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Increased survivin expression in high-grade oral squamous cell carcinoma: a study in Indian tobacco chewers

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BACKGROUND: Oral cancer is one of the five leading sites of cancer in the Indian population. In the present study we analyzed the expression of apoptosis regulating genes, viz. survivin, Bcl-2, Bax and p53 in precancerous and cancerous lesions of the buccal mucosa of Indian tobacco chewers.

METHOD: Paraffin-embedded tissue samples from 38 patients with primary oral squamous cell carcinoma (OSCC) and 17 patients with leukoplakia were used. The expression of survivin, Bcl-2, Bax, and p53 was evaluated using immunohistochemical staining method.

RESULTS: Thirty-six percent OSCC were found to be positive for nuclear p53 staining while none of the precancerous lesions showed p53 positivity. Survivin, Bcl-2 and Bax expression was found to increase with increased grade of malignancy. Increase in survivin expression was statistically most significant ($P < 0.001$).

CONCLUSION: Increased expression of anti-apoptotic survivin in high-grade tumors suggests that survivin is likely to contribute significantly to apoptosis resistance in response to therapy.

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Introduction

Carcinoma of the oral cavity is one of the most frequent malignant tumors worldwide (1), with major predominance in South-East Asia and India (2). In India, cancer of the oral cavity is one of the five leading sites of cancer (3) and accounts for 19% of the total cancer cases in men and 7% of that in women (4). Among the oral tumors, 90% are squamous cell carcinoma (SCC) (5), which arise from the mucosal lining. This high incidence of oral cancer in India is due to the widespread habits of tobacco chewing and smoking (6). The clinicopathological profile of Indian oral cancers shows significant differences from the oral cancers in several developed countries of the world, including the USA, UK, France and Japan, where it is associated with tobacco smoking with/without alcohol consumption. A variety of tobacco habits are prevalent in India and they differ from region to region (7). Many people extensively use smokeless tobacco in the form of nass, naswar, khaini, mishri, pan masala, gutkha, betel-quid (betel leaf coated with slaked lime wrapped around areca nut and catechu) or smoke tobacco in the forms of bidi, chutta, the reverse type of smoking and hooka (8). In India, the buccal mucosa (cheek) is the primary site for cancer development as against the tongue and the floor of the mouth in Western countries (9), which may be due to the habit of keeping the betel-quid and tobacco in contact with the cheek for a long time. There are about 300 carcinogenic compounds present in tobacco (10–12) out of which tobacco-specific nitrosamines have been identified as the most important carcinogens in tobacco. The other carcinogenic compounds in tobacco metabolites are polycyclic aromatic hydrocarbons, α-particle-emitting ^{210}Po (polonium), trace metals, carbon monoxide, hydrogen cyanide, and phenols.

A precancerous lesion is defined as morphologically altered tissue in which cancer is more likely to occur than in its apparently normal counterpart (13). Oral leukoplakia (OL) is defined clinically as a white keratotic plaque that cannot be removed by manual manipulations. Despite the increased risk associated with having leukoplakia, many people with this condition never get oral cancer (13). According to the most commonly used Modified-Broder's grading system (14, 15), oral squamous cell carcinomas (OSCCs) are graded based on the degree of differentiation and keratinization of tumor cells into the following three categories:

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(i) well-differentiated squamous cell carcinoma (WDSCC), (ii) moderately differentiated squamous cell carcinoma (MDSCC), and (iii) poorly differentiated squamous cell carcinoma (PDSCC).

The treatment of oral cancer depends on the appropriate treatment of both the primary site and the regional lymphatics (16). Generally surgery alone or with radiation is administered to oral cancer patients (17, 18). The 5-year survival rate is in the range of 20–43% as most patients are in stage III and IV (18). Histological grade has been found to be predictive of recurrence in patients with early stage OSCC of the buccal mucosa (17).

Survivin belongs to the group of inhibitor of apoptosis (IAP) family proteins (19). IAP family members inhibit apoptosis by inhibiting one or more caspases (20). Survivin also plays role in proliferation by regulating mitosis (21). Survivin is known to be expressed specifically in various human cancers (19, 22) and is likely to be involved in tumor cell resistance to radiation and chemotherapeutic drugs (23). Survivin expression in oral cancer resulting from tobacco chewing has not been studied so far. In the present study we analyzed survivin expression in paraffin sections of precancerous and cancerous lesions of the buccal mucosa of the oral cavity in patients with tobacco chewing habit for a minimum period of 10 years.

