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IMMUNOHISTOCHEMICAL STUDY OF p53 AND bcl-2 EXPRESSION IN ORAL EPITHELIAL DYSPLASIA AND ORAL SQUAMOUS CELL CARCINOMA

Dissertation submitted to

THE TAMIL NADU Dr. M.G.R. MEDICAL UNIVERSITY

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in

ORAL AND MAXILLOFACIAL PATHOLOGY

by

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DECLARATION

The dissertation titled **IMMUNOHISTOCHEMICAL STUDY OF p53 AND bcl-2 EXPRESSION IN ORAL EPITHELIAL DYSPLASIA AND ORAL SQUAMOUS CELL CARCINOMA** is a bonafide record of work done by me under the guidance of **Dr. K. RANGANATHAN**, Professor, Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital, Chennai.

This dissertation is submitted to **THE TAMIL NADU Dr. M.G.R. MEDICAL UNIVERSITY**, in partial fulfillment for the degree of **MASTER OF DENTAL SURGERY** in the branch of **ORAL AND MAXILLOFACIAL PATHOLOGY**. It has not been submitted partially or fully, for the award of any other degree or diploma.

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INTRODUCTION

Cancer is one of the main causes of death all over the world; its relative position varying with age, sex and geographic location.³⁴ Globally, oral cancer is the sixth most common cause of cancer related death.⁵⁵ In India, cancer of the oral cavity is one of the five leading types of cancer in either sex and comprises about 40% of all the cancers.⁸⁵ Over 80% of malignant lesions of the oral cavity are squamous cell carcinomas of the lining mucosa and it is for this reason the term oral cancer has become synonymous with oral squamous cell carcinoma (OSCC). OSCC has a relatively unfavorable prognosis, with an overall five-year survival rate of 35-50%.³⁴ The survival rate has virtually not changed over the past three decades and the increase in the incidence of OSCC accentuates the mortality from cancer.

Oral epithelial dysplasias are considered potentially malignant lesions as they have a higher probability of developing into squamous cell carcinomas when compared to normal oral mucosa. Oral epithelial dysplasias are classified as mild, moderate, or severe depending on the extent of dysplastic changes. The percentage of these lesions that progress to OSCC is accepted to be directly proportional to the severity of the dysplastic changes.⁴⁸ Although the figures are variable, they range between 0.3% and 17.5% and a consensus range has been regarded to be around 3 – 6%.³⁵ Furthermore, transformation into OSCC in moderate or severe dysplasia appears to be at least double (2.3 times) than in mild dysplasia or hyperplasia.⁷³

The pioneer work by Fearon and Vogelstein characterizing the genetic alterations in colorectal cancers has become a paradigm for other neoplasms.²² OSCC is one of the few cancer types which is easily accessible to obtain biopsies at all stages of cancer progression. Consequently, it is possible to define a genetic progression model of this disease. The frequency of genomic alterations in tumor and histopathologically defined precursor lesions has formed the basis for the description of the first genetic progression model for OSCC by Califano et al ⁹ and has led to the belief that OSCC may follow a progression pattern preceded

by lesions exhibiting dysplasia.⁵⁸

Many surrogate markers have been used in the recent years to identify dysplastic lesions that have a propensity to convert to OSCC. The most frequently involved and best studied biomarker *p53* is a tumor suppressor gene that participates in cell proliferation control and plays a role in deletion of cells with DNA damage by induction of apoptosis. Aberrant *p53* expression is considered one of the most common genetic events in OSCC. The protein expression of *bcl-2* gene, an anti-apoptotic marker, is found to occur early in oral carcinogenesis.⁸⁹ Abnormal expression of *bcl-2* leads to extended cell survival and can facilitate the acquisition of additional mutations and eventual clonal expansion. Studies have revealed that *p53* inversely interacts with *bcl-2* in regulation of apoptosis. Therefore, much attention has been paid to a simultaneous investigation of *p53* and *bcl-2* abnormalities in order to obtain more accurate information for evaluation of malignant neoplasms.⁹³

The present study aims to assess the expression of a tumor suppressor protein (*p53*) and an apoptotic marker (*bcl-2*) in normal oral mucosa, oral epithelial dysplasia and OSCC using immunohistochemistry (IHC).

AIMS & OBJECTIVES

AIMS & OBJECTIVES

- To assess p53 expression in normal oral mucosa, oral epithelial dysplasia and OSCC by IHC.
- To assess bcl-2 expression in normal oral mucosa, oral epithelial dysplasia and OSCC by IHC.
- To evaluate the correlation between p53 and bcl-2 expression in normal oral mucosa, oral epithelial dysplasia and OSCC.

HYPOTHESIS

- p53 is over expressed in oral epithelial dysplasia and OSCC.
- bcl-2 is over expressed in oral epithelial dysplasia and OSCC.
- There is an inverse correlation between p53 and bcl-2 expression in oral epithelial dysplasia and OSCC.

REVIEW OF LITERATURE

EXPRESSION OF p53 IN OSCC, ORAL EPITHELIAL DYSPLASIA AND NORMAL MUCOSA

Field J.K, Spandidos D.A and Stell P.M (1992) in their study, correlated p53 protein expression with smoking and drinking habits of 43 head and neck squamous cell carcinoma (HNSCC) patients and found that those who smoked and drank heavily showed increased p53 expression. They concluded that smoking and drinking have a synergistic effect and point to a genetic link in the aberrant expression of p53.²³

Ogden G.R, Kiddie R.A, Lunny D.P et al (1992) assessed p53 expression using IHC in normal, benign, premalignant and malignant oral tissues and found p53 positivity in 54% of oral cancers and no expression in normal, benign and premalignant samples. They concluded that p53 expression appears to correlate with oral malignancy.⁵⁹

Kaur J, Srivastava A and Ralhan R (1994) detected p53 immunoreactivity in 55% of leukoplakia cases and 75% OSCCs from patients with betel, areca nut and/or tobacco chewing habit. They attributed this higher frequency of p53 over expression to the patient habits and concluded that p53 aberrations may be an early event in the development of OSCC in India.³⁹

Shin D.M, Kim J, Ro J.Y et al (1994) studied p53 immunohistochemical expression in 33 HNSCC with adjacent normal, hyperplastic and/or dysplastic epithelium. They detected positive p53 expression in 45% HNSCC, 21% adjacent normal epithelium, 29% cases of hyperplasia, and 45% samples of dysplasia and concluded that p53 alterations can occur very early in HNSCC and may be used for risk assessment and in chemoprevention trials.⁷⁶

Piffko J, Bankfalvi A, Ofner D et al (1995) in their study, used a panel of four anti-p53 antibodies (CM1, Pab 1801, DO7, and Pab 240) and compared the expression of p53 using conventional (without pretreatment) antigen retrieval, with wet autoclaving and microwave irradiation. They observed that, wet autoclave pretreatment was significantly superior for all the panel of antibodies used and concluded that it is a reliable and highly reproducible method for p53 antigen retrieval in routinely processed archival material.⁶⁶

Kerdpon D, Rich A.M and Reade P.C (1997) correlated the immunohistochemical expression of p53 in normal oral mucosa, oral mucosal hyperplasia, dysplasia and OSCC with the clinical outcome and observed no p53 expression in normal mucosa; positive nuclear staining in 36% hyperplasia, 85% dysplasia and 95% OSCC. They further observed that, none of the p53 negative dysplasias progressed; 11% of the p53 positive dysplasias underwent neoplastic transformation, 19% recurred following excision, while 50% of the severe dysplasia resolved. They concluded that p53 expression increases from hyperplasia to dysplasia to OSCC, which may indicate an involvement of p53 in proliferation as well as in neoplastic transformation.⁴⁰

Cruz I.B, Snijders P.J.F, Meijer C.J.L.M et al (1998) investigated p53 immunohistochemical expression in 35 oral premalignant lesions and 11 OSCC that developed from them in a period of 16 years; 6 benign lesions and 11 normal mucosa served as controls. They noted that the combined use of histologic assessment of dysplasia (moderate/severe) with p53 expression patterns (suprabasal positivity) showed highest sensitivity for the detection of lesions that progressed to carcinoma (91%). When used individually, the p53 expression pattern showed higher specificity than assessment of dysplasia and higher positive predictive value for correct prediction of malignant transformation. They concluded that suprabasal p53 expression is an early event in oral carcinogenesis and an indicator of a developing carcinoma and further recommended combined p53 immunohistochemical analysis and histologic assessment to increase the sensitivity of detection of dysplastic lesions that will progress to carcinoma.¹⁷

Murti P.R, Warnakulasuriya K.A.A.S, Johnson N.W et al (1998) compared the immunohistochemical expression of p53 in 22 premalignant lesions that transformed to OSCC in 4-25 years against 68 similar lesions that did not transform over the same time period. Their results showed p53 immunoreactivity in 29% of the premalignant lesions that showed malignant transformation as against 31% of the lesions that did not transform; with 9 lesions becoming p53 positive as they progressed to OSCC. They concluded that p53 over expression peaks close to cancer rather than early in the natural history of oral precancer.⁵⁶

Chiang C.P, Huang J.S, Wang J.T et al (1999) studied the expression of p53 protein in OSCC of areca quid chewers and tobacco smokers using IHC. They noted p53 expression in 58% OSCC; p53 positivity was higher in patients without chewing or smoking habits than in patients with these habits. They found no significant correlation between p53 expression and the clinicopathologic parameters. Their analysis showed significantly better prognosis for patients with p53 negative tumors as compared to those with p53 positive tumors. They concluded that p53 may serve as an adjuvant marker of poor prognosis in patients with OSCC in Taiwan.¹⁴

Van Oijen M.G.C.T, Van de Craats J.G and Slootweg P.J (1999) compared the expression of p53 protein in the tumor adjacent normal mucosa of HNSCC patients with smoking habit and those without the habit. Their results showed that, p53 over expression occurred more frequently in tumor adjacent normal mucosa of smokers (50%) as compared to non-smokers (20%) with HNSCC. They concluded that an increase in focal p53 expression might represent an early alteration in the development of HNSCC in smokers and that other factors such as alcohol abuse or genetic mutagenic sensitivity might also play a role.⁸⁸

Cruz I.B, Meijer C.J.L.M, Snijders P.J.F et al (2000) investigated p53 immunoexpression patterns in OSCC, corresponding adjacent non-malignant mucosa and respective lymph node metastases. They observed that 17% of non-malignant mucosal samples adjacent to OSCC showed suprabasal p53 staining and this was significantly associated with moderate/severe dysplasia. In 86% of these carcinomas, p53

immunoexpression was seen in more than 50% of the neoplastic cells and in the remaining cases, p53 immunoexpression was found in more than 25% of the neoplastic cells. In all the p53 negative carcinomas that showed p53 immunoexpression in the non-malignant adjacent mucosa, p53 staining was never detected suprabasally. Lymph node metastases showed the same patterns of p53 immunoexpression as the carcinomas from which they derived. They concluded that immunostaining in non-malignant mucosa of the resected margins of OSCC might be a valuable predictor for local recurrences and may therefore have implications for the management of patients who have received surgical treatment for OSCC.¹⁶

Shin D.M, Charuruks N, Lippman S.M et al (2001) analyzed p53 protein expression and chromosome 9 and 17 polysomy in 48 HNSCC and their adjacent normal epithelium (31 sites), hyperplastic (24 sites) and dysplastic lesions (26 sites), and 7 normal controls. They observed p53 immunoreactivity in 58% carcinomas, 19% adjacent normal epithelium, 29% of the hyperplastic lesions and 46% dysplastic lesions, and no expression in the normal controls. They also observed an increase in the degree of chromosome polysomy from adjacent normal epithelium to carcinoma; lesions with dysregulated p53 expression showed nearly 2-4-fold increased levels of polysomy. They concluded that altered p53 expression is associated with increased genetic instability during head and neck carcinogenesis.⁷⁵

Gonzales Moles M.A, Galindo P, Gutierrez J et al (2002) evaluated immunohistochemical expression of p53 in 78 OSCCs and 53 cases of non-tumoral adjacent epithelium and concluded that the expression of p53 occurs early in oral carcinogenesis and that p53 does not help as an objective marker of the presence or severity of epithelial dysplasia.²⁶

Pillay M, Vasudevan D.M, Rao C.P et al (2003) evaluated immunohistochemical expression of p53 in 110 cases of OSCC, 35 dysplastic lesions, 15 hyperplastic lesions and 50 samples of normal mucosa and observed p53 over expression in 36% OSCC and 17% dysplasia; no expression in hyperplasia and normal mucosa. They concluded that p53 may be involved in only certain cases of OSCC and may be used to detect early cases.⁶⁷

EXPRESSION OF p53 IN RELATION TO OTHER MARKERS

Yan J.J, Tzeng C.C and Jin Y.T (1996) correlated p53 protein expression in 60 OSCC of tongue and buccal mucosa with PCNA expression and clinicopathologic parameters. Their results showed strong p53 immunoreactivity in 45% of cases; 48% of p53 positive OSCC demonstrated p53 over expression in adjacent hyperplastic or dysplastic epithelium. They found no association of p53 over expression with PCNA scores and tumor grade, size, staging, vascular invasion, lymph node metastasis, and early local recurrence. However, they noted p53 expression to be relatively higher in nonsmokers than in heavy smokers and in non-betel quid chewers than in heavy chewers. They concluded that inactivation of p53 protein may occur early in oral carcinogenesis and could possibly be used to assess risk and local recurrence.⁹²

Kushner J, Bradley G and Jordan R.C.K (1997) examined the relationship between p53 expression and Ki-67 expression using IHC in 40 epithelial dysplasias from the floor of the mouth. They noted a significant correlation between the labeling indices of p53 and Ki-67. They concluded that altered p53 protein expression is probably an early event in oral carcinogenesis in the floor of the mouth and is associated with dysregulation of cell proliferation.⁴⁶

Warnakulasuriya K.A.A.S, Tavassoli M and Johnson N.W (1998) examined the relationship between p53 protein expression and expression of p21, p27 and p16 proteins in 24 OSCC by IHC and detected p53 expression in 10 of 24 carcinomas. They observed that p21, p27 and p16 expression did not correlate with that of p53 and concluded that p21 / p27 positivity can be independent of p53 status and that p16 and p53 may act in separate pathways.⁹⁰

De Rosa I, Staibano S, Muzio L.L et al (1999) analyzed silver-stained nucleolar organizer regions (AgNORs) and immunohistochemical expression of PCNA, p53 and c-myc in 44 malignant and potentially malignant lesions of the lower lip. They concluded that the number and size of AgNORs and the percentage

of PCNA-positive cells are sensitive markers and their combined evaluation with the p53 and c-myc status might help in prognostication of malignant and potentially malignant lesions of the lip.¹⁸

Saito T, Nakajima T and Mogi K (1999) studied the immunohistochemical expression of cell cycle-associated proteins p16, pRb, p53, p27 and Ki-67 in precancerous and cancerous lesions of the oral cavity including verrucous carcinomas (VC). They observed increased expression of pRb, p53 and Ki-67 and decreased expression of p27 as the OSCC progressed, with variable expression of these markers in VC; expression of p16 was low in dysplasia and OSCC, but high in VC. They concluded that variations in p53 expression between OSCC and VC might be due to the differences in cell proliferation or inactivation of *p53* gene and that increased expression of p16 and pRb in VC as compared to OSCC may be attributed to human papilloma virus (HPV) infection.⁷⁰

