Original Article

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Correlation between Histolologic Grading and p53 Protein Expression at Invasive Tumour Front in Oral Squamous Cell Carcinoma

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Abstract

Aim/Background: In the search to refine the prediction of aggressiveness in individual tumours, different biological markers and malignancy grading systems have been used. The aim of the present study was to evaluate the association between histologic grading and expression of p53 at the invasive tumour front (ITF) in oral squamous cell carcinoma (OSCC). *Methods:* The expression of p53 protein at ITF was assessed by immunohistochemistry. Thirty OSCC patients included 17 men and 13 women with an age range of 30-67 years and a mean age of 51.4 years. Ten normal oral mucosa samples served as control. The data for mean p53 labeling index (LI) was analyzed for difference between low and high grade carcinomas at ITF and centre of the tumour. *Results:* In low grade carcinomas, the mean p53 LI at ITF was 48.24 (±34.0177); and mean p53 LI at centre was 42.1200 (±31.3014). In high grade carcinomas, the mean p53 LI at ITF was 52.3733 (±38.0615); and mean p53 LI at centre was 45 (±40.2120). The mean p53 LI at ITF was more as compared to mean p53 LI at centre. The mean p53 LI at ITF and centre in high grade carcinomas was more as compared to the mean p53 LI at ITF and centre in low grade carcinomas. The difference was statistically non-significant. *Conclusion:* There is a high incidence of expression of p53 protein in OSCC. The cells present at ITF have different molecular characteristics when compared with those in the superficial areas of the tumour.

Keywords: Invasive Front Grading; Oral Squamous Cell Carcinoma; p53.

Introduction

Oral cancer is among the ten most common cancers in the world, accounting for 3-5% of all malignancies; and representing the third most common form of malignancy in developing countries [1,2]. Even now, half of the patients afflicted with the disease die within the first two years of diagnosis and oral cancer is expected to become a public health problem in foreseeable future [3].

There is a strong need for development of diagnostic tools that can help the clinician to define the most appropriate management for the individual patient. One such finding is the recognition of the significance of structural and functional features of the most advanced parts of a carcinoma, namely the invasive tumor front (ITF), in determining the biological aggressiveness of oral cancer [3].

There are various grading systems for carcinoma ranging from the Broder's system of grading to the invasive front grading (IFG) developed by Bryne M et al that is shown to have more prognostic value [4].

Evidence exists to suggest that cells present at the ITF have different characteristics compared with superficial areas of the tumour, making ITF the most important area of the tumour prognostication. These changes at the ITF include: aberrant expression of tumor suppressor genes and oncogenes; increased expression of proliferation related molecules; aberrant expression of adhesion related molecules; overproduction of matrix metalloproteinases; initiation of angiogenesis [3,5].

One of the crucial events in carcinogenesis is inactivation of tumour suppressor genes [6]. p53 is a tumour-suppressor gene that is one of the most frequent targets for genetic mutation in human

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cancers. Like many other malignancies, oral cancer has a high frequency of p53 mutation and its tumourigenesis is thought to be related to p53 mutation [7]. Mutation of the p53 gene prolongs the half-life of the protein and/or alters its conformation whereby immunostaining of the protein is rendered possible. Therefore, detectable levels of p53 are assumed to be synonymous with the presence of p53 mutation [8]. Few studies have described the correlation between IFG and expression of p53 in oral squamous cell carcinoma (OSCC) [9]. The purpose of the present study was to evaluate the association between histologic grading and expression of p53 at the ITF in OSCC.

Materials and Methods

The study included histopathologically diagnosed cases of OSCC. Samples were retrieved from the registry. Two sections (4μm) of each specimen were prepared; one section was stained with hematoxylin and eosin and the other section was stained immunohistochemically to assess the expression of p53 protein. Forty samples were included in the study which was categorized in three groups. Group I- OSCC, Bryne's grade ≤ 8 (n=15), Group II- OSCC, Bryne's grade > 8 (n=15), Group III- normal oral mucosa (n=10). Thirty OSCC patients included 17 men and 13 women with an age range of 30-67 years and a mean age of 51.4 years. The tumours were located in the buccal mucosa (18 cases), in the gingiva (6 cases), in the tongue (3 cases), lower lip (2 cases) and in the oral floor (one case). The histologic grade of malignancy at the ITF was determined by the method of Bryne M et al [10].