The Bcl-2 oncogene belongs to a family of oncogenes, implicated in cancer development by inhibiting apoptosis (24). Bcl-2 localizes on the mitochondrial outer membrane and prevents apoptosis by suppressing the release of caspase-activating protein cytochrome-*c* from mitochondria (25). Overexpression of Bcl-2 has been reported in a number of human cancers including oral cancer (26–28), although correlation with tumor differentiation and clinical outcome are conflicting and depend on tumor type and site. Bax, the proapoptotic member of the Bcl-2 family inhibits the anti-apoptotic action of Bcl-2. Recent data have focused on simultaneously examining two apoptosis-related proteins Bcl-2 and Bax (29). Several lines of evidence suggest that the Bcl-2 and Bax expression ratio is a better discriminant of prognosis than levels of any one protein alone. Investigations into the sequential expression of Bcl-2 and Bax in precancerous and cancerous lesions of the oral mucosa are lacking and therefore their possible role in oral tumor progression remains unknown.

The levels of p53 in normal cells are low, but following cellular stress, p53 protein levels increase leading to the activation of a large number of downstream target genes involved in cell cycle regulation and apoptosis. In a large portion of oral and other types of human cancers, the p53 tumor suppressor gene is mutated resulting in the loss of p53 control of cell cycle progression and apoptosis (27, 30, 31). Thus the aberrant cellular expression of p53 is a good prognostic biomarker of the stage and malignant vigor of cancer (32).

In the present study, along with survivin expression, we also studied the expression of apoptosis regulators, viz. Bcl-2, Bax, and p53.

Material and Methods

Tissue specimens

Patients with cancerous lesions

The present study includes 38 patients (26 male and 12 female) with primary oral squamous carcinoma of the buccal mucosa ranging in age from 30 to 75 years (median age, 52.5 years). Histopathologically the tumors were categorized as WDSCC (13 cases), MDSCCA (14 cases), and PDSCCA (11 cases).

Patients with precancerous lesions

Premalignant lesions consisting 17 leukoplakias (13 male and 4 female) of 23–60 years of age (median age, 41.5 years) were studied.

All the samples were obtained from the Department of Oral Medicine, Diagnosis and Radiology, Nair Hospital Dental College, Mumbai. Informed consent was obtained from the patients. All the samples were obtained by biopsy or surgery before treatment and were fixed in 10% buffered formalin and embedded in paraffin for immunohistochemical analysis. All the patients were tobacco chewers for a minimum period of 10 years.

Immunohistochemical analysis

Tissue sections were cut at 5 μm and mounted on poly L-lysine-coated glass slides (Sigma, St Louis MO, USA). Antigen retrieval was then performed by heating slides immersed in citrate buffer, pH 6, in a microwave oven (33). Primary rabbit polyclonal antibodies against Bcl-2 (sc-492), Bax (sc-493), p53 (sc-6243), and survivin (sc-10811) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and used at a dilution of 1:100 in 1% BSA. Biotinylated anti-rabbit IgG was used as a secondary antibody and the bound antibody was detected using streptavidin-conjugated horseradish peroxidase (Amersham Pharmacia, Uppsala, Sweden) with 3,3′-diaminobenzidine as a substrate and Harris' hematoxylin as the counterstain. The high-grade human breast carcinoma specimen showing strong expressions for the markers under study was used as a positive control (27, 34, 35). Negative control was done by staining the positive control breast cancer specimen with the secondary antibody alone. The correlation between the level of expression and the histological grade was analyzed using the chi-squared test.

Results

The immunohistochemical analysis of survivin, p53, Bax, and Bcl-2 expression in precancerous and cancerous lesions of the oral cavity was carried out on the basis of the percentage of cells showing staining in the different layers of the oral mucosa. The level of expression was scored as follows: 0 = negative, less than 5% of cells staining; +1 = weak staining, between 5% and 25% of the cells staining; +2 = moderate staining, between 25% and 50% of the cells staining; +3 = strong staining, more than 50% of the cells staining.