Brennan P.A, Conroy B and Spedding A.V (2000) studied the expression of inducible nitric oxide synthase (iNOS) and p53 in 36 cases of oral epithelial dysplasia of varying grades and observed statistically significant correlation between iNOS immunoreactivity and grade of dysplasia and between p53 and iNOS staining. They concluded that further research can fully establish the relationship between iNOS and p53 in both dysplasia and OSCC.⁶

Chang K.W, Lin S.C, Kwan P.C et al (2000) detected p53 and p21^{WAF1} immunohistochemical expression (51% and 75% respectively) in 53 cases of oral verrucous leukoplakia and followed-up with histopathologic examination for 3 ½ years. They observed that 42% of the cases developed into OSCC, 26% cases recurred and 32% were disease-free. They noted a significant correlation between p53 and p21^{WAF1} expression and the frequency of OSCC progression and concluded that their aberrant expression may affect the outcome of oral verrucous leukoplakia.¹¹

Huang J.S, Ho T.J, Chiang C.P et al (2001) correlated immunohistochemical expression of MDM2 and

p53 with the clinicopathologic parameters in 52 OSCC. They observed 69% and 61% of positive expression for MDM2 and p53 respectively, 48% of the cases with co-expression and 17% with no expression. They also observed a significant correlation between MDM2 and p53 expression in 38 cases with areca quid chewing habit but no significant correlation with other clinicopathologic parameters. They concluded that MDM2 may play a role in the carcinogenesis of areca quid chewing associated OSCC in Taiwan.³¹

Valente G, Pagano M, Carrozo M et al (2001) evaluated immunohistochemical expression of p53 and MIB1 in 28 cases of oral lichen planus (OLP) undergoing malignant transformation, followed by sequential biopsies for up to 96 months. They categorized the 28 cases into 3 groups: Group 1 (n=15) with no dysplastic changes or neoplastic transformation, Group 2 (n=7) with synchronous OLP and OSCC, and Group 3 (n=6) in which OSCC developed several months or years after diagnosis of OLP. They observed that groups 2 and 3 showed a higher percentage of p53 positivity as compared to group 1, whereas MIB1 expression did not show any statistical differences among the three groups. They concluded that p53 expression may be of practical value to detect malignant potential of OLP.⁸⁷

Kuo M.Y.P, Huang J.S, Kok S.H et al (2002) correlated immunohistochemical expression of p21^{WAF1} in 43 OSCC with p53 expression, clinicopathologic parameters and overall patient survival. They observed that 72% of OSCC had positive p21^{WAF1} nuclear staining and 63% cases expressed p53. They found no significant correlation between p21^{WAF1} and p53 expression; p21^{WAF1} and patient's age, sex, habits, cancer location, or TNM status. They however, observed a significant correlation between p21^{WAF1} expression and poor overall survival and when p53 and p21^{WAF1} were evaluated together, the 5-year overall survival was lowest in p53 + p21^{WAF1} + patients and highest in p53 - p21^{WAF1} - patients. They concluded that the combined evaluation of p21^{WAF1} and p53 expression may be useful in assessing the prognosis of OSCC patients in Taiwan.⁴²

Pande P, Soni S, Kaur J et al (2002) did a prospective study to evaluate the prognostic significance of impairments in the expression of cell cycle regulatory proteins (p53, pRb, p16, MDM2 and p21),

transcription factor Ets-1 and metastasis in 105 habitual betel and tobacco chewers with OSCC during the period 1988-1999. Their results showed overexpression of p53 in 66% cases, MDM2 in 69% cases, p21 in 54% cases and Ets-1 in 61% cases; decreased expression of pRb in 55% cases and p16 in 69% cases. Loss of pRb was found to be the most significant predictor of advanced tumor stage and over expression of Ets-1 an independent risk factor for lymph node metastasis. They concluded that pRb loss and p53 over expression may serve as adverse prognosticator for disease free survival of the patients.⁶¹

Soni S, Pande P, Shukla N.K et al (2002) correlated immunohistochemical expression of Ets-1, P-glycoprotein (P-gp) and p53 with the clinicopathologic parameters in 40 OSCCs and observed Ets-1 positivity in 68% cases, P-gp over expression in 68% cases and p53 positivity in 65% cases. They also noted a significant correlation between the concomitant expression of Ets-1, P-gp and p53 with poor prognosis in OSCC.⁷⁸

Chen Y.K, Huse S.S and Lin L.M (2003) analyzed immunohistochemical expression of p53 and two new members of the p53 gene family p63 and p73 (the three share a structural homology) in 40 well-differentiated OSCC and 10 normal controls and concluded that p63 and p73 may be involved in the development of OSCC probably in connection with p53.¹³

Kurokawa H, Matsumoto S, Murata T et al (2003) studied the expression of syndecan-1, p53, and Ki-67 using IHC in 43 cases of leukoplakia with or without dysplasia and 22 normal controls. Their results showed a strong expression of syndecan-1 on the surface keratinocytes in normal epithelium with a gradual loss of positivity as the extent of dysplasia increased. In normal controls, p53 and Ki-67 expression was confined to the basal cells; increased expression with significant changes in the labeling index was observed as the dysplasia progressed. They concluded that decreased expression of syndecan-1 and overexpression of p53 and Ki-67 are associated with dysplastic changes.⁴⁴

Farhadieh R.D, Smee R, Ow K et al (2004) correlated immunohistochemical expression of KAI1 / CD82

(KAI1 is a newly discovered metastasis suppressor gene) and p53 proteins with clinicopathologic parameters in 57 cases of OSCC. They observed down-regulation of KAI1 / CD82 in 73.7% cases and positive p53 expression in 45.6% cases and found no correlation between their expression or clinicopathologic parameters.²¹

Hafian H, Venteo L, Sukhanova A et al (2004) analyzed immunohistochemical expression of DNA topoisomerase-I, DNA topoisomerase-II α , p53 and Ki-67 in 36 OSCCs, 18 epithelial hyperplasias and 18 mild dysplasias and correlated their expression with clinicopathologic parameters. p53 immunostaining was found to be uninformative with significant correlation only between DNA topoisomerase-I and differentiation parameter. They concluded that overexpression of this protein in poorly differentiated OSCCs suggests their higher sensitivity to drug treatment.²⁸

Iamaroon A, Khemaleelakul U, Pongsiriwet S et al (2004) studied the association between Epstein-Barr virus (EBV) and OSCC using in-situ hybridization and evaluated the immunohistochemical expression of p53 and Ki-67 in normal oral mucosa, oral hyperkeratosis, dysplasia and OSCC. They detected increased p53 and Ki-67 expression in OSCC than in dysplasia, hyperkeratosis and normal mucosa with a significant correlation between the labeling indices of p53 and Ki-67 positive cells in OSCC. They found out that none of the OSCC cases expressed EBV-encoded RNA transcripts. They concluded that p53 and Ki-67 may play a role in carcinogenesis and p53 overexpression may promote cell proliferation in OSCC. They further concluded that EBV does not appear to be a risk factor for OSCC particularly in the population of northern Thailand.³²

Lim S.C, Zhang S, Ishii G et al (2004) correlated immunohistochemical expression of p53, cyclin D1, Ki-67, EGF receptor, COX-2, MUC 1, laminin-5 γ 2, E-cadherin and β -catenin with clinicopathologic parameters in 56 patients with T₁₋₂N₀M₀ invasive squamous cell carcinoma (SCC) of the tongue who did not undergo elective neck dissection. They found no significant correlation between p53 expression and any of the clinicopathologic parameters and concluded that patients with stage I and II invasive SCC of

tongue with tumor thickness > 4mm, mode of invasion grade 3 or 4 and low E-cadherin expression should be considered a high-risk group for late cervical metastasis when elective neck dissection has not been performed.⁴⁸

Lee J.J, Kuo M.Y.P, Cheng S.J et al (2005) studied the immunohistochemical expression of p53 and PCNA in 56 OLP cases in relation to its clinical behavior and the patients' oral habits and compared it with the expression in normal oral mucosa, epithelial hyperkeratosis, dysplasia and OSCC. They observed that, the labeling index (LI) of p53 (28.6%) and PCNA (27.6 +/- 8.8%) in OLP was similar to that in hyperkeratosis, higher than in normal controls and lower than that in dysplasia and OSCC; however, the LI of p53 and PCNA were significantly increased in areca quid chewers with atrophic OLP and were similar to that in dysplasia and OSCC. With these results they concluded that patients with areca quid chewing habit having atrophic OLP may have a higher disease activity in view of higher expression of p53 and PCNA.⁴⁷

p53 AND MUTATION

Boyle J.O, Hakim J, Koch W.M et al (1993) investigated mutations in the conserved regions of *p53* in 65 OSCCs, 24 carcinoma in-situ lesions and 13 severe dysplasias. They detected mutations in 15% dysplasia, 21% carcinoma in-situ and 43% OSCC and concluded that the incidence of *p53* mutations increases with progression of OSCC.³

Brennan J.A, Boyle J.O, Koch W.M et al (1995) correlated *p53* mutations in 129 HNSCC patients with their habits (smoking and alcohol consumption) and observed mutations in 52% of the patients who smoked cigarettes and used alcohol, 33% who smoked but abstained from alcohol and 17% of the patients who neither smoked nor drank. They further observed that 100% of the mutations in patients who never smoked or drank occurred at the sites containing cytidine phosphate guanosine dinucleotides probably representing

endogenous mutations. They concluded that tobacco and alcohol use may be associated with a higher frequency of *p53* mutations in patients with HNSCC.⁷

Kusama K, Okutsu S, Takeda A et al (1996) evaluated the expression of p53 protein and gene alterations in normal oral mucosa, oral epithelial dysplasia and OSCC using IHC and temperature gradient gel electrophoresis (TGGE) respectively. Their results showed no p53 protein expression in normal controls, 27.3% positivity in dysplasia and 33.3% positivity in OSCC. TGGE analysis showed gene alterations in exons 5-8 in 3 out of 3 dysplasias and 17 out of 19 OSCCs which were immunohistochemically positive for p53. They concluded that *p53* gene mutation may be involved in the early stages of oral carcinogenesis.⁴⁵

Mao E.J, Schwartz S.M, Daling J.R et al (1996) correlated detection of HPV-16 using polymerase chain reaction (PCR) and *p53* mutations using polymerase chain reaction – single strand confirmation polymorphism (PCR-SSCP) in oral premalignant and malignant lesions (64 patients) and normal mucosa (6 patients) with clinicopathologic parameters. They detected HPV in 31% of cases with oral lesions, with none of the normal controls showing HPV infection. Mutations in exons 5-8 were observed in 37.5% of cases with lesions and in one normal. Around 33% of premalignant lesions and 42% OSCC showed *p53* mutations (mainly G:T transversions) in exons 7 and 8. Samples from 6 patients with oral lesions (out of which 4 were poorly differentiated OSCC) were found both to be HPV-16 positive and to contain *p53* mutations. They concluded that *p53* mutations might occur early in oral carcinogenesis.⁵²

Gopalakrishnan R, Weghorst C.M, Lehman T.A et al (1997) studied expression of p53 protein and mutations in the *p53* gene using IHC and PCR-SSCP respectively, in 10 cases each of normal oral mucosa, proliferative verrucous leukoplakia and OSCC. They observed minimal p53 immunostaining in normal controls, and overexpression in 80% verrucous leukoplakias and 70% OSCCs. *p53* mutations were detected within exons 5-8 in 40% OSCC with 2 of the 4 mutated carcinoma samples lacking p53 expression. No mutations were detected in leukoplakia cases. HPV-16 and HPV-16 & 18 were identified in 2 of 7 p53 positive OSCCs and 2 of 8 p53 positive proliferative verrucous leukoplakias respectively. One p53 negative

OSCC was positive for HPV-16 and had a mutation in exon 6 of *p53*. They concluded that *p53* protein status, *p53* mutations and HPV infection do not provide means to differentiate between leukoplakia and carcinoma and do not provide a predictive test for progression of leukoplakia to carcinoma.²⁷

Kuo M.Y.P, Huang J.S, Hsu H.C et al (1999) studied *p53* mutations in the conserved regions (exons 5-9) in 37 OSCCs using PCR-SSCP and DNA sequencing analysis. They detected *p53* mutations in 5.4% cases with one case showing a point mutation at codon 266 and the other showing a missense point mutation at codon 177. Both the patients with *p53* mutation did not have areca quid chewing habit. They concluded that *p53* mutations might not play a role in the pathogenesis of OSCC in Taiwan.⁴³

Tjebbes G.W.A, Leppers vd Straat F.G.J, Tilanus M.G.J et al (1999) assessed *p53* mutations in primary OSCC and matched lymph node metastases and observed that in all cases of OSCC and matched nodes the mutations were identical. They concluded that *p53* mutations develop in carcinogenesis before metastasis occur and are maintained during metastasis.⁸⁶

Shahnavaz S.A, Regezi J.A, Bradley G et al (2000) correlated *p53* immunohistochemical expression with *p53* mutational status (direct DNA sequencing) in 24 sequential biopsies of epithelial dysplasias and carcinomas from the same site in the oral cavity. Mutations of the *p53* gene were identified in 9 of 24 samples; eight were missense mutations and occurred at a splice site. In six patients, mutations occurred late after the transformation of epithelial dysplasia to carcinoma. In the other two patients, with progressive dysplasia (no invasive carcinoma), *p53* missense mutations occurred at the carcinoma in-situ stage in one case and moderate dysplasia in another. They did not find a correlation between *p53* gene mutations and the level of *p53* protein expression. They concluded that during oral carcinogenesis, *p53* gene mutations seem to occur relatively late in the progression to carcinoma.⁷⁴

Cruz I.B, Snijders P.J.F, Van Houten V et al (2002) studied 55 cases of OSCC for *p53* protein expression and mutations associated with smoking habits. They concluded that in their study, *p53* negativity was not

informative for *p53* mutations, 25% *p53* immunopositive cells appeared to be a good cut off value to predict *p53* mutations and *p53* immunostaining patterns appeared to be predictive for *p53* mutations associated with the smoking habits of the patients.¹⁵

Hafkamp H.C, Speel E.J.M, Haesevoets A et al (2003) studied the frequency of HNSCC demonstrating HPV-16/18 integration as identified by fluorescence in-situ hybridization and investigated their *p53* status by IHC and single-strand confirmation polymorphism analysis of exons 5-8. They used 47 cases of HNSCC and 27 premalignant lesions for their study and observed HPV integration in 21% HNSCC cases including 67% tonsillar carcinomas with *p16^{INK4A}* immunoreactivity in all the HPV positive cases; none of the premalignant lesions were positive for HPV-16/18 DNA. 64% of HNSCC showed overexpression of *p53* protein including 8 out of 10 HPV positive tumors. None of the HPV positive cases showed *p53* mutations in exons 5-8. They concluded that, tonsillar carcinomas, a subset of HNSCC harbor HPV-16 and show increased immunohistochemical expression of *p16^{INK4A}* and *p53* in patients with no significant tobacco and alcohol consumption habits.²⁹