Immunohistochemical Method for Detection of p53 Antigen

Four μm sections were mounted on silane coated slides. Antigen retrieval was conducted by autoclaving the sections at 121°C in 0.01 M citrate buffer (pH 6.0) for 10 min. The sections were incubated in Peroxide block (3% H_2O_2 in water) for 5 min at room temperature to eliminate endogenous peroxidase activity and then incubated with Power Block (casein and proprietary additives in PBS with 0.09% sodium azide) for 10 minutes at room temperature to block non-specific immune reactions. Sections were covered with prediluted monoclonal primary anti-p53 antibody (DO7 diluted 1:100; BioGenex CA), and incubated in a humidifying chamber at room temperature for 60 minutes and then kept overnight at 4°C. The sections were then washed three times with wash buffer. The Super Sensitive Polymer-HRP Detection System (BioGenex, CA) was used for application of the secondary antibody, according to the manufacturer's instructions, and the reaction products were visualized by immersing the sections for 5–20 min in 0.03% diaminobenzidine (DAB) solution containing 2 mM hydrogen peroxide. The sections were then counterstained with Mayer's hematoxylin. Known squamous cell carcinoma samples showing good p53 expression acted as a positive control. The negative control was done by omission of primary antibody.

Assessment of Immunohistochemically Stained Sections

The tissue sections were evaluated for p53 positivity at invasive front and other areas of the section by scanning the sections at a 100× magnification under a light microscope. All stained nuclei were scored positive regardless of intensity of staining. Cells that lacked a clear nucleus were excluded. Cell counts were made at 400× magnification with conventional light microscope in at least 10 randomly selected fields. Minimum of 1000 cells were counted in each section [9]. The number of positively stained nuclei was expressed as a percentage of the total number counted. The p53 labeling index (LI) was calculated from the ratio of the number of tumour cells stained by p53 to the total number of tumour cells counted per section.

Statistical Analysis

The data was analyzed by SPSS 10.0© software. The data for mean p53 LI was analyzed for difference by use of Paired t test within the group at ITF and centre and Unpaired t test between the two groups (I and II). Pearson correlation coefficient test was used to analyse p53 LI at ITF and total IFG score and also p53 LI at ITF and each of the individual parameters of Bryne's grading system in Groups I and II.

Results

p53 Labeling Index

The pattern of p53 immunostaining was classified as negative (-) when < 10% of the cells were reactive for p53 and positive (+) when >10% of the cells were

reactive for p53.

Group I & II showed nuclear p53 staining found only in epithelial cells. The overlying/adjacent epithelium showed p53 positivity in basal and suprabasal cell layers. p53 positive cells were found to be present throughout the tumour tissue or consisted of groups of positive cells scattered among negative cells. In group I, p53 staining was found mostly in the peripheral cells in areas showing keratin pearl formation (Figure 1,2).

However in group II, the pattern of p53 staining was constantly observed (Figure 3,4). In group III p53 positive cells were present mainly in the basal cell layer (Figure 5,6).

Comparison of p53 Positive and Negative Cases in Three Groups

In group I, p53 antigen was positive in 10 (66.67%) cases at ITF as well as centre. The overlying/ adjacent epithelium showed p53 positive in 6 (40%) cases. In group II, p53 antigen was positive in 11 (73.33%) cases at ITF and in 10 (66.67%) cases at centre. The overlying/ adjacent epithelium showed p53 positive in 5 (33.33%) cases. In group III, p53 antigen was positive in 4 (40%) cases at basal cell layer while it was negative in all 10 (100%) cases in the suprabasal cell layer.

Mean p53 Labeling Index

In group I, the mean p53 LI at ITF was 48.24 (±34.0177) with range of 0-90.5; and mean p53 LI at centre was 42.1200 (±31.3014) with range of 0-90. The mean p53 LI of overlying/adjacent epithelium was 22.46 (±28.5357) with range of 0-82. In group II, the mean p53 LI at ITF was 52.3733 (±38.0615) with range of 0-100; and mean p53 LI at centre was 45 (±40.2120) with range of 0-100. The mean p53 LI of overlying/ adjacent epithelium was 22.746 (±32.479) with range of 0-80.2. In group III, the mean p53 LI at basal cell layer was 7.68 (±8.5983) with a range of 0-21.1 and mean p53 LI at suprabasal layer was 0.67 (±2.1187) with a range of 0-6.7.

Comparison of Mean p53 LI at ITF and Centre in Group I and Group II

In group I and group II, the mean p53 LI at ITF was more as compared to mean p53 LI at centre. Paired samples t test shows statistically significant difference for mean p53 LI between ITF and centre in group I and group II (Table 1,2).

Comparison of Mean p53 LI at ITF in Group I and Group II

The mean p53 LI at ITF in group II was more as compared to the mean p53 LI at ITF in group I. The difference was statistically non-significant (0.756) as revealed by unpaired t test (Table 3).

Comparison of Mean p53 LI at Centre in Group I and Group II

The mean p53 LI at centre in group II was more as compared to the mean p53 LI in group I. The difference was statistically non-significant (p=0.840) as revealed by unpaired t test (Table 4).

The Pearson correlation coefficient test was used to evaluate the relationship between mean p53 LI at ITF and total invasive front grading (IFG) score and the relationship between mean p-53 LI at ITF and each of the parameters of Bryne's grading system in group I and group II.