In OL, survivin expression was found to be localized mainly in the parakeratin/kertin layer and the prickle

cell layer. About 11 of 17 (65%) OL cases showed cytoplasmic survivin positivity (Fig. 1A). Ten of thirteen (77%) WDSCCs were found to exhibit weak survivin expression, predominantly in the keratinocytes (Fig. 1B). Out of the 14 MDSCC cases, 12 (86%) showed weak to moderate cytoplasmic survivin expression (Fig. 1C). All PDSCC showed moderate to strong survivin expression. Two out of the 11 PDSCC cases (18%) showed distinct nuclear expression of survivin (Fig. 1D). The degree of survivin expression was found

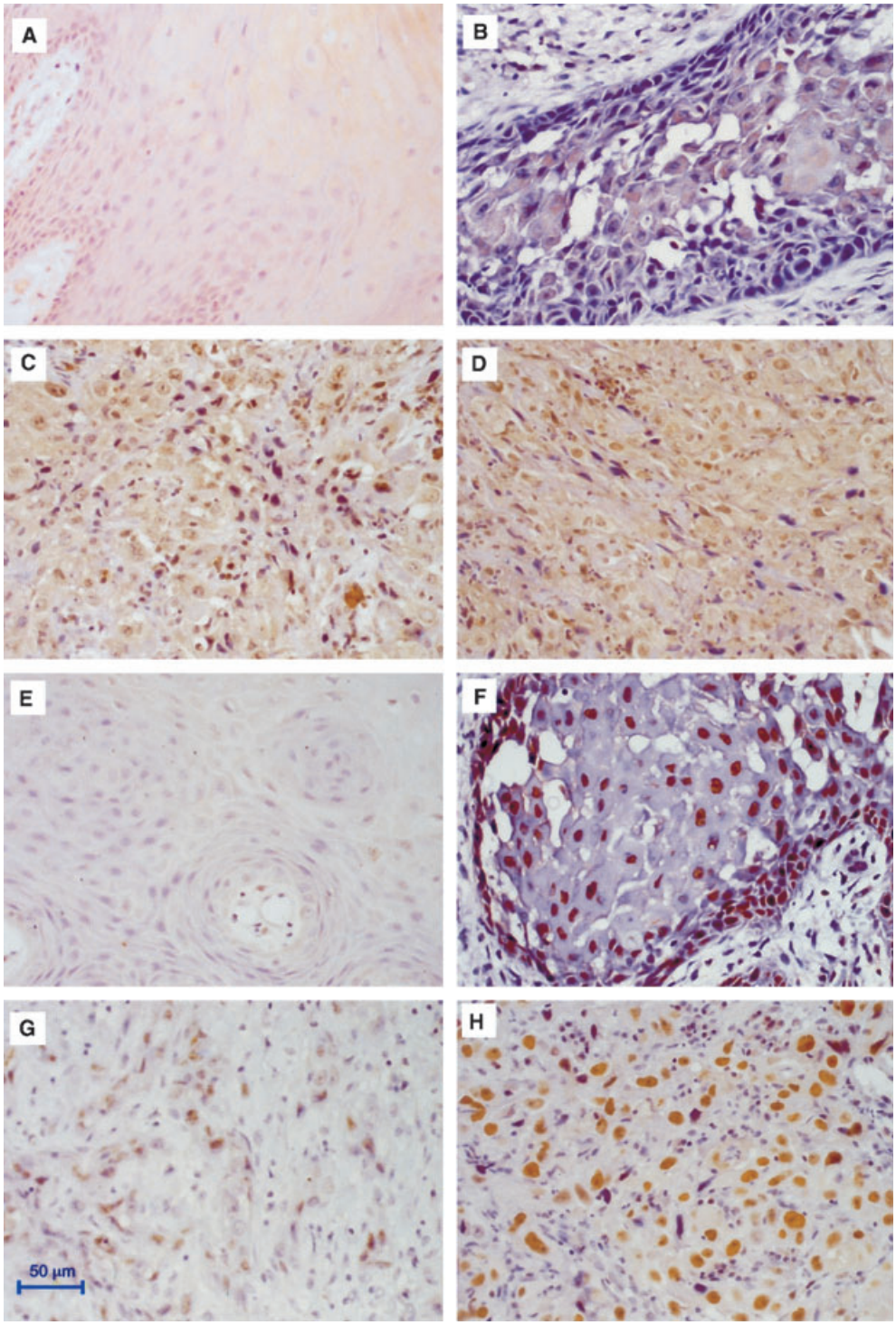

Figure 1 Representative photomicrographs of the immunochemical staining for Survivin (A–D) and p53 (E, H) in a case of oral leukoplakia (OL) (A, E), well-differentiated squamous cell carcinoma (WDSCC) (B, F), moderately differentiated squamous cell carcinoma (MDSCC) (C, G), and poorly differentiated squamous cell carcinoma (PDSCC) (D, H). Immunohistochemical staining shows positive staining in brown (DAB as substrate), counterstained with hematoxylin. All photographs were taken with a digital camera at 40× magnification under identical conditions.

