IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)
e-ISSN: 2279-0853, p-ISSN: 2279-0861.Volume 13, Issue 11 Ver. I (Nov. 2014), PP 78-81
www.iosrjournals.org

Expression of apoptotic markers in patients with oral squamous cell carcinoma (OSCC)

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Abstract: *Oral cancer is one of the ten most common cancers worldwide and accounts for 30-40% of all cancers detected in India. In normal circumstances, the process of apoptosis effectively eliminates genetically damaged cells from tissues to guard against their continued growth and progression toward malignancy. Dysregulation of apoptosis has been involved in carcinogenesis. Hence oral cancer tissues were examined and markers (bcl-2 and bax expression along with p53) were analysed using immunohistochemistry. In the present study we observed overexpression of p53 and bcl2 and decreased expression of bax in patients with oral squamous cell carcinoma. Thus altered apoptotic mechanism can be accounted for oral carcinogenesis.*
Key words: *P53, immunohistochemistry, apoptosis, bcl2, bax*

I. Introduction

Oral cancer is one of the ten most common cancers worldwide and accounts for 30-40% of all cancers detected in India. Despite advances in surgery, radiotherapy and chemotherapy over the past 20-30 years, no significant improvement in the prognosis for oral cancer has been observed. So early detection of oral cancer reduce the morbidity and mortality associated with malignant transformation of oral carcinogenesis [1].

So far, the only accepted method to quantify the risk of progression to OSCC of a potentially malignant lesion is the presence/absence of histological findings of dysplasia, although the presence of dysplasia does not always indicate malignant transformation and its absence do not preclude it [2]. Moreover, histological assessment of dysplasia is extremely subjective and prone to inter and intra-observer variation and additionally some potentially malignant lesions do not show dysplastic alterations [3]. These considerations have stimulated intense research in this area, and many studies have been conducted so far, in an attempt to find out molecular markers that are associated with OSCC and that can predict malignant transformation when found in epithelial precursor lesions, especially in cases without signs of dysplasia [4].

In normal circumstances, the process of apoptosis effectively eliminates genetically damaged cells from tissues to guard against their continued growth and progression toward malignancy. P53 gene exists on chromosome 17p and behaves as a tumor suppressor. P53 gene not only participates in cell proliferation control, but also plays a role in elimination of cells with DNA damage by induction of apoptosis. B cell lymphoma 2 (Bcl2) is the first gene shown to be involved in apoptosis and was regarded as a proto oncogene that suppresses the cell death rather than stimulating cell proliferation. The human Bcl2 protein is an intracellular, integral membrane protein with a molecular weight of about 26KD. Bcl2 associated X protein (Bax) promotes apoptosis and its chromosomal location is 19q.

The accumulation of p53 protein in response to DNA damage *in vitro* is well established and appears to induce growth arrest and apoptosis by the transcriptional regulation of other genes [5]. However mutation of this gene can inactivate this tumor suppressor activity. Mutations and alterations in the p53 gene have been implicated in almost all human cancers and p53 status is therefore one of the most important biomarkers for a variety of cancer types. In normal cells, the p53 protein has a very short half life (6-20 min) and cannot be detected immuno histo chemically. In contrast, the mutant forms are more stable and thus have an extended half life (6hours) and can be detected using immunohistochemical techniques [6].

Overexpression of Bcl-2 has been reported to protect tumor cells from apoptosis [7] whereas increased Bax expression promotes apoptosis induced by cytotoxic drugs and radiation [8]. Induction of apoptotic process of p53 through up regulation of Bax and down-regulation of Bcl-2 has been suggested to determine death of cells following an apoptotic stimulus [9]. Anti-apoptotic (Bcl-2) and pro-apoptotic proteins (Bax) determine the fate of damaged cells and imbalance in Bcl-2 to Bax ratio result in carcinogenesis [10] . So in this study, we have investigated the expression of the apoptosis- related proteins Bax, Bcl-2, and p53 in OSCC to explore the possible relationship among these apoptotic markers in oral carcinogenesis.