Comparison of Mean p53 LI at ITF and Total IFG Score and Each of the Parameters of Bryne's Grading System in Group I

Statistically significant positive correlation was found between the mean p53 LI at ITF and the total invasive front grading score (r=0.950, p=0.000). Statistically significant positive correlation was found between mean p53 LI at ITF and degree of keratinization (r=0.912, p=0.000) and nuclear polymorphism (r=0.943, p=0.000). No statistically significant correlation was found between the mean p53 LI at ITF and number of mitoses (r=0.233, p=0.402), pattern of invasion (r=0.306, p=0.267) and lymphoplasmocytic infiltration (r=0.251, p=0.367).

Comparison of Mean p53 LI at ITF and Total IFG Score and Each of the Parameters of Bryne's Grading System in Group II

Statistically significant positive correlation was found between the mean p53 LI at ITF and the total invasive front grading score (r=0.962, p=0.000). Statistically significant positive correlation was found between mean p53 LI at ITF and nuclear polymorphism (r=0.891, p=0.000) and pattern of invasion (r=0.775, p=0.001). There was no statistically significant correlation between the mean p53 LI and the degree of keratinization (r=0.376, p=0.168), number of mitoses (r=0.328, p=0.233), and lymphoplasmocytic infiltration (r=0.141, p=0.616).

Table 1: Comparison of mean p53 LI at ITF and centre in Group I

p53 LI	N	Mean	Standard Deviation	t value	p value	Result
at ITF	15	48.2400	34.0177	3.837	0.002	p≤0.5, Significant
at Centre	15	42.1200	31.3014			

Table 2: Comparison of mean p53 LI at ITF and centre in Group II

p53 LI	N	Mean	Standard Deviation	t value	p value	Result
at ITF	15	52.3733	38.0615	4.514	0.0000	p≤0.5, Significant
at Centre	15	45.0000	40.2120			

Table 3: Comparison of mean p53 LI at ITF in Group I and Group II

p53 LI at ITF	Group	N	Mean	Standard Deviation	t value	p value	Result
	Group I	15	48.2400	34.0177	- 0.314	0.756	p > 0.5, Not Significant
	Group II	15	52.3733	38.0615			

Table 4 : Comparison of mean p53 LI at centre in Group I and Group II

p53 LI at Centre	Group	N	Mean	Standard Deviation	t value	p value	Result
	Group I	15	42.1200	31.3014	-0.204	0.840	p > 0.5, Not Significant
	Group II	15	45.0000	40.2120			

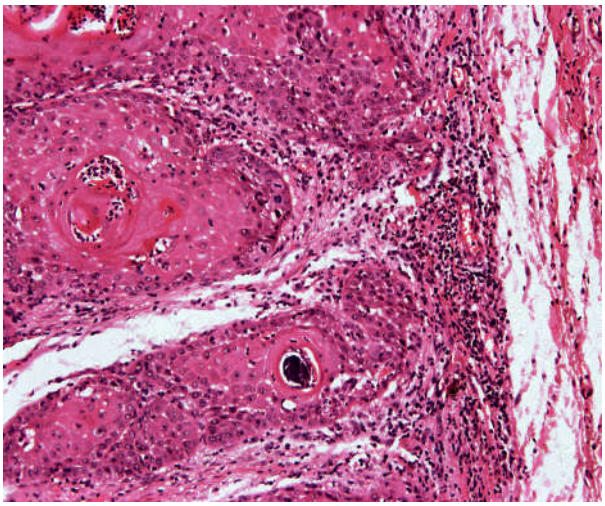

Fig. 1: Invasive tumour front of oral squamous cell carcinoma, Bryne's grade ≤ 8 (Group I). H & E stain. (100x magnification)

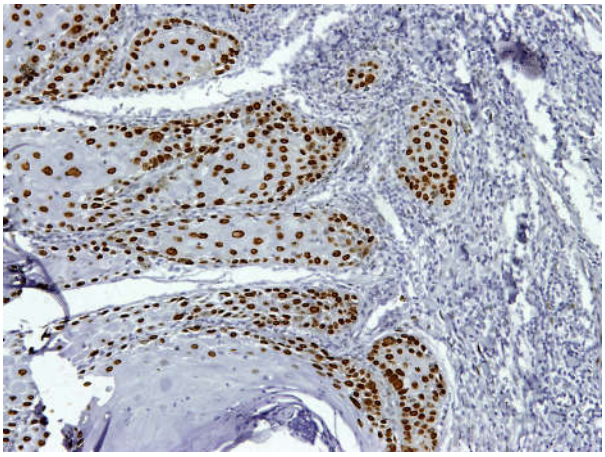

Fig. 2: p53 expression at invasive tumour front of oral squamous cell carcinoma, Bryne's grade ≤ 8 (Group I). (100x magnification)