to increase with increasing grade of malignancy (Fig. 2A).

p53 staining was found to be localized to the tumor cell nuclei. None of the OL cases expressed p53 (Fig. 1E) whereas 13 of 36 (36%) OSCC showed p53 nuclear expression. Three out of twelve (25%) WDSCCs were found to be p53 positive and the p53-positive nuclei were localized primarily in tumor islands (Fig. 1F). Six out of fourteen (42.86%) MDSCCs and 4 out of 10 (40%) PDSCCs showed p53-positive nuclei throughout the tumor tissue (Fig. 1G,H).

Bcl-2 staining was found to be localized in the cytoplasm of the cells. OL showed weak to moderate Bcl-2 expression in the keratin and prickle cell layer (Fig. 3A). In WDSCC 12 of 13 cases (92.3%) showed weak to moderate Bcl-2 expression (Fig. 3B). In MDSCC 8 of 14 cases (57.14%) showed moderate Bcl-2 expression in the keratinocytes (Fig. 3C) and 100% of PDSCCs showed strong Bcl-2 expression throughout the tumor tissue (Fig. 3D). Thus Bcl-2 expression was also found to be increasing with grade of malignancy (Fig. 2B).

Bax expression was found to be localized in the cytoplasm of the precancerous as well as cancerous cells. OL showed Bax-positive cells in the prickle cell layer and the basal layer (Fig. 3E). In WDSCC Bax expression was predominantly seen in the keratinocytes (Fig. 3F). Eleven of thirteen (84.62%) MDSCC tumors showed Bax immunopositivity. In this group Bax staining was seen in the cells of the tumor islands (Fig. 3G). Ten of eleven (90.91%) PDSCC cases showed moderate to strong Bax expression throughout the epithelium (Fig. 3H). Bax expression was also found to be higher in high-grade tumors (Fig. 2C).

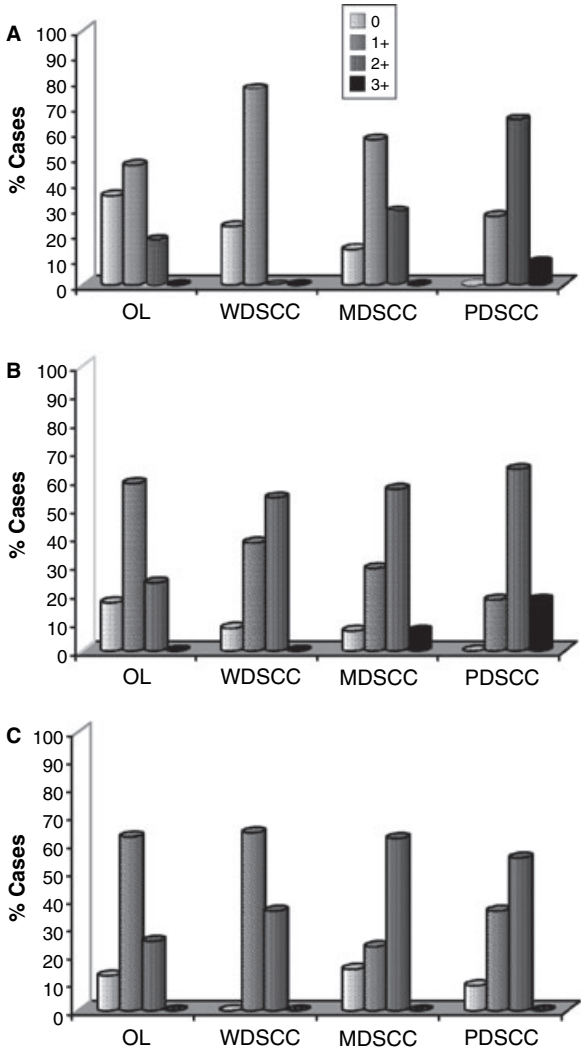

Figure 2 Summary of the results of immunohistochemical staining for survivin (A), Bcl-2 (B), and Bax (C).

Discussion

In the present study, we studied the expression of survivin and other apoptosis regulators, viz. Bcl-2, Bax, and p53 in precancerous and cancerous lesions in the buccal mucosa of tobacco chewing-induced oral cancers.

