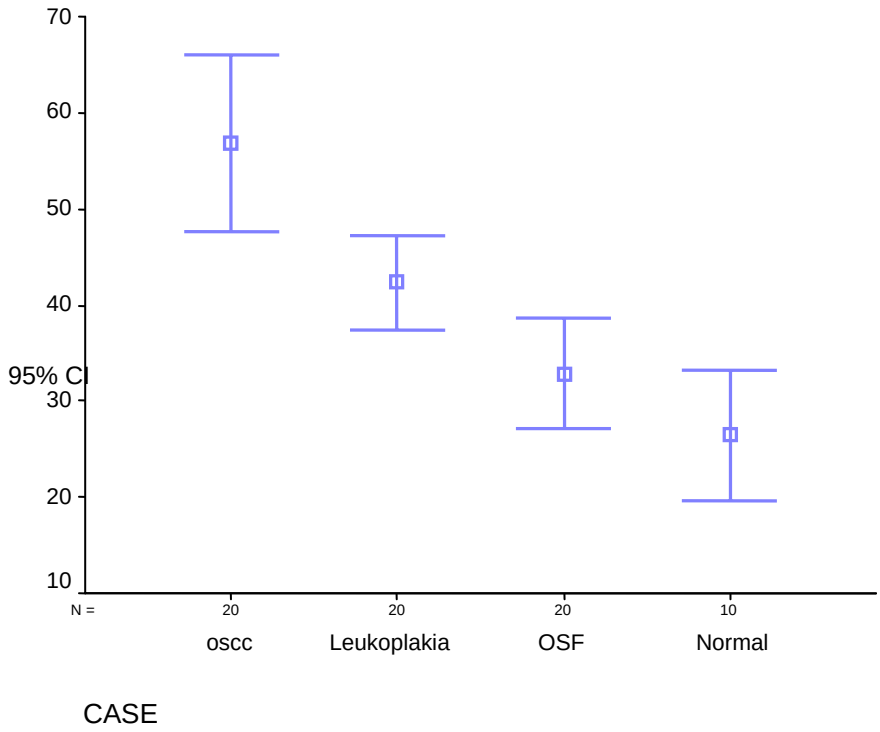
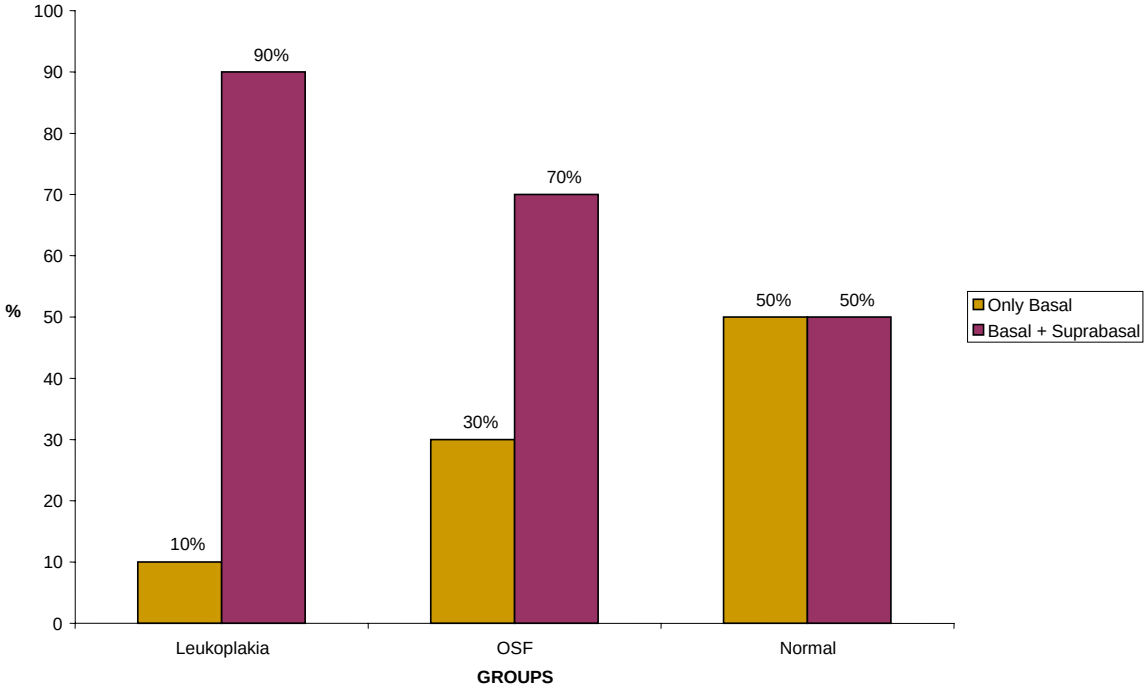