Yamazaki Y, Chiba I, Hirai A et al (2003) assessed *p53* mutations in 121 OSCC by PCR-SSCP and correlated it with clinicopathologic parameters. They detected *p53* mutations in 42% cases and observed that samples containing specific mutations, especially in DNA-binding surface regions (L2, L3 and LSH motif) and conserved regions (2-5) had poorer prognosis as compared to the cases with mutations outside these regions. They concluded that specific mutations of *p53* might serve as an important prognostic factor in OSCC.⁹¹

Stock R.S, Mawrin C, Motsch C et al (2004) correlated allele constitution of codon 72 in *p53* with apoptotic regulation in 54 HNSCC by evaluating the association between *p53* loss of heterozygosity, *p53* mutations and *p53* protein expression, and the expression of three apoptosis-related proteins (*fas*, *fasL* and *bcl-2*). They observed *p53* loss of heterozygosity in 45.2% cases with a loss of the proline allele and up-regulation of *bcl-2* protein expression. Lack of co-expression of *fas* / *fasL* was observed. *p53* mutations

were also detected in 29.6% cases, preferentially at the arginine allele. They concluded that apoptosis correlates with the codon 72 allelic status of *p53* in HNSCC and homozygous proline 72 appears to be an important regulator of apoptosis.⁸¹

EXPRESSION OF bcl-2 IN OSCC, ORAL EPITHELIAL DYSPLASIA AND NORMAL MUCOSA

Gallo O, Boddi V, Calzolari A et al (1996) analyzed using IHC, the prognostic significance of bcl-2 expression on 5-year disease-free and overall survival in 71 irradiated patients with early stage HNSCC and observed 21% immunoreactivity. They also noted a statistically significant association between tobacco exposure and bcl-2 expression, and higher rate of bcl-2 immunoreactive tumors, in relapse patients. They concluded that in their study, bcl-2 expression was the most important indicator for disease-free survival and overall survival within 5 years of radiotherapy.²⁵

Singh B.B, Chandler F.W Jr., Whitaker S.B et al (1998) did immunohistochemical analysis to study the dysregulation of bcl-2 expression during progression from oral epithelial dysplasia to OSCC. Positive immunoreactivity was observed in 25% of mild dysplasias, 32% of moderate dysplasias, 56% of severe dysplasias, 16% of well differentiated carcinomas, 25% of moderately differentiated carcinomas and 50% of poorly differentiated carcinomas. Their study showed a higher percentage of immunoreactivity for severe epithelial dysplasias when compared to the mild and moderate dysplasias and squamous cell carcinomas. They concluded that bcl-2 may play a role in early stages of oral tumor progression.⁷⁷

McAlinden R.L, Maxwell P, Napier S et al (2000) examined 65 sequential biopsies of potentially malignant oral mucosal lesions from 12 patients using IHC and observed bcl-2 immunoreactivity in only 1 patient. They concluded that bcl-2 was not expressed early in oral premalignant lesions.⁵³

Muzio L.L, Mignogna M.D, Pannone Get al (2003) analyzed bcl-2 expression in 90 OSCCs and 10 normal mucosa samples using IHC to assess its clinicopathological implications. They observed 17% of

OSCC samples showing consistent cytoplasmic positivity of the peripheral cells of differentiating epithelial tumor islands. Normal mucosa showed a cytoplasmic pattern of bcl-2 positivity in the basal cell layers. They observed a direct correlation between bcl-2 positivity and increasing tumor stage, but this did not reach statistical significance. They further noted that patients with absent or low bcl-2 positive OSCC manifested poor overall survival rates when compared to the patients with moderate or high bcl-2 positive OSCC, but the difference was not statistically significant. They concluded that bcl-2 immunoreactivity may be useful for better characterizing and predicting the prognosis of OSCC and further studies may be of use to understand this correlation.⁵⁷

EXPRESSION OF bcl-2 IN RELATION TO OTHER MARKERS

Jordan R.C, Catzavelos G.C, Barrett A.W et al (1996) correlated bcl-2 and bax protein expression with tumor differentiation in adjacent serial sections of 30 OSCCs and found bcl-2 expression confined to basal keratinocytes and dendritic cells and bax expression throughout the thickness of normal epithelium. They observed moderate or marked immunostaining for bcl-2 in 60% and for bax in 63% OSCC; strong immunostaining for bcl-2 in 86% poorly differentiated carcinomas and strong bax immunoreactivity in 72% of the well-differentiated carcinomas; upregulation of bcl-2 protein in dysplastic epithelium adjacent to OSCC with reduced bax immunostaining. They concluded that, alterations of bcl-2 and bax may play a role in the development of OSCC.³⁶

Drachenberg C.B, Blanchaert R, Ioffe O.B et al (1997) did a comparative study of OSCC and VC for the immunohistochemical expression of bcl-2, p53 and Her-2/neu, and in-situ end-labeling of DNA to identify apoptosis. They detected minimal apoptosis in rare keratinizing cells of VC (0-3%); p53 positivity (4/8) and Ki-67 positivity (8/8) confined to the nuclei of the basal proliferating cells. bcl-2 (4/8) was expressed only in the cytoplasm of rare tumor cells. In contrast, they observed that OSCC displayed higher apoptosis rates (5-10%), with p53 (5/8) and Ki-67 (8/8) positive nuclei distributed randomly throughout the tumor; bcl-2 showed patchy cytoplasmic staining (4/8) or strong cytoplasmic and nuclear positivity (2/8)

in the less differentiated OSCC. Her-2/neu was negative in all the cases of OSCC and VC. They concluded that, the different levels and patterns of gene expression and cell turnover between OSCC and VC correlate with the different biology in behavior and prognosis of these tumors.²⁰

Kannan K, Lakshmi Latha P.N and Shanmugam G (1998) assessed 39 biopsies of OSCC for bcl-2 expression by IHC and observed 23% positivity with no correlation to p53 expression. They concluded that overexpression of either of these genes may substitute each other in the development of OSCC in Indians.³⁸

Ravi D, Ramadas K, Mathew B.S et al (1998) analyzed the significance of angiogenesis (assessed by immunohistochemical expression of endothelial cell marker CD38) in relation to apoptosis measured by terminal deoxynucleotidyl-mediated dUTP nick end labeling (TUNEL), expression of apoptosis regulatory p53, bax and bcl-2 proteins and tissue proliferation defined by cyclin D1 expression in 22 hyperplastic lesions, 16 dysplasias and 87 OSCCs. They concluded that increased angiogenesis, decreased apoptosis and deregulated proliferation occur simultaneously during tumor progression in the oral mucosa and the presence of mutant p53, increased bcl-2 expression and altered bax expression are also involved in this process.⁶⁹

Staibano S, Mignogna M.D, Muzio L.L et al (1998) studied the prognostic role of bcl-1, bcl-2, bax, PCNA, and DNA-ploidy in a series of 25 OSCCs and observed that low positivity for PCNA with a high positivity for bcl-2 was related to a better prognosis and high positivity for PCNA, bax, and bcl-1 correlated with worse prognosis. They also detected the presence of aneuploid cells in all the cases which did not correlate with the clinicopathologic parameters or with the overexpression of bcl-1, bcl-2, bax, and PCNA.⁸⁰

Hotz M.A, Bosq J, Zbaeren P et al (1999) assessed the differential expression pattern and the prognostic importance of the apoptosis-regulating proteins p53 and bcl-2 family members bcl-2, bcl-1, bax and bak in 26 patients with locally advanced HNSCC. They also measured apoptosis by TUNEL and quantified it by flow cytometry. They observed an inverse correlation between the apoptotic fraction and patient

outcome and concluded that their findings, which is of potential interest, requires confirmation.³⁰

Pena J.C, Thompson C.B, Recant W et al (1999) correlated the immunohistochemical expression of bcl-2, bcl-xL and p53 with clinicopathologic parameters in 42 patients with locally advanced HNSCC. They concluded that patients whose tumors demonstrate bcl-2 immunoreactivity may be treated successfully with less toxic therapy.⁶²

Piffko J, Bankfalvi A, Joos U et al (1999) studied immunohistochemical expression of p53, MDM2, bcl-2, WAF1, MIB1, epidermal growth factor receptor (EGF-R), various isoforms of adhesion molecule CD44 and AgNOR-associated protein expression in 100 OSCCs with adjacent normal mucosa. They found no correlation between p53, MDM2, bcl-2, and WAF1 immunophenotypes of the respective tumors and adjacent normal mucosa, but observed a statistically significant sequential increase from normal to dysplastic mucosa to OSCC for MIB1 and Ag NORs. CD44 isoforms showed variable expression patterns in OSCC, with decreased expression of v4 and v9 isoforms and strong expression of v5 and v6 variants as compared to the normal mucosa. They found favorable prognosis in four patients with marked bcl-2 expression and suggested that bcl-2 could regulate cell growth via routes other than suppressing apoptosis. They further concluded that proliferation markers – MIB1 and AgNORs, and selected CD44 isoforms may be useful markers for the assessment of precancerous lesions and for screening patients at high risk for the development of OSCC.⁶⁵

Schoelch M.L, Le Q.T, Silverman S Jr. et al (1999) evaluated the expression of apoptosis-associated proteins in premalignant and malignant oral epithelial lesions and observed that, p53 and especially bak and bcl-x were expressed early; bax expression was largely absent; and bcl-2 and MDM2 showed sporadic expression in oral premalignant and malignant lesions. They concluded that apoptosis-associated proteins are altered in variable patterns in both premalignant and malignant oral epithelial lesions.⁷²

Yao L, Iwai M and Furuta I (1999) correlated immunohistochemical expression of *bcl-2* and *p53* in 52 primary tongue SCC with clinicopathologic parameters, patient's prognosis and apoptosis index. They observed *bcl-2* expression in 50% of cases and *p53* expression in 60% of cases. The frequency of *bcl-2* expression correlated with tumor histologic grade and marginally with mode of tumor invasion but not with lymph node involvement; whereas *p53* expression was associated with mode of tumor invasion and lymph node status and not with tumor histologic grade. They concluded that the combined evaluation of *bcl-2* and *p53* may help in the assessment of tumor aggressiveness.⁹³

Badaracco G, Venuti A, Bartolazzi A et al (2000) correlated immunohistochemical expression of *p53* and *bcl-2* and identification of HPV by PCR with clinicopathologic parameters in 30 patients with head and neck cancer. They detected HPV positivity in 33% cases and positive *p53* protein expression in 70% cases (9/10 HPV-positive tumors and 12/20 HPV-negative tumors). Only 4 cases were *bcl-2* positive, irrespective of the presence of either HPV or *p53*. They found no correlation between their findings and clinicopathologic parameters. They concluded that *p53* and *bcl-2* expression and the presence of HPV infection are independent events in head and neck cancers.²

Chen Y, Kayano T and Takagi M (2000) used reverse transcriptase-PCR to evaluate the expression of *bcl-2* and *bax* mRNAs and the ratio of *bcl-2/bax* mRNA, and employed IHC to investigate the *bcl-2* and *bax* encoded proteins in OSCC and adjacent histologically normal epithelium. They observed that the *bcl-2* mRNA or *bax* mRNA expression was not consistent with their protein expression in some cases. Higher expression of *bcl-2* mRNA and stronger immunostaining of *bcl-2* protein in OSCC was observed in comparison to the adjacent normal epithelium and these findings were more prominent in poorly differentiated carcinomas. No significant differences in *bax* mRNA and protein were observed between OSCC and the adjacent normal epithelium; however, the poorly differentiated carcinomas showed very weak *bax* immunostaining. The ratio of *bcl-2/bax* mRNA was higher in OSCC when compared to the adjacent normal oral epithelium with higher ratios seen in poorly differentiated carcinomas. They concluded that their study gives indirect evidence of post-transcriptional control of *bcl-2* and *bax* expression

suggesting that dysregulated expression of bcl-2 and bax may be related to the histological grade of OSCC.¹²

Macluskey M, Chandrachud L.M, Pazouki S et al (2000) studied the contribution of apoptosis (measured by in-situ end-labeling of DNA), proliferation (assessed by immunohistochemical staining of Ki-67) and angiogenesis (assessed by immunohistochemical staining of von Willebrand factor) in 17 dysplasias, 18 OSCCs and 12 normal controls. They concluded that oral carcinogenesis is accompanied by angiogenesis and increases in both epithelial proliferation and apoptosis and the net epithelial growth results from proliferation starting earlier and proceeding at a higher rate than apoptosis.⁵¹

Tanda N, Mori S, Saito K et al (2000) compared immunohistochemical expression of p53, p21, MDM2, and bcl-2 and apoptosis by TUNEL in leukoplakia and OLP. They observed higher expression of MDM2 and bcl-2 in leukoplakia as compared to OLP. p53 and p21 were expressed more in OLP. No significant difference in the number of apoptotic cells was observed between the two lesions.⁸⁴

Ravi D, Ramadas K, Mathew B.S et al (2001) evaluated using IHC, the predictive value of pre-treatment status of p53, bax, bcl-2 and cyclin D1 in relation to response to radiotherapy (followed up for 36 months) in 69 OSCCs. Mutant p53 was also detected using a mutant p53-specific ELISA; extent of apoptosis was defined morphologically and by the TUNEL assay and angiogenesis was evaluated by CD34 antigen expression. They observed that increased immunohistochemical expression of p53 and detection of mutant p53 was associated with poor response to radiotherapy and therefore poor prognosis. Decreased angiogenesis and increased p53 and bax expression in less vascularized tumors correlated with recurrence and poor prognosis. Increased bcl-2 expression in cells around the vasculature also indicated poor prognosis.⁶⁸

Sulkowska M, Famulski W, Chyczewski L et al (2001) evaluated the immunohistochemical expression of p53 and bcl-2 proteins in the proliferating epithelium in relation to leukoplakia and with regard to the

lesions associated with and not associated with squamous cell carcinomas. From their results they noted that, leukoplakias coexisting with squamous cell carcinomas showed a higher p53 and bcl-2 expression. They also observed a correlation between the degree of epithelial dysplasia and p53 and bcl-2 expression; with increased expression of both the proteins in cases of severe dysplasias.⁸²

Chang K.C, Su I.J, Tsai S.T et al (2002) evaluated the immunohistochemical expression of p53 and bcl-2 and the role of HPV in 232 samples of betel quid-related oral epithelial lesions (verrucous hyperplasia, epithelial dysplasia, VC and OSCC) and their results showed p53 staining in 30% of dysplasia, 38% of OSCC and absence of staining in verrucous hyperplasia and VC with the p53-positive OSCC having a higher recurrence rate than p53-negative ones; bcl-2 expression was negligible for all types of lesions. HPV-6/11 was detectable in 10% of dysplasia and 13% of OSCC, but in neither verrucous hyperplasia nor VC and HPV-16/18 was negative for all types of lesions. They concluded that p53, but not bcl-2, may play a role in tumor progression of betel quid-related oral epithelial lesions and the consistent absence of the malignant-type HPV in all the lesions suggests that HPV plays an insignificant role in the tumorigenesis of these lesions, although a co-operative role may exist between the benign-type HPV and betel quid chewing.¹⁰