Survivin, a novel member of the IAP family, is a bifunctional protein that suppresses apoptosis and regulates cell division (21). Survivin expression has been detected at high levels in embryonic tissues, but at low or non-detectable levels in normal adults with the exception of the thymus, basal colonic epithelium, endothelial cells, and neural stem cells (22, 36). In our study, the majority of OLs, the pre-cancerous lesions were found be survivin positive. Normal oral mucosa have been reported not to express survivin. Therefore, survivin expression is likely to be the result of dysplastic transformation of oral epithelium. Survivin expression has also been reported to occur in various preneoplastic and benign lesions including polyps of the colon, breast adenomas, Bowen's disease and hypertrophic actinic keratosis (22), suggesting that re-expression of survivin may occur early during malignant transformation. In a study by Lo Muzio et al. survivin was found to be expressed in 33% (10/30 cases) of precancerous oral lesions that did not progress to malignancy while 94% (15/16 cases) of precancerous oral lesions that evolved into SCC were survivin positive (37). In another study, 37% of precancerous oral lesions were found to be survivin positive (38). The malignant conversion rate of leukoplakias in Indian tobacco chewers is considered to be low and has been reported to be in the range of less than 1% to 33% in a period of 10 years (39). It is unlikely therefore that the survivin expression in leukoplakias is indicative of progression to malignant transformation.

Although OLs showed survivin positivity, the expression in most cases was weak. The OSCCs showed higher survivin expression that was found to increase with increasing grade of malignancy. The increase in survivin expression with the increasing grade of malignancy was found to be statistically significant by chi-square analysis ($P < 0.001$). Survivin has been shown to be expressed in various types of cancers like lung (40), breast (41), gastric cancer (42), etc. as well as oral cancer