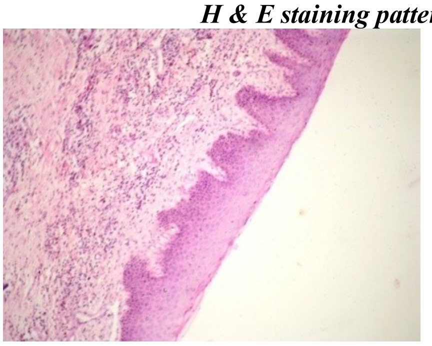
II. Materials And Methods

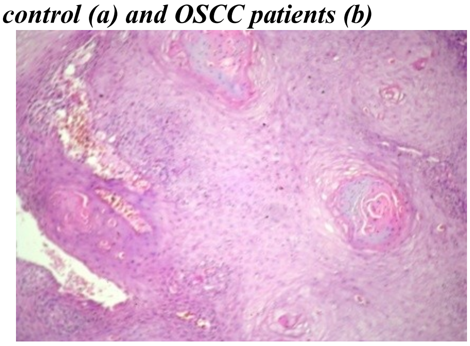
Thirteen age- and sex-matched healthy controls formed the first group (HC, n =13). Clinically diagnosed and histopathologically confirmed cases of oral cancerous cases (OSCC, n = 13) formed the second group. The samples were collected from the Department of Oral surgery, Rajah Muthiah Dental College. The age ranged from 40 to 60 years. The study was approved by Institutional Human Ethical Committee of Rajah Muthiah Medical College and informed consent was obtained from all subjects before sample collection. Patients with diabetes mellitus or any other systemic diseases were excluded from the study.

All subjects were subjected to biopsy procedure and specimens were fixed in 10% of neutral buffered formalin and embedded in paraffin wax and H&E staining done for all the samples before proceeding to immunohistochemistry staining. Formalin fixed paraffin embedded blocks were sectioned at 4µm and transferred to precoated slide [(Bio Genex Optiplus ™) Bio Genex, Fremont, CA]. The sections were deparaffinised in xylene and rehydrated in ethanol solution. Antigen retrieval was performed in 10mM citrate buffer (pH6.0) using a pressure cooker (10min) and cooled at room temperature. Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 30 min. Non specific binding was blocked with protein block solution for 8 min at room temperature. Following rinsing with wash buffer, sections were incubated for 1hr with antihuman p53 mouse monoclonal antibody (Novocastra, New castle, UK RTU -ready to use), bcl2 for 30 min at a 1:50 dilution (Path insitu, Minnesota, USA), Bax incubated for 30 min (Biogenex San Ramon CA). The sections were then incubated with their corresponding secondary antibodies, (Horseradish Peroxidase, Thermoscientific, Fremont, CA) for 30 minutes at room temperature. The antigen- antibody complex was detected using 3, 3' diaminobenzidine (DAB) (Sigma, St.Louis, MO, USA). The immunostained slides were counterstained with mayer's hematoxylin for 5 minutes and covered with a mounting medium. Negative controls were employed in which the primary antibody was replaced by phosphate buffered saline. Positively stained cells were counted under a microscope using 200X magnification in a minimum of five selected areas with frequent positive staining.

III. Results

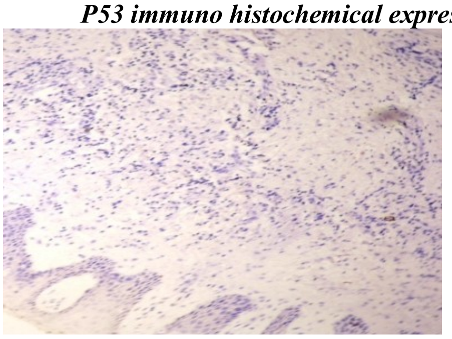
H & E staining pattern in control (a) and OSCC patients (b)

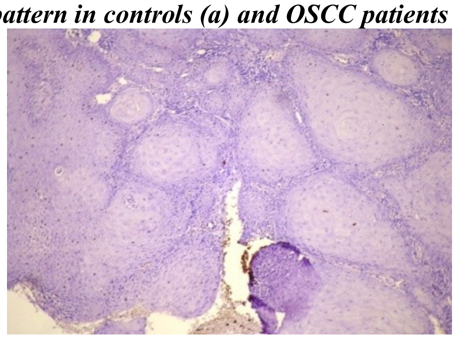




(a) ***(b)***

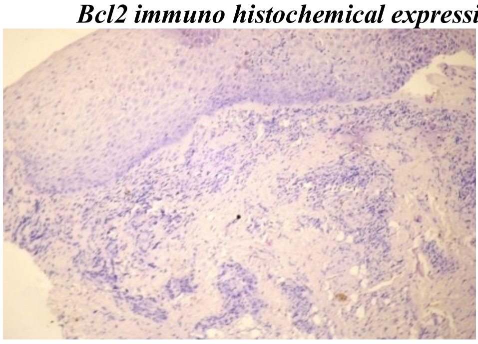
P53 immuno histochemical expression pattern in controls (a) and OSCC patients (b)