Loro L.L, Johannessen A.C and Vintermyr O.K (2002) studied expression of bcl-2 and bax in normal oral epithelium, focal epithelial hyperplasia and oral epithelial dysplasia in relation to apoptosis and proliferation using IHC, TUNEL method, and in-situ hybridization. Their study revealed that *bcl-2* mRNA and protein were markedly decreased in the basal cells of moderate and severe epithelial dysplasias when compared to the basal layers of normal oral epithelia and correlated with a 3-4-fold increase in apoptosis and increased proliferation. *bax* mRNA and protein were not significantly altered in normal, hyperplastic, and dysplastic epithelia and from normal epithelium to severe epithelial dysplasia, there was an inverse relationship between the bcl-2/bax ratio and apoptosis. They concluded that bcl-2 suppression may have a role in oral tumorigenesis.⁵⁰

Piattelli A, Rubini C, Fioroni M et al (2002) studied immunohistochemical expression and relationship of p53, bcl-2, MIB-1 and apoptotic index (AI) in normal oral epithelium, dysplasia and OSCC. They observed a positive correlation between p53 and MIB-1 overexpression with AI; an inverse correlation between bcl-2 and MIB-1 expression, bcl-2 and p53 expression and bcl-2 expression and AI. They concluded that the study of apoptosis could be important to understand oral carcinogenesis.⁶³

Teni T, Pawar S, Sanghvi V et al (2002) studied expression of bcl-2 and bax in chewing tobacco-induced 63 OSCCs and 31 oral premalignant lesions. They observed overexpression of bcl-2 in 56% and bax in 43% OSCC and bcl-2 positivity in 16% and bax positivity in 55% of premalignant lesions. When they combined these results with the earlier results on expression of p53 in the same samples, they observed that 30% of OSCC demonstrated a p53+bcl2+ pattern and 14% exhibited p53+bcl+bax+ pattern. None of the premalignant lesions showed concurrent dysregulation of p53, bcl-2 or bax, but showed an overexpression of bax in the absence of p53 and bcl-2 proteins. Significant correlation was observed between positive nodal status and bcl2+ and p53+bcl2+ in OSCC and survival rate in patients was higher with p53- OSCC in comparison to p53+ OSCC. Based on these results they concluded that the aberrant bcl-2 expression and loss of p53 function may play a role in the tumorigenesis of OSCC by allowing escape from apoptosis and enabling additional genetic alterations to accumulate.⁸⁵

Vora H.H, Shah N.G, Patel D.D et al (2003) correlated immunohistochemical expression of p53 (18%), bcl-2 (26%), cyclin D1 (62%), c-myc (75%), p21ras (73%), c-erb B2 (50%), and cytokeratin-19 (29%) with clinicopathologic parameters, in anterior and posterior tongue tumors from patients with early and locally advanced-stage disease. They observed a significant positive correlation between bcl-2 and cytokeratin-19 expression but no association between bcl-2 positivity and p53 expression. They found overall stage as the most significant prognostic indicator of disease outcome but however concluded that, immunostaining of c-myc in the tumors of locally advanced-stage tongue cancer patients might be a potential adjunct to clinical staging.⁸⁹

MATERIALS & METHODS

The material for this study consisted of archival, formalin-fixed, paraffin-embedded specimens of patients from the Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital, Chennai. Ten cases of normal oral mucosa (control), twenty cases of oral epithelial dysplasia and twenty cases of OSCC constituted the three study groups.

The OSCC cases were histologically graded as well differentiated, moderately differentiated and poorly differentiated depending on the extent of differentiation of the neoplastic cells. All OSCCs were carcinomas from buccal mucosa. The epithelial dysplasia cases, also from buccal mucosa, were histologically graded as mild, moderate and severe, according to the extent of dysplastic changes. Site matched normal controls were obtained during the extraction of the lower third molars.

ARMAMENTARIUM

Instruments / Equipments

1. Aluminum foil
2. APES coated slides
3. Autoclave
4. Beakers
5. Coplin jars
6. Cover slips
7. Cyclomixer
8. Electronic timer
9. Hot air oven

10. Light microscope
11. Measuring jar
12. Micropipettes
13. Pasteur pipettes
14. Rectangular steel trays with glass rods
15. Refrigerator
16. Slide carrier
17. Slide warmer
18. Sterile gauze
19. Tooth forceps
20. Weighing machine (DHONA 200D)

Reagents

1. Distilled water
2. Laxbro solution
3. 1 N Hydrochloric acid
4. APES (3- amino propyl tri ethoxy silane)
5. Acetone
6. Xylene
7. Absolute alcohol
8. Alcohol 70%
9. Hydrogen peroxide 3%
10. Citrate buffer (pH 6)
11. Phosphate buffer saline (pH 7)
12. Hematoxylin
13. Ammonia
14. DPX

Antibodies

1. DAKO™ Monoclonal Mouse Anti–Human p53 Protein (Clone DO7)
2. DAKO™ Monoclonal Mouse Anti–Human BCL2 Oncoprotein (Clone 124)
3. DAKO™ LSAB + System HRP
 - Biotinylated Link
 - Streptavidin Peroxidase
 - Chromogen DAB (3-3¹ Diaminobenzidine Tetrahydrochloride)

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Procedure

Before taking the sections on to the slides, all the slides were APES coated. Pre-coating procedure of the slides was as follows:

Pre-treatment of Slides

- Slides were first washed in tap water for a few minutes.
- They were then soaked in detergent solution (Laxbro) for an hour.
- After 1 hour each slide was brushed individually using the detergent solution and was transferred to distilled water.
- Slides were washed in two changes of distilled water.
- Later slides were washed in autoclaved distilled water.
- The slides were then immersed in 1 N Hcl overnight.
- The following day the slides were washed in two changes of autoclaved distilled water.
- All the slides were then transferred to slide trays, wrapped in aluminum foil and baked in a hot air oven for 4 hours at 180° C.

APES Coating Procedure

The slides were allowed to cool down and were then coated with APES using the following procedure:

- Slides were dipped in a coplin jar containing acetone for 2 minutes.
- The slides were then dipped in a coplin jar containing APES for 5 minutes.
- Following this, the slides were dipped in two changes of distilled water for 2 minutes each to remove excess APES and were left to dry.

Tissue Sectioning

Tissue sections were made using a rotary manual microtome. The ribbons of tissue sections were transferred on to the APES coated slides 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). Circles were drawn with a glass marking instrument around the tissue, so that the reagents were localized in the area of interest.

IHC Procedure

The slides with the tissue sections were treated with two changes of xylene to remove paraffin wax. They were then put in descending grades of alcohol for dehydration. The slides were then washed in two changes of distilled water for rehydration. Following rehydration, the slides were treated with 3% hydrogen peroxide for 30 minutes to quench endogenous peroxidase activity of cells that would otherwise result in nonspecific staining. The slides were put in two changes of distilled water. The slides were then transferred to the citrate buffer and autoclaved for antigen retrieval at 15 lbs pressure for 15 minutes. The slides were left to cool down at room temperature. The slides were washed in two changes of distilled water following which they were dipped in two changes of PBS for 5 minutes each. The slides were then blotted carefully, without touching the tissue section to remove excess PBS. Staining for p53 was done by adding primary antibody, monoclonal mouse anti-human p53 (DO7) to the P tissue on the slide; to the N tissue PBS was added and the slide was placed in a humidifying chamber and left in the refrigerator overnight. Similarly, for bcl-2 staining, primary antibody monoclonal mouse anti-human BCL2 (Clone 124) was added on to the P tissue

and PBS added to the N tissue and the slide was placed in a humidifying chamber and left in the refrigerator overnight. The following day, slides were washed in three changes of cold PBS, 5 minutes each. Following this, a drop of biotinylated link from the secondary antibody kit (LSAB+) was added on to both the tissue sections (P and N) of the slides and the slides were incubated for 20 minutes (for p53 staining) and 40 minutes (for bcl-2 staining). After the respective incubation, slides were washed in three changes of cold PBS, 5 minutes each. A drop of Streptavidin from the secondary antibody kit (LSAB+) was added to both the tissue sections on the slides and the slides were incubated for 20 minutes (for p53 staining) and 40 minutes (for bcl2 staining). The slides were then washed in 3 changes of cold PBS and the excess PBS was wiped off using gauze. A drop of freshly prepared substrate chromogen (DAB) was added to both the tissue sections and the slides were washed after 5 minutes in running distilled water to remove excess DAB. The slides were then counterstained using hematoxylin. Following counterstaining, the slides were transferred to 70% alcohol, 100% alcohol and 2 changes of xylene in a sequential order. The tissue sections were then mounted with DPX and the slides were observed under microscope. The slides were checked for positive staining in both the tissue sections (P and N).

Procedure for p53

APES coated glass slides with P and N sections



Placed in Xylene I (8 minutes)



Placed in Xylene II (10 minutes)



Placed in 100% Alcohol (5 minutes)



Placed in 70% Alcohol (5 minutes)



Washed in Distilled water twice (5 minutes each)



Placed in 0.3% Hydrogen Peroxide (30 minutes)



Placed in Citrate buffer and autoclaved



Washed in Distilled water twice (3 minutes each)



Washed in PBS twice (5 minutes each)



Primary antibody to p53 added to P section and PBS added to N section
and incubated overnight



Washed in cold PBS thrice (5 minutes each)



Added biotinylated link to both P and N sections
and incubated at room temperature (20 minutes)



Washed in cold PBS thrice (5 minutes each)



Added Streptavidin to both P and N sections
and incubated at room temperature (20 minutes)

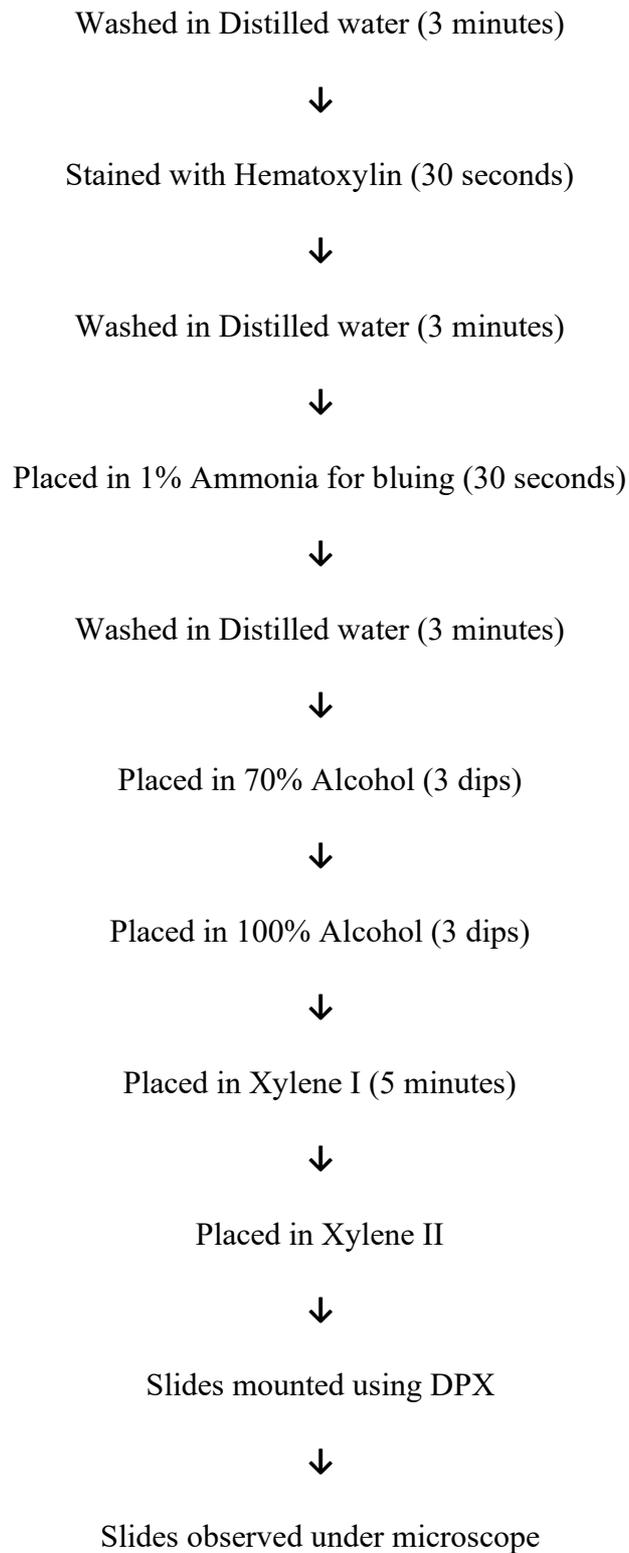


Washed in cold PBS thrice (5 minutes each)



Added DAB and incubated at room temperature (5 minutes)





Procedure for bcl-2

APES coated glass slides with P and N sections



Placed in Xylene I (8 minutes)



Placed in Xylene II (10 minutes)



Placed in 100% Alcohol (5 minutes)



Placed in 70% Alcohol (5 minutes)



Washed in Distilled water twice (5 minutes each)



Placed in 0.3% Hydrogen Peroxide (30 minutes)



Placed in Citrate buffer and autoclaved



Washed in Distilled water twice (3 minutes each)



Washed in PBS twice (5 minutes each)



Primary antibody to bcl-2 added to P section and PBS added to N section

and incubated overnight



Washed in cold PBS thrice (5 minutes each)



Added biotinylated link to both P and N sections
and incubated at room temperature (40 minutes)



Washed in cold PBS thrice (5 minutes each)



Added Streptavidin to both P and N sections
and incubated at room temperature (40 minutes)



Washed in cold PBS thrice (5 minutes each)



Added DAB and incubated at room temperature (5 minutes)



Washed in Distilled water (3 minutes)



Stained with Hematoxylin (30 seconds)



Washed in Distilled water (3 minutes)



Placed in 1% Ammonia for bluing (30 seconds)



Washed in Distilled water (3 minutes)



Placed in 70% Alcohol (3 dips)



Placed in 100% Alcohol (3 dips)



Placed in Xylene I (5 minutes)



Placed in Xylene II



Slides mounted using DPX



Slides observed under microscope

STATISTICAL ANALYSIS

There were three study groups: normal oral mucosa, oral epithelial dysplasia, and OSCC.

Analysis of p53 expression was done by evaluating the labeling index (LI). LI for each slide was calculated by dividing the number of positive cells by the total number of cells counted in the slide and expressed as percentage. A total of thousand cells were counted in each slide.

$$\text{LI} = \frac{\text{Number of positive cells}}{1000} \times 100$$

Analysis of bcl-2 expression was done by evaluating the staining intensity. Slides were assessed for mild (+), moderate (++), intense (+++), or no expression (-).

Data entry and descriptive statistical analysis was performed using SPSS version 10.0.5[®]. Mean of LI and standard deviation (SD) were calculated to assess p53 expression and percentage of intensity was calculated to assess bcl-2 expression.

- Analysis of variance (ANOVA) was done to compare the mean LI of p53 between normal oral mucosa, oral epithelial dysplasia and OSCC.
- ANOVA was done to compare the LI of p53 between varying grades of oral epithelial dysplasia.