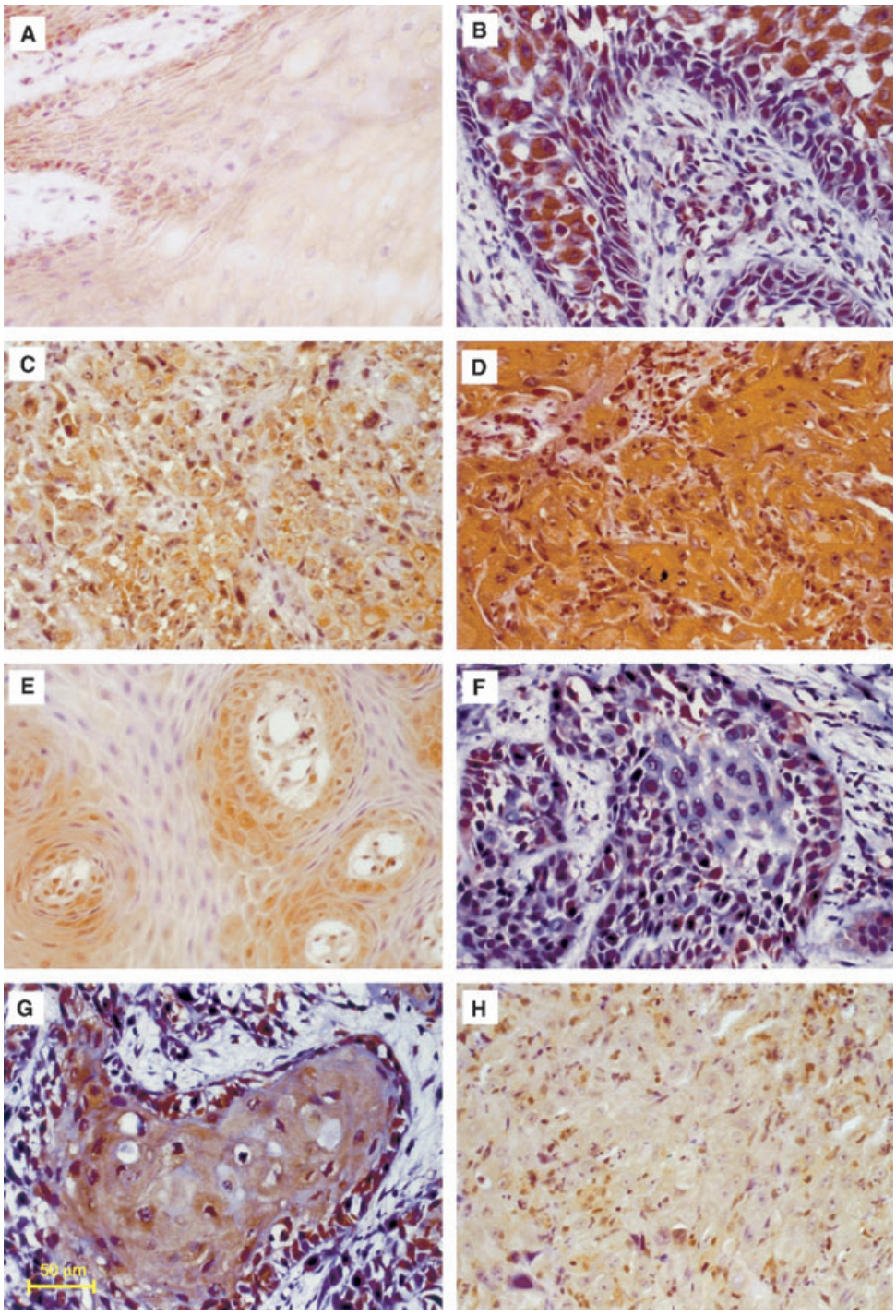

Figure 3 Representative photomicrographs of the immunochemical staining for Bcl-2 (A–D) and Bax (E, H) in a case of oral leukoplakia (OL) (A, E), well-differentiated squamous cell carcinoma (WDSCC) (B, F), moderately differentiated squamous cell carcinoma (MDSCC) (C, G), and poorly differentiated squamous cell carcinoma (PDSCC) (D, H). Immunohistochemical staining shows positive staining in brown (DAB as substrate), counterstained with hematoxylin. All photographs were taken with a digital camera at 40× magnification under identical conditions.

(43). In the majority of solid tumors, survivin expression has been found to correlate with tumor progression (22). Higher survivin expression has been reported to indicate poor prognosis (40–43).

p53 plays an important role in regulating cell proliferation and survival following cellular stress (44). In normal cells, the levels of p53 protein are low, but following cellular stress, p53 protein levels increase leading to the activation of a large number of downstream target genes involved in cell cycle regulation and apoptosis. p53 gene is the most commonly mutated gene seen in various types of cancers including oral cancer (45). In most cases mutations in p53 result in overexpression and increased stability of non-functional p53

protein (46). Therefore, nuclear p53 expression is considered to be indicative of mutant p53 protein. p53 has been reported to regulate survivin expression (47). In our study none of the 17 OL cases showed p53 positivity while survivin expression although weaker, was present in the majority of leukoplakias (65%).

Another anti-apoptotic gene Bcl-2 has also been reported to be overexpressed in various types of cancers (48). Bax, a 21-Kd protein that shares homology with Bcl-2, is a pro-apoptotic protein that can form homodimers or heterodimers (49). When Bcl-2 is overexpressed, it heterodimerizes with Bax and death is repressed. The ratio of Bcl-2 to Bax appears to serve as a rheostat to determine the susceptibility to apoptosis (50). In our study, Bcl-2 and Bax was found to be expressed in most cases of both precancerous and cancerous lesions. The expression of both Bcl-2 and Bax was found to increase with the increase in histologic grade. The Bax expression in high-grade tumors was found to be moderate while the Bcl-2 was found to be expressed strongly in about 20% of poorly differentiated SCCs. Higher Bcl-2 expression in high-grade tumors was found to be statistically significant ($P < 0.025$). It therefore appears that with the increasing grade the ratio of Bcl-2/Bax increases that correlates with decrease in differentiation and possible increase in resistance to apoptosis.

The correlation between histologic grade and survivin expression was found to be statistically more significant when compared with Bcl-2 expression. Furthermore, two out of eleven PDSCCs showed nuclear expression of survivin. Nuclear survivin expression has been shown to be associated with poor prognosis in non-small cell lung cancer (51) and in esophageal squamous cell cancer (52). Nuclear localization in our studies was found only in two poorly differentiated tumors. Higher survivin expression and its nuclear localization thus appears to correlate with higher grade of malignancy. A study with larger number of samples along with clinical follow-up data is required to check whether survivin would serve as a prognostic marker. Its increased expression in poorly differentiated tumors is likely to contribute to resistance to treatment. Survivin may serve as a therapeutic target that could increase the effectiveness of radiation and chemotherapeutic agents in treatment of oral cancer patients.

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