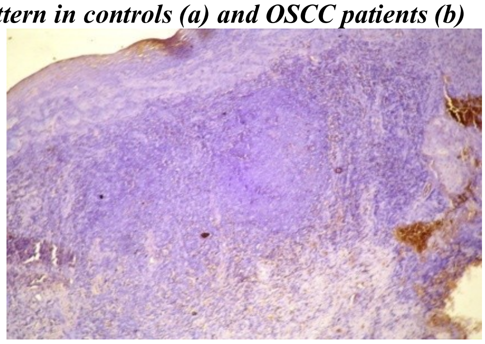




(a) ***(b)***

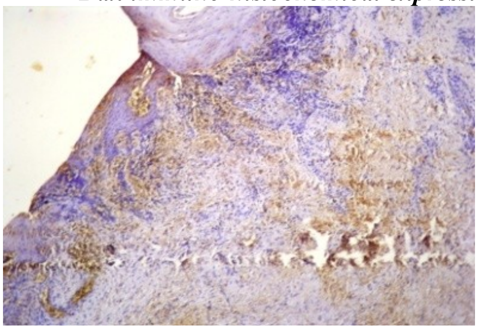
Bcl2 immuno histochemical expression pattern in controls (a) and OSCC patients (b)



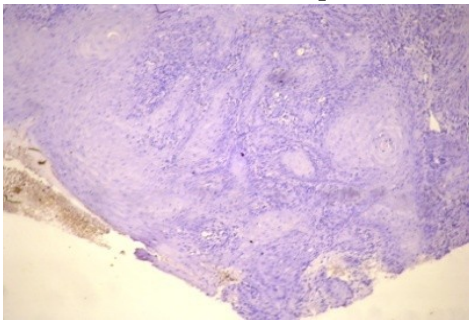


(a) ***(b)***

Bax immuno histochemical expression pattern in controls (a) and OSCC patients (b)



(a)



(b)

Table: 1Comparison of staining intensity in OSCC patients with controls

Samples	P53				Bcl2				Bax			
Staining intensity	0	1+	2+	3+	0	1+	2+	3+	0	1+	2+	3+
Controls	13	0	0	0	13	0	0	0	6	7	0	0
OSCC	0	3	5	5	2	2	6	3	10	3	0	0

0-No expression, 1+- mild, 2+- moderate, 3+- strong

IV. Discussion

The identification of molecular markers that provide an insight into the potential behaviour or aggressiveness of tumors is a necessary step for the improvement of cancer treatment. As per our study, all the OSCC samples showed mutant p53 expression where as none of the control samples showed expression. Bcl2 was expressed in 85% of OSCC samples where as control samples were negative for bcl2 expression. Bax expression was low in OSCC samples (23%) when compared with control samples (57%). This indicates of a lesser pro apoptotic bax expression which might have contributed to oral cancer.

It is widely accepted that OSCC develops as a result of accumulation of genetic errors in the same tissue [11] and that mutations in the TP53 gene leading to loss of function is one the most common genetic damage found in human tumour and in OSCC. TP53 mutations may result in an over-production of P53 inactive proteins which accumulate in the epithelium or due to delayed/ partial degradation [12].

The balance between cell proliferation and apoptosis determines growth in both normal tissue and in cancer. This balance is controlled by many factors of which the Bcl-2 family, particularly Bcl-2, and Bax, play an important role [13]. The Bcl-2 protein, an anti-apoptotic marker and its over-expression has been reported in several tumors including breast, thyroid, lung and skin carcinomas [14]. In oral carcinomas, over expression of Bcl-2 from 7% to 60% has been reported from developed countries while Ravi *et al.,* and Kannan *et al.,* reported 100% and 23% Bcl-2 expression respectively, in oral cancers from India[15,16,17]. It has been suggested that apoptosis is a barrier against cancer. In our study all normal epithelium was negative for Bcl-2 expression. Lack of Bcl-2 expression in normal has also been reported by other investigators [18].

The Bcl-2 gene is a proto-oncogene whose protein product inhibits apoptosis. Its role is associated with keeping cells alive, but not by stimulating them to proliferate, as other proto-oncogenes do. Even though increased Bcl-2 expression does not seem to be a required factor for the progression of the neoplastic process, it may play a significant role in early carcinogenesis. The proapoptotic protein Bax plays an important role in defense against cancer as it mediates apoptosis in response to genotoxic stress. It has been shown that Bax can bind to Bcl-2, resulting in inactivation of the antiapoptotic action of Bcl-2 [19]. Bax is expressed in normal epithelium. It has been reported that patients with oral cancer along with Bax expression had a better prognosis than those without Bax expression [20]

Bax expression was noticed in the cytoplasm uniformly in all cell layers of the normal squamous epithelium. Decreased expression of Bax in the cancerous tissues may reduce apoptotic cell death as well as accelerate their growth [21]. A positive association between loss of Bax immune positivity and shorter survival of patients with metastatic breast adeno carcinoma has been shown [22]. An inverse correlation between p53 and Bax immuno staining has been reported in oral carcinoma [23]. Our results support these observations.

V. Conclusion

Apoptotic mechanism is dysregulated in oral carcinogenesis as evidenced by expression of mutant p53 protein along with increased Bcl2 and decreased Bax expression. Further, exonic studies of the mutant p53, in p53 expressed samples can be explored for common mutation.

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