- ANOVA was done to compare the LI of p53 between varying grades of OSCC.
- Chi-square test was done to compare the percentage expression of bcl-2 between normal oral mucosa, oral epithelial dysplasia and OSCC.

RESULTS

p53

All the cases (10 normal controls, 20 dysplasias, 20 OSCCs) were assessed for p53 expression. Staining was nuclear with a variable number of positive cells among the three groups.

Normal Oral Mucosa

Of the ten cases, three were positive for p53 expression. In all the three cases, a scattered pattern of staining was observed limited to the basal layer of cells (Fig. 1 & 2). The mean LI was 1.10 with SD of 1.80 (Table 1, Graph 1 & 2).

Oral Epithelial Dysplasia

All the twenty cases of oral epithelial dysplasia showed p53 expression with a variation in number of positive cells and extent in varying grades of dysplasia. Mild dysplasia showed basal cell staining while, moderate and severe dysplasias exhibited staining of basal and suprabasal cells (Fig. 3 - 8). The mean LI for mild dysplasia was 14.15 with SD of 5.53; for moderate dysplasia it was 27.74 with SD of 10.0 and for severe dysplasia it was 37.13 with SD of 1.33 (Table 2, Graph 3 & 4). The difference in mean LI between the varying grades of dysplasia was statistically significant ($p = 0.001$) (Table 2). The overall mean LI for oral epithelial dysplasia was 20.63 with SD of 10.65 and the difference in mean LI between normal oral mucosa and oral epithelial dysplasia showed statistical significance ($p = 0.00$).

OSCC

All the twenty cases of OSCC showed p53 positive expression with some of the cases showing intense staining in the epithelial islands (Fig. 9 - 14). The mean LI for well differentiated OSCC was 51.39 with SD of 9.55, for moderately differentiated OSCC it was 61.23 with SD of 23.62 and for poorly differentiated OSCC the mean LI was 55.83 with SD of 22.25 (Table 3, Graph 5 & 6). The difference in mean LI between the three grades of OSCC was not statistically significant ($p = 0.52$) (Table 3). The overall mean LI for OSCC was 53.53 with SD of 13.67. The difference in mean LI between normal oral mucosa, oral epithelial dysplasia and OSCC; between normal oral mucosa and OSCC and between oral epithelial dysplasia and OSCC was statistically significant ($p = 0.00$).

bcl-2

Expression of bcl-2 in the three study groups was assessed based on staining intensity. Staining was both nuclear and cytoplasmic.

Normal Oral Mucosa

Of the ten cases, only one showed positive bcl-2 expression. Staining intensity was mild (+) with only a few basal cells showing positivity (Fig. 15, Table 4, Graph 7).

Oral Epithelial Dysplasia

Of the twenty cases, two cases of mild dysplasia showed positivity with mild (+) expression confined to the cells of the basal layer (Fig. 16 & 17, Table 4, Graph 7).

OSCC

Two out of the twenty cases of OSCC were positive for bcl-2. One case of well differentiated OSCC showed a mild (+) expression with a few positive cells in one focus of malignant epithelium (Fig. 18). The other case, a poorly differentiated OSCC showed a moderate (++) expression with positive cells distributed throughout the epithelial islands (Fig. 19 & 20). The percentage expression in each group and the overall percentage expression was both 10%, with 90% of the cases showing no expression (Table 4).

Of the total number of fifty cases studied, four cases (one normal, two dysplasias and one OSCC) showed mild expression and one case of OSCC showed a moderate expression for bcl-2 (Graph 7). The normal oral mucosa case that showed bcl-2 positivity was negative for p53. Both the oral epithelial dysplasia cases that were positive for bcl-2 were mild dysplasias with p53 LI of 13.10 and 13.32. The two bcl-2 positive OSCC cases, one a well differentiated OSCC and the other a poorly differentiated OSCC, had p53 LI of 51.80 and 70.20 respectively.

TABLES & GRAPHS

Table 1: Mean Labeling Index of p53 in Study Groups

Antibody	Study Group	<i>n</i>	Mean	SD	p value
p53	Normal	10	1.10	1.80	0.00*
	Dysplasia	20	20.63	10.65	
	OSCC	20	53.53	13.67	

* $p < 0.05$ - Statistically significant

Table 2: Mean Labeling Index of p53 in Oral Epithelial Dysplasia

Antibody	Grade	<i>n</i>	Mean	SD	p value
p53	Mild	9	14.15	5.53	0.001*
	Moderate	8	27.74	10.00	
	Severe	3	37.13	1.33	
	Total	20	20.63	10.65	

* $p < 0.05$ - Statistically significant

Table 3: Mean Labeling Index of p53 in OSCC

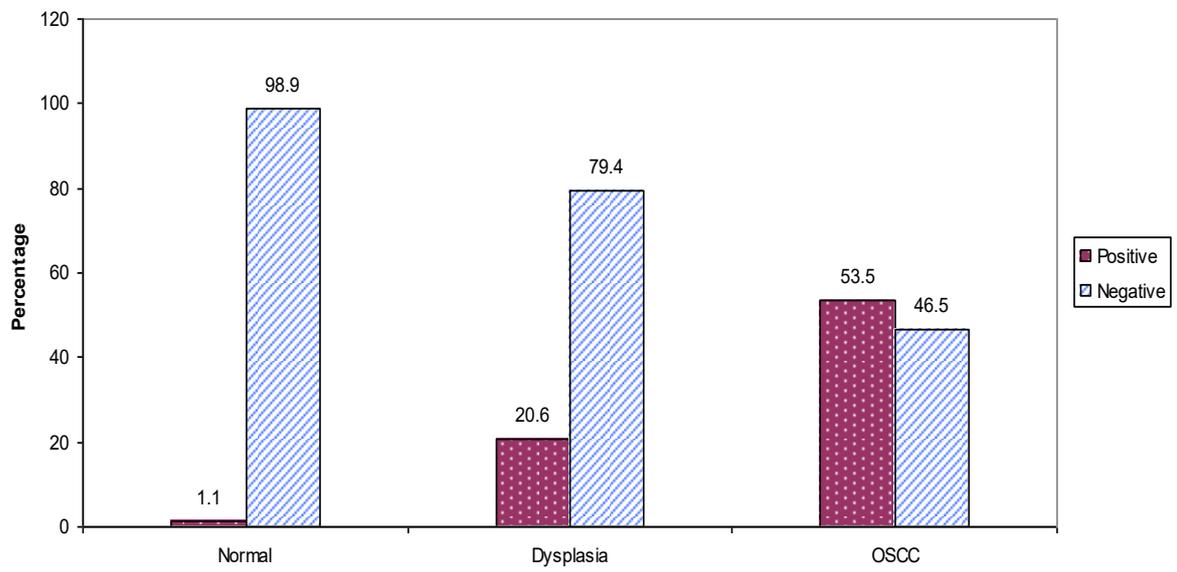
Antibody	Grade	<i>n</i>	Mean	SD	p value
p53	Well differentiated	14	51.39	9.55	0.52
	Moderately differentiated	3	61.23	23.62	
	Poorly differentiated	3	55.83	22.25	
	Total	20	53.53	13.67	

Table 4: Percentage Expression of bcl-2 in Study Groups *

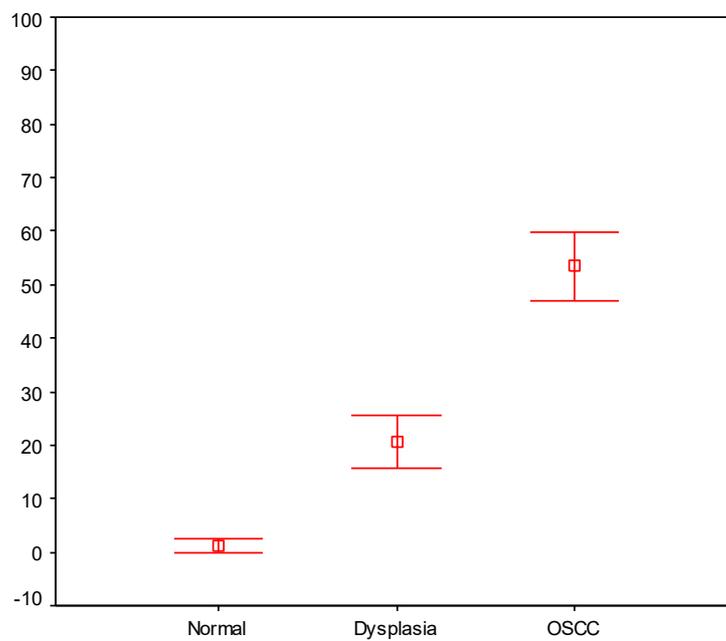
Antibody	Study Group	<i>n</i>	Mild	Moderate	No expression
bcl-2	Normal	10	10% (1/10)	-	90% (9/10)
	Dysplasia	20	10% (2/20)	-	90% (18/20)
	OSCC	20	5% (1/20)	5% (1/20)	90% (18/20)
	Total	50	8% (4/50)	2% (1/50)	90% (45/50)

* No case had intense staining for bcl-2

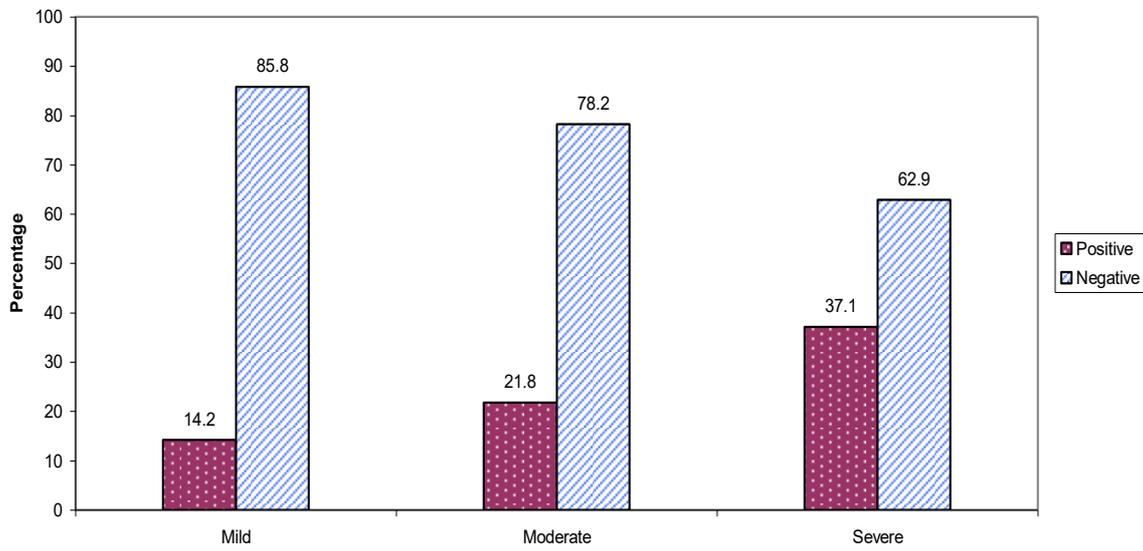
Graph 1: Percentage Positivity & Negativity of p53 in Study Groups



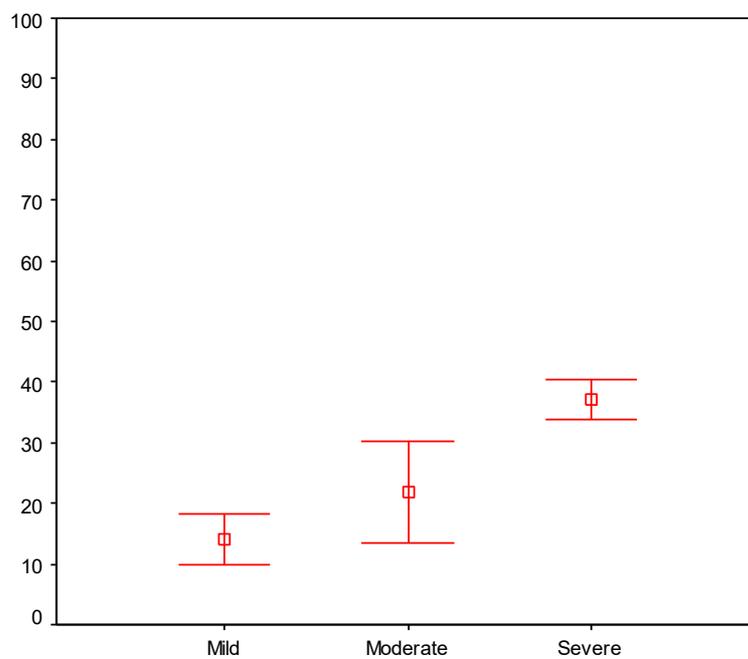
Graph 2: Labeling Index of p53 in Study Groups



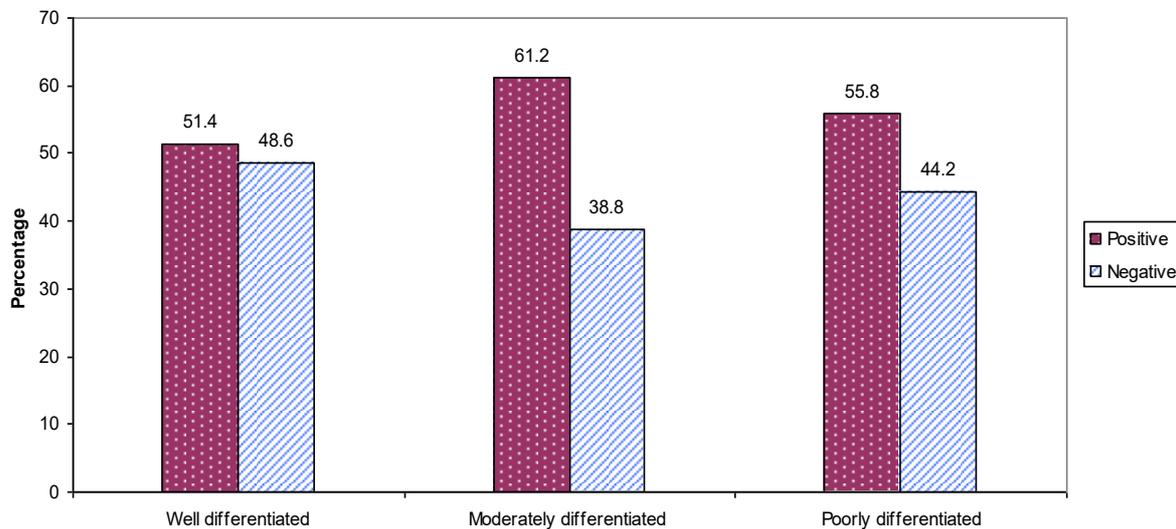
Graph 3: Percentage Positivity & Negativity of p53 in Oral Epithelial Dysplasia