Table 9: Comparison of p63 localization among study groups

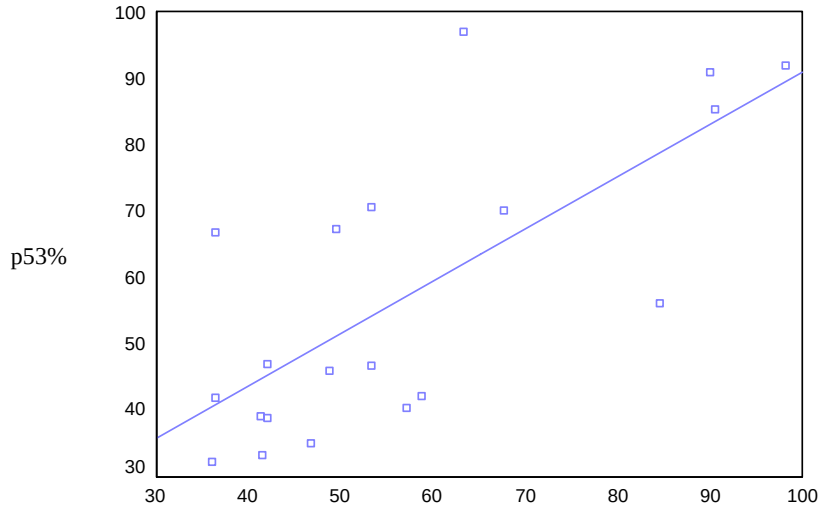
Groups	Localization				p- value
	Only Basal		Basal + Suprabasal		
	n	%	n	%	
Leukoplakia(GroupII)	2	10	18	90	0.05
OSF(GroupIII)	6	30	14	70	
Normal(GroupIV)	5	50	5	50	

$p<0.05$ is statistically significant at 5% level

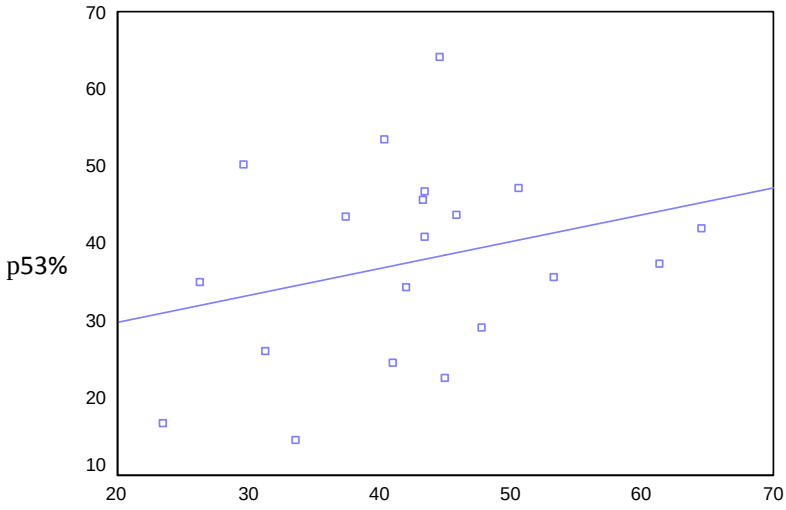
GRAPH 9: COMPARISON OF p63 LOCALIZATION AMONG STUDY GROUPS



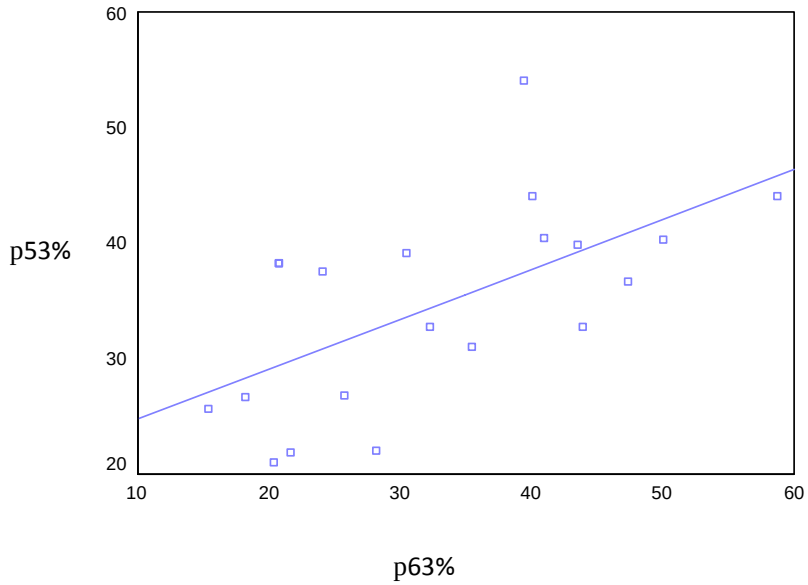
GRAPH 10: CORRELATION BETWEEN p53 AND p63 IN OSCC



GRAPH 11: CORRELATION BETWEEN p53 AND p63 IN LEUKOPLAKIA



GRAPH 12: CORRELATION BETWEEN p53 AND p63 IN OSF



GRAPH 13: CORRELATION BETWEEN p53 AND p63 IN NORMAL