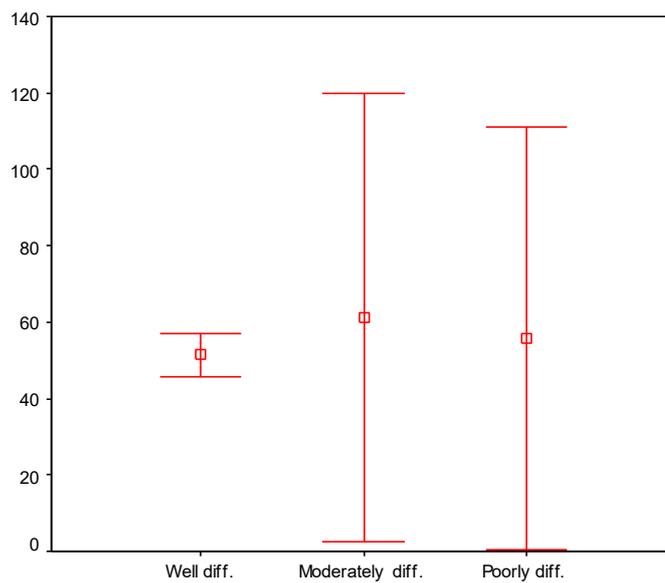
Graph 4: Labeling Index of p53 in Oral Epithelial Dysplasia



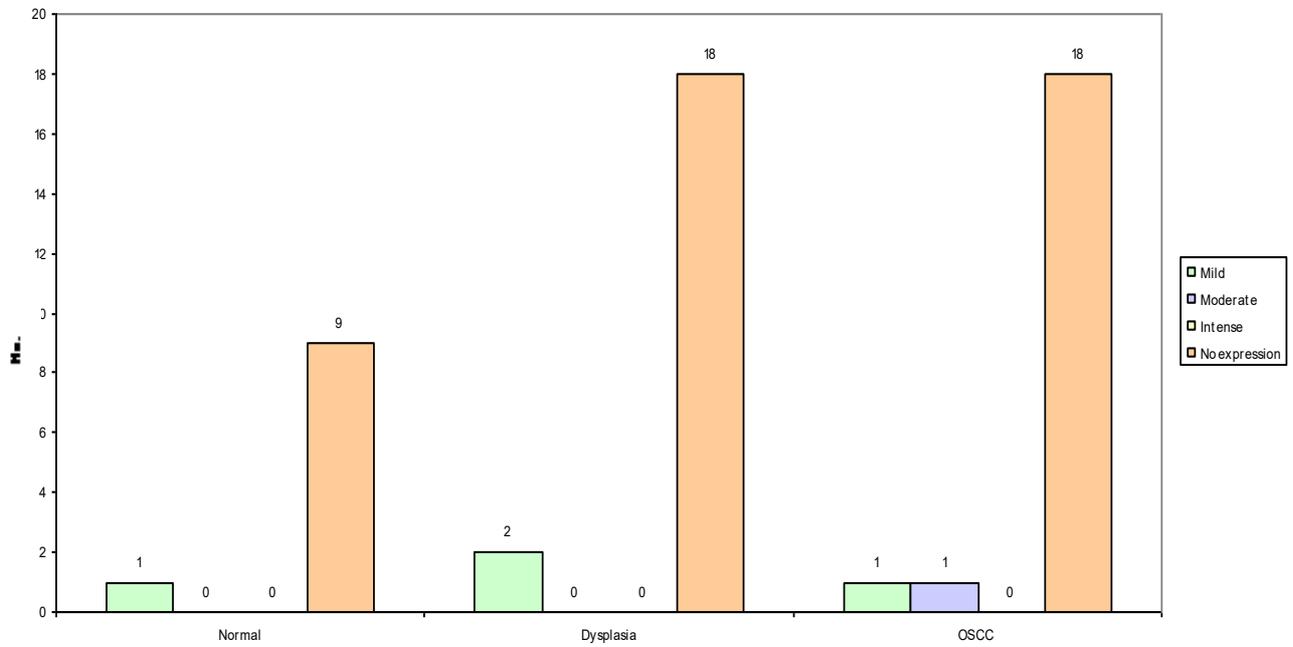
Graph 5: Percentage Positivity & Negativity of p53 in OSCC



Graph 6: Labeling Index of p53 in OSCC



Graph 7: Comparison of bcl-2 expression in Study Groups



PHOTOMICROGRAPHS

Figure 1: p53 expression in normal oral mucosa (10 X)

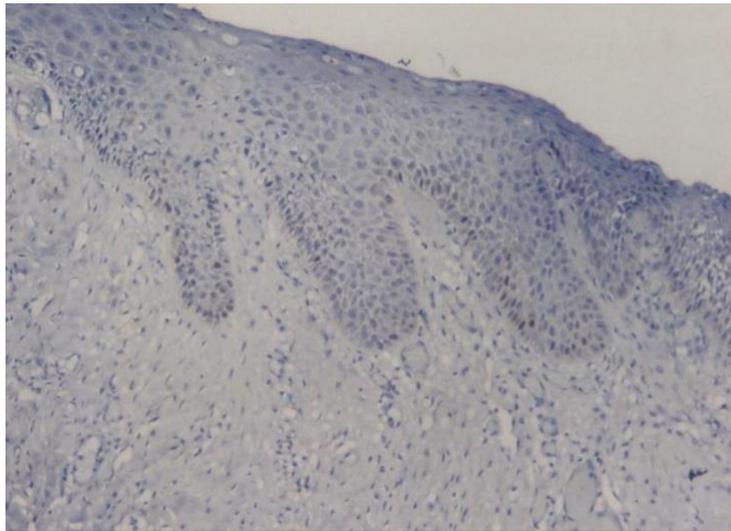


Figure 2: p53 expression in normal oral mucosa (40 X)

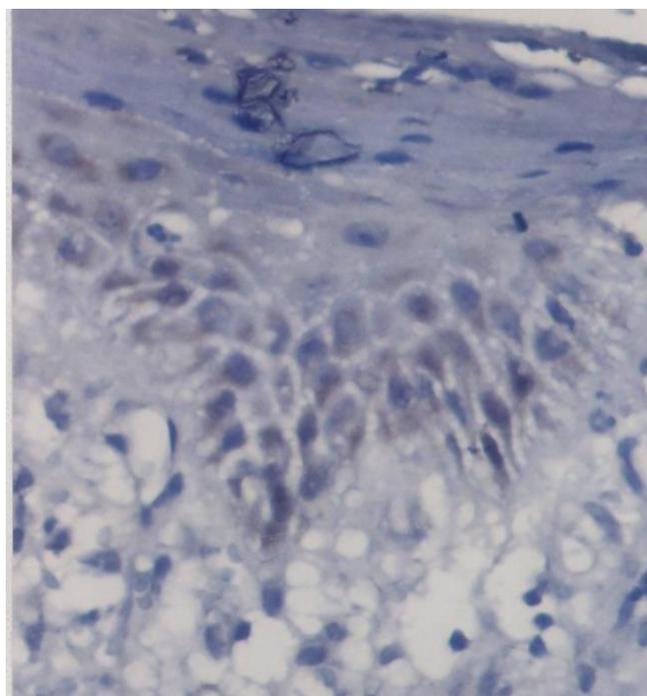


Figure 3: p53 expression in mild epithelial dysplasia (40 X)

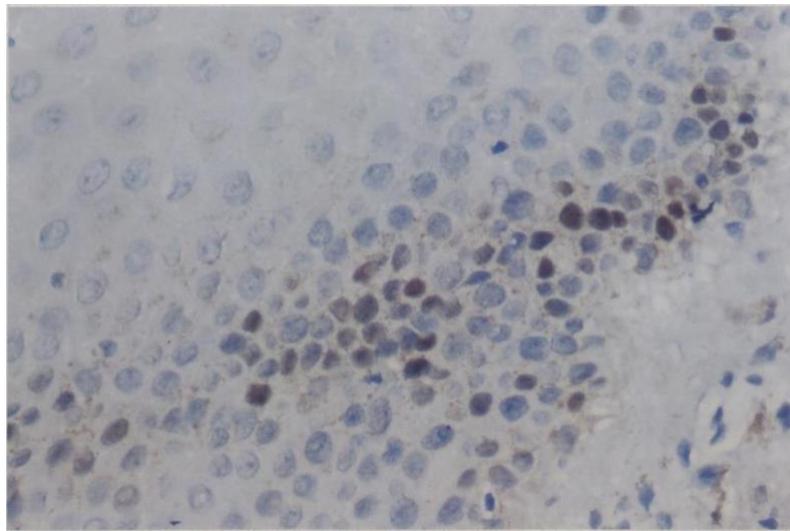


Figure 4: p53 expression in mild epithelial dysplasia (40 X)

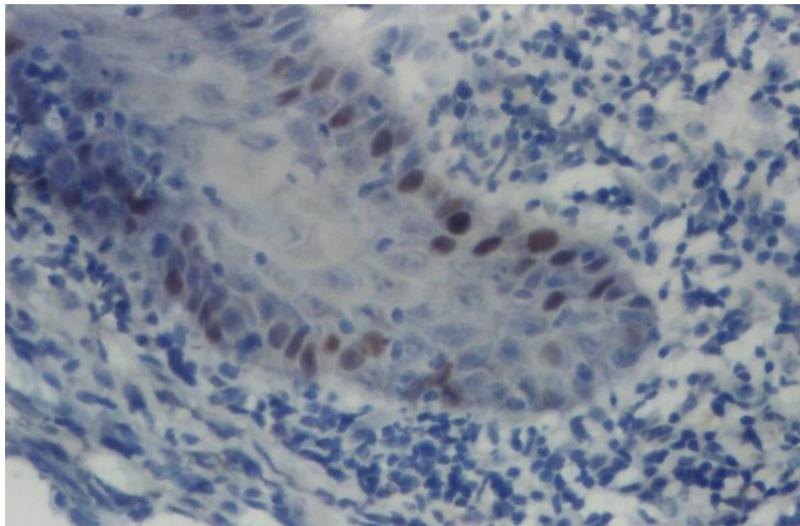


Figure 5: p53 expression in moderate epithelial dysplasia (10 X)

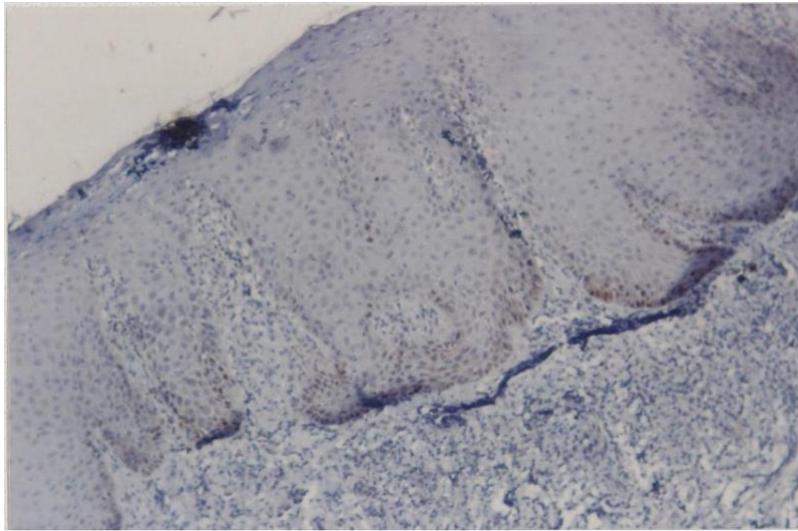


Figure 6: p53 expression in moderate epithelial dysplasia (40 X)

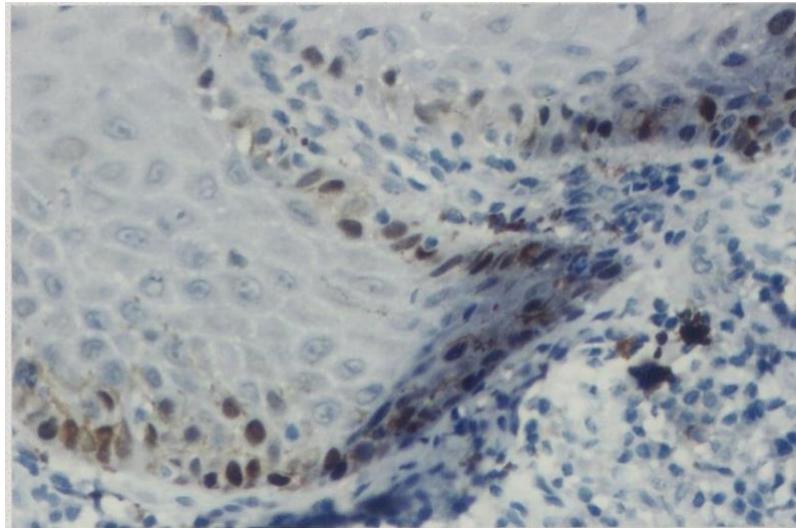


Figure 7: p53 expression in severe epithelial dysplasia (10 X)

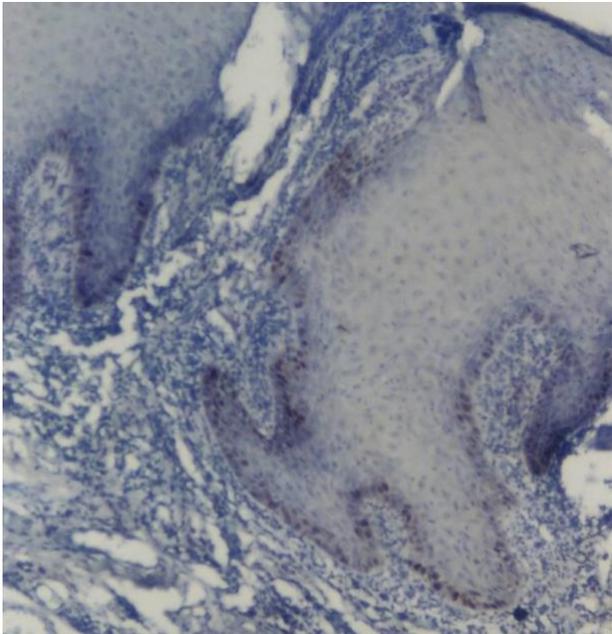


Figure 8: p53 expression in severe epithelial dysplasia (40 X)

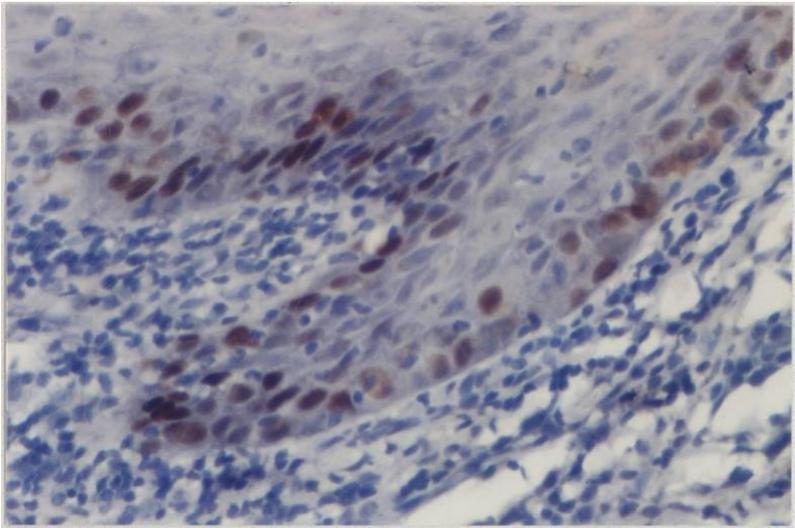


Figure 9: p53 expression in well differentiated OSCC (10 X)

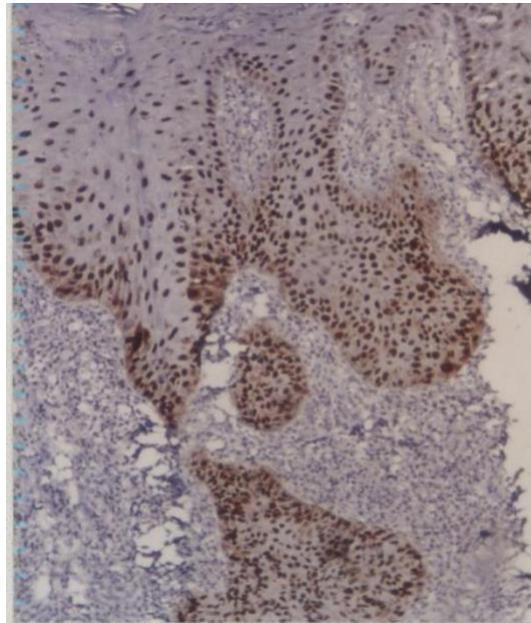


Figure 10: p53 expression in well differentiated OSCC (40 X)

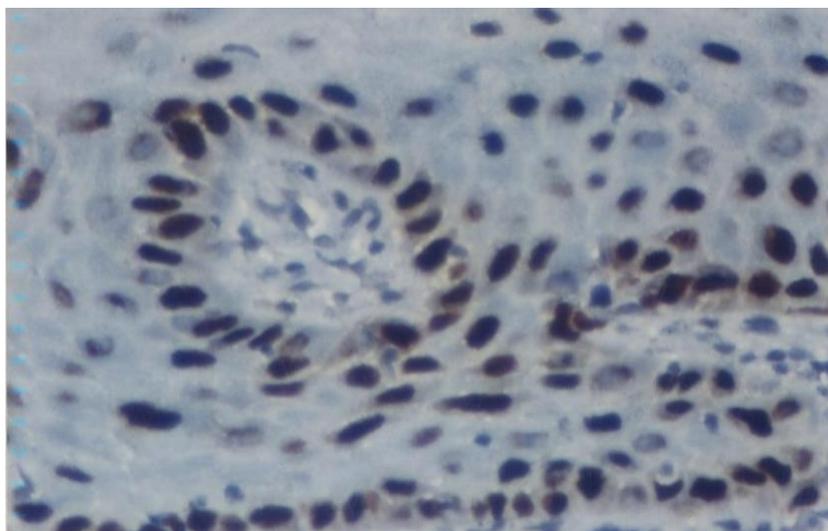


Figure 11: p53 expression in moderately differentiated OSCC (10 X)

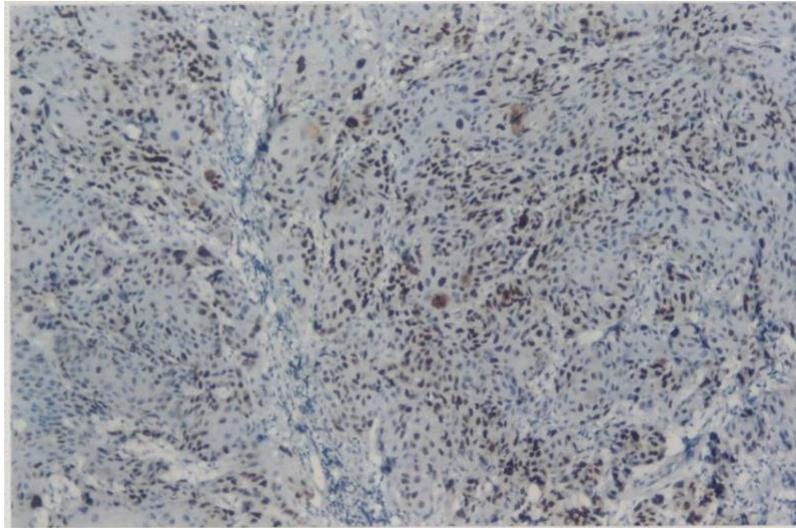


Figure 12: p53 expression in moderately differentiated OSCC (40 X)

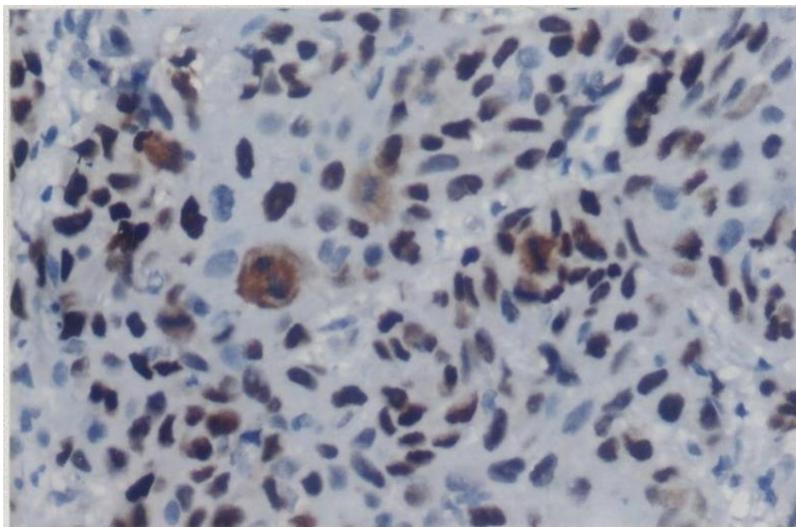


Figure 13: p53 expression in poorly differentiated OSCC (40 X)

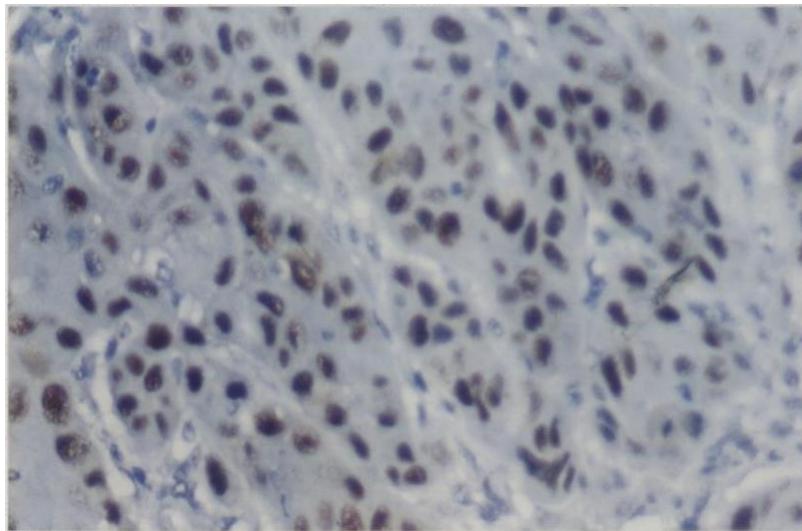


Figure 14: p53 expression in poorly differentiated OSCC (40 X)

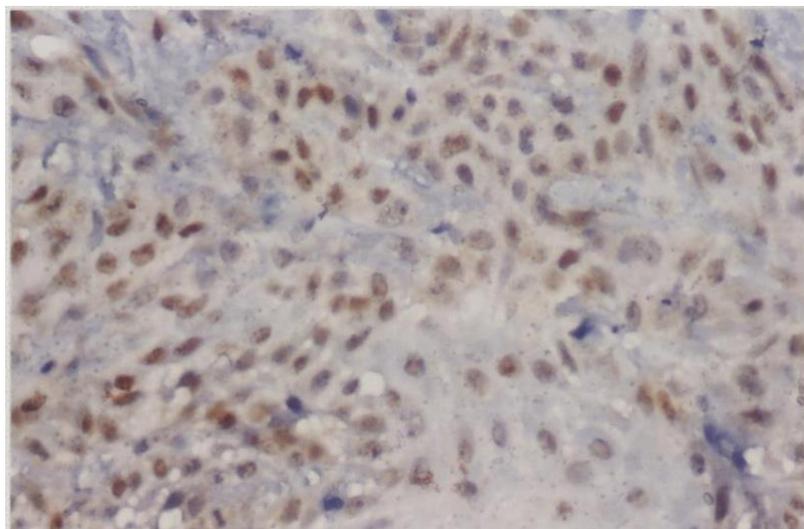


Figure 15: bcl-2 expression in normal oral mucosa (40 X)

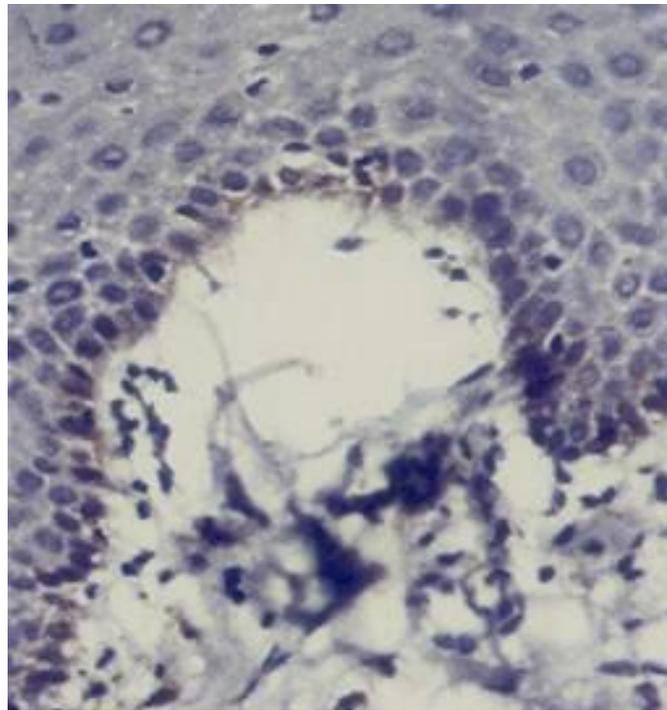


Figure 16: bcl-2 expression in mild epithelial dysplasia (40 X)

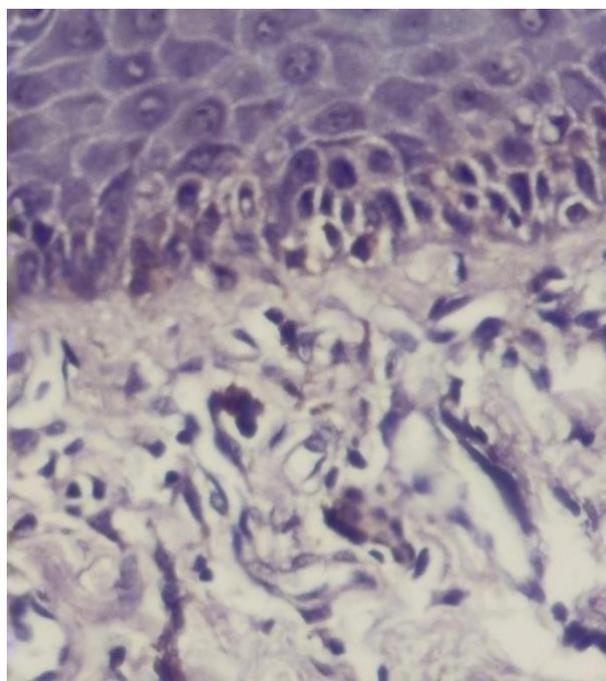


Figure 17: bcl-2 expression in mild epithelial dysplasia (40 X)

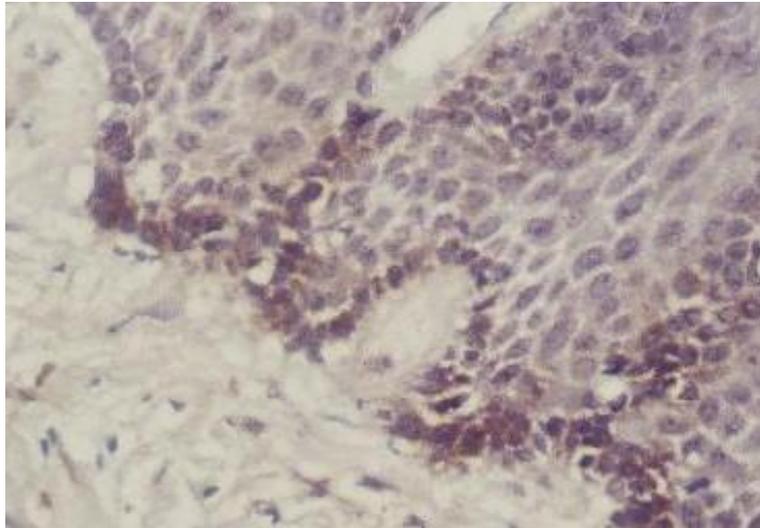


Figure 18: bcl-2 expression in well differentiated OSCC (40 X)

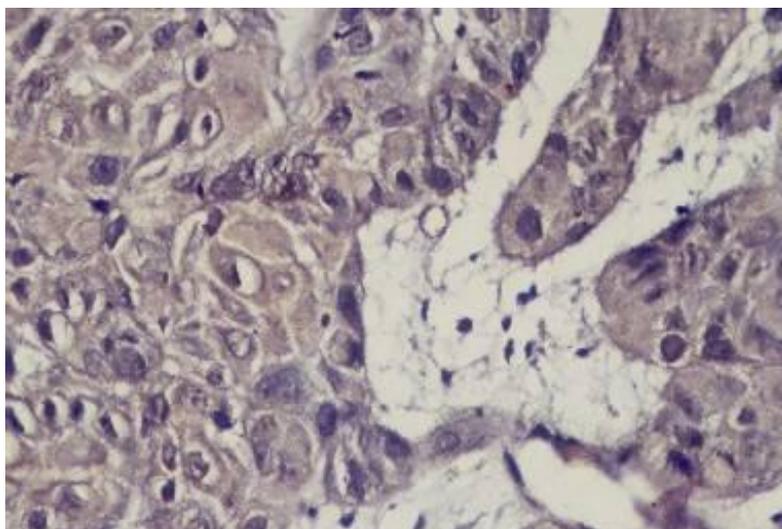


Figure 19: bcl-2 expression in poorly differentiated OSCC (10 X)

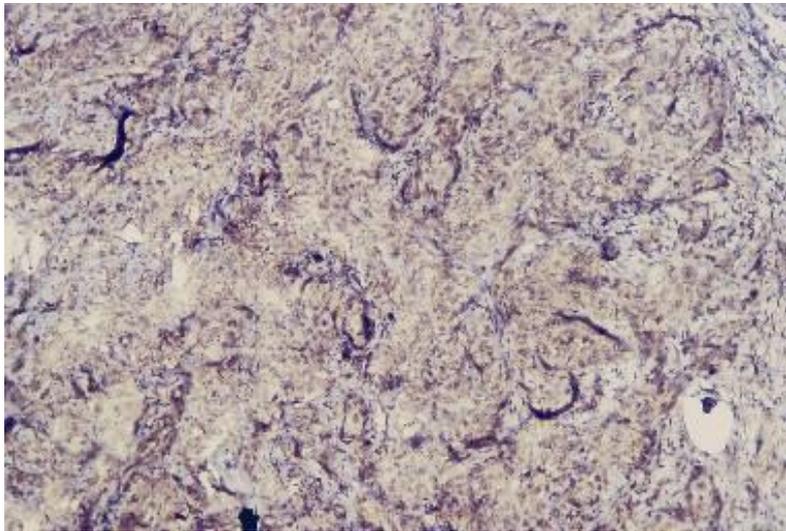
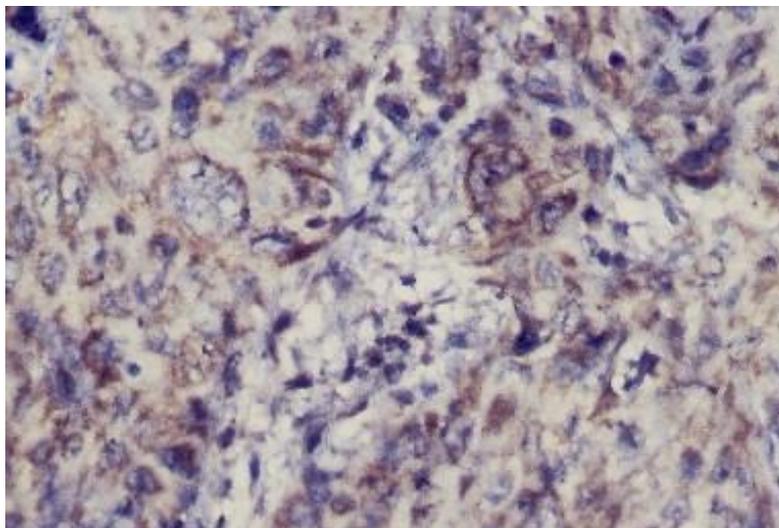


Figure 20: bcl-2 expression in poorly differentiated OSCC (40 X)



DISCUSSION

Development of OSCC, considered a multi-hit process, involves a number of aberrant genetic events. A minimum of five events in humans is required to transform a normal cell into a cancer cell.⁴ Determining which event/s may lead to malignant transformation can be of practical significance in its prevention.

The *p53* gene plays a tumor suppressor role in its normal state (wild-type), but in its mutated form it is believed to exert tumor initiating and promoting influences, largely through sequence-specific DNA binding and transcriptional regulation.⁵⁶ This gene, which mediates its function through its protein product p53, has been the subject of study in several body sites^{1,8,24,33,37} and a spectrum of oral tumors/lesions^{5,19,54,60,71,79,83,87,90} in order to help understand the process of carcinogenesis, to evaluate potential risk or as a prognostic marker. Immunohistochemical studies of p53 rely on the principle that the majority of changes in *p53* gene are missense mutations, which result in conformational change and stabilization of the transformed protein. The stabilized protein can then be detected by IHC in tissue sections, in contrast to the wild-type which has a short half-life and cannot be readily detected. The advantage of immunohistochemical staining is the direct demonstration of the spatial relationship of cells that have altered protein expression, which is of particular importance in the study of clonal expansion of altered cell populations during multi-step carcinogenesis.⁴⁶

The bcl-2 protein, an anti-apoptotic marker located on the outer mitochondrial membrane, endoplasmic reticulum and the nuclear envelope, promotes cell survival and increases the risk of cells acquiring other changes (such as chromosomal abnormalities and viral infections) that can result in malignant transformation or overt tumor progression.⁵⁷ Enhanced bcl-2 expression has been demonstrated in several tumors/lesions.^{1,8,41,54,64,94,95}

In the present study, of the ten cases of normal oral mucosa, three stained positive for p53 with staining being limited to the cells of the basal layer. This is in agreement with the previous studies^{17,32,44,46,65,76} that have reported p53 expression confined to the basal cell layer of normal mucosa. Cruz I.B et al hypothesized that p53 protein expression in the basal cells of normal mucosa is due to physiologic stabilization of wild-type p53 (rather than mutant p53) in an epithelium subjected to genotoxic stress. They further reasoned that its expression does not occur in the more differentiated cells of the superficial layer.¹⁷ Piffko J et al suggested that p53 expression in normal mucosa could represent a controlled inhibition of cell proliferation due to DNA damage.⁶⁵ Whereas, Kushner J et al believed that the detection of p53 positive cells in normal mucosa could be due to the enhanced sensitivity of immunohistochemical staining coupled with antigen retrieval techniques.⁴⁶ Although different reasons have been suggested for p53 expression in normal mucosa, its expression in the basal layer of normal mucosa is not considered to be existent and not an artefact. Cruz I.B et al¹⁶, Piffko J et al⁶⁵ Shin D.M et al^{75,76} detected increased p53 expression in tumor-adjacent histologically normal epithelium. Piffko J et al suggested that the detection of p53 in peri-tumoral normal mucosa may serve as a marker for field cancerization.⁶⁵ While Shin D.M et al were of the opinion that p53 expression in tumor-adjacent mucosa may serve as an important biomarker in assessing the risk of tumor development in carcinogen-exposed normal mucosa.⁷⁶ In contrast, Gonzales-Moles M.A et al²⁶, Gopalakrishnan R et al²⁷, Kerdpon D et al⁴⁰, Ogden G.R et al⁵⁹, Shin D.M et al⁷⁵ and Yao L et al⁹³ did not detect any significant p53 expression in the normal controls.

The results of this study showed a differing pattern of p53 expression in varying grades of oral epithelial dysplasia. In mild dysplasias staining was seen predominantly in cells of the basal layer. Staining of both basal and suprabasal cells was seen in moderate and severe dysplasias. These observations were similar to the results of Cruz I.B et al¹⁷, Kerdpon D et al⁴⁰ and Murti P.R et al⁵⁶. The authors suggested that this pattern of staining could be, in part, due to a sensitive immunohistochemical technique using monoclonal antibody DO-7 and heat pre-treatment antigen retrieval technique, allowing the detection of both wild-type and mutant p53 protein. Murti P.R et al further emphasized that the increased levels of wild-type p53 protein, probably as a result of DNA damage due to oral tobacco/ betel-quid use, may lead to non-specificity

of p53 as a risk marker.⁵⁶ Based on their study, Murti P.R et al, were also of the view that p53 overexpression may be a late event in the development of OSCC.⁵⁶ Although Cruz I.B et al¹⁷ and Kerdpon D et al⁴⁰ did not arrive at any statistically significant association between p53 expression and the degree of dysplasia, they however suggested that p53 alterations may occur early in oral carcinogenesis. Cruz I.B et al also emphasized that sampling of heterogenous lesions may account for the discrepancies found in different studies related to the strength of association between dysplasia and p53 staining.¹⁷

We observed that the mean LI of p53 for each grade of oral epithelial dysplasia was significantly higher than that of normal controls and there was an increase in mean LI with increasing grade of dysplasia. Also, the staining was suprabasal and basal in dysplasia as opposed to only basal cells staining in normal mucosa. The difference in mean LI between the varying grades of dysplasia and between the normal controls and dysplasia showed statistical significance. These observations were consistent to that of Iamaroon A et al³², Kurokawa H et al⁴³ and Kushner J et al⁴⁶ who reported statistically significant higher LI of p53 in dysplasias than in normals. However, Kushner J et al did not observe any statistically significant difference of p53 LI between the grades of epithelial dysplasia.⁴⁶

We detected p53 expression in all the twenty cases of OSCC with some cases showing intense staining in the epithelial islands. The mean LI of p53 for each grade of OSCC was significantly higher than that of dysplasia and normal controls. We did not observe statistically significant difference in mean LI between the three grades of OSCC. These findings are similar to those of Chang K.C et al¹⁰, Iamaroon A et al³² and Kerdpon D et al⁴⁶ who observed a step-wise increase of p53 positivity in the sequence of normal oral mucosa - oral epithelial dysplasia - OSCC. These results might indicate an involvement of p53 in neoplastic transformation as well as in proliferative events. Cruz I.B et al¹⁶ and Shin D.M et al^{75,76} observed similar findings in OSCC and adjacent dysplastic and histologically normal epithelium and these results support the concept of oral carcinogenesis as a multi-step process.

Our study showed bcl-2 expression in only one out of the ten cases of normal oral mucosa, with only a few

basal cells showing positivity. This observation is similar to that reported by Muzio L.L et al ⁵⁷, Piffko J et al ⁶⁵ and Singh B.B et al ⁷⁷. These authors suggested that bcl-2 protein when present in the basal cell layers of normal epithelium probably plays a role in the regulation of terminal differentiation of keratinocytes by protecting the stem cells from apoptosis. Chen Y et al ¹², Singh B.B et al ⁷⁷ and Yao L et al ⁹³ observed similar pattern of bcl-2 positivity in tumor-adjacent histologically normal epithelium and reasoned that this could precede the overt appearance of oral preneoplastic and neoplastic lesions. Loro L.L et al detected increased expression of bcl-2 protein in the basal cell layers in normal controls when compared to oral epithelial dysplasias and a high proportion of bcl-2 positive cells in the suprabasal cell layers of both normal controls and dysplasias. They suggested that in normal oral epithelium, bcl-2 expression may correlate more closely with apoptosis than with cell proliferation.⁵⁰ In contrast to these data, Chang K.C et al ¹⁰, Kannan K et al ³⁸ and McAlinden R.L et al ⁵³ did not detect bcl-2 expression in the normal mucosa.

In the present study, only two out of the twenty cases of oral epithelial dysplasias stained positive for bcl-2 with mild expression (+) confined to the basal cells. This was comparable to the results of Teni T et al who reported bcl-2 positivity in 6 out of 31 oral premalignant lesions, all of which showed mild (+) staining.⁸⁵ Loro L.L et al observed a progressive loss of bcl-2 expression in the basal cell layers of oral epithelial dysplasias from mild to moderate to severe dysplasia when compared to the basal cell layers of normal controls. They hypothesized that bcl-2 is dysregulated early in oral carcinogenesis.⁵⁰ On the other hand, Singh B.B et al detected highest percentage of bcl-2 positivity in severe epithelial dysplasias rather than mild or moderate dysplasias and OSCC and suggested that bcl-2 plays a role in the progression of dysplasia to OSCC.⁷⁷ Whereas, Chang K.C et al ¹⁰ and McAlinden R.L et al ⁵³ could not detect immunoreactivity for bcl-2 in any of their cases of oral epithelial dysplasia.

We observed bcl-2 expression in two of the twenty cases of OSCC with one case, a well differentiated OSCC showing mild (+) expression and the other, a poorly differentiated OSCC showing moderate (++) expression. The percentage expression in each of the three study groups (normal oral mucosa, oral epithelial dysplasia and OSCC) was found to be 10%. Overall, wide variation in results have been obtained so far in

studies dealing with *bcl-2* expression in OSCC, with different authors reporting varying degrees of percentage expression ranging from 0 to 56%.^{2,10,12,25,38,53,57,65,77,80,85,89,93} Chen Y et al demonstrated higher *bcl-2* mRNA expression and stronger *bcl-2* protein immunostaining in OSCC in comparison with the adjacent normal oral epithelium, highlighting an inverse correlation between *bcl-2* expression and tumor differentiation.¹² Singh B.B et al demonstrated *bcl-2* overexpression in non-dysplastic basal/ parabasal cells contiguous with tumors and increased expression in severe epithelial dysplasia than in mild/ moderate dysplasia and OSCC and suggested that *bcl-2* may play a role early in oral carcinogenesis.⁷⁷ Staibano S et al found no significant variation of *bcl-2* expression in their series of OSCC cases and hypothesized that the *bcl-2* correlated block of the apoptotic process may be absent, at least on the level of immunohistochemical investigation.⁸⁰ In contrast, Chang K.C et al¹⁰, McAlinden R.L et al⁵³ and Piffko J et al⁶⁵ observed no significant *bcl-2* expression in OSCC. Chang K.C et al emphasized that the lack of *bcl-2* expression is a result of methodological or geographical differences, such as a positive cut off value (5 or 10%), antibody source or antigen retrieval. They also suggested that other gene products in the cell cycle regulation, such as enhanced translation of MDM2-P2 transcripts may be alternatively involved.¹⁰ Also, McAlinden R.L et al suggested that the *bcl-2* expression in mature lymphocytes within the locality may be much higher than the epithelial cell component leading to an erroneous under-representation within the epithelia due to the sensitivity limits of the assay.⁵³

The discrepancies in *bcl-2* expression, from the various studies may reflect subtle inherent differences in upstream genetic events between the different population groups and environmental influences.⁸⁵ Thus the very sparse *bcl-2* staining in our study could be due to actual absence as described by Chang K.C et al¹⁰, Loro L.L et al⁵⁰, McAlinden R.L et al⁵³, Piffko J et al⁶⁵, Yao L et al⁹³ or sparse expression not determined by IHC. Furthermore, Chen Y et al reported that *bcl-2* expression may not reflect m-RNA activity and suggested that post-transcriptional regulation could be a possible mechanism controlling the expression of *bcl-2* in OSCC.¹²

In this study, there was no correlation between the expression of p53 and *bcl-2* in normal oral mucosa, oral

epithelial dysplasia and OSCC. The results are similar to those of Kannan K et al ³⁸ and Vora H.H et al ⁸⁹ who found no association between p53 and bcl-2 expression. In contrast, Teni T et al ⁸⁵ and Yao L et al ⁹³ observed overexpression of both p53 and bcl-2 in their studies and suggested that combined evaluation of both these proteins may provide an insight into the multi-step process of oral carcinogenesis. These findings highlight the fact that oral carcinogenesis may involve different genetic aberrations. Growing evidence however indicate that, bcl-2 and p53 expression are inversely related.³⁸ Kannan K et al observed overexpression of p53 and bcl-2 in their series, to be mutually exclusive of each other and proposed that overexpression of any one of these proteins may substitute each other in the induction of carcinogenesis in Indian OSCCs.³⁸

In the present study, we observed alterations in the expression of p53 and bcl-2 in oral epithelial dysplasia and OSCC when compared to the normal oral mucosa. However, p53 alterations seem to be better defined and significant as compared to that of bcl-2. Our study showed a consistent increase in p53 expression from normal oral mucosa to oral epithelial dysplasia to OSCC and a similar increase in the increasing grades of oral epithelial dysplasia. In contrast, bcl-2 expression was only around 10% in the three study groups and ranged from mild to moderate with no intense staining. In addition, there was no significant correlation between p53 and bcl-2 expression. These findings, especially of bcl-2, should be interpreted in the light of the fact that it could be due to the small sample size. If studies in larger sample size yield similar results which are corroborated by mRNA studies, the findings could imply that oral carcinogenesis may involve multiple genes, acting not necessarily together and also apoptosis as indicated by bcl-2 expression may not be a significant step in oral carcinogenesis.

SUMMARY & CONCLUSIONS

In the present Immunohistochemical study

- Increased p53 expression in the sequence normal oral mucosa - oral epithelial dysplasia - OSCC was observed.
- Increased p53 expression in the sequence mild - moderate - severe was observed in oral epithelial dysplasia.
- No significant increase of bcl-2 expression could be detected in oral epithelial dysplasia and OSCC when compared to normal oral mucosa.
- No association was observed between p53 expression and bcl-2 expression in normal oral mucosa, oral epithelial dysplasia and OSCC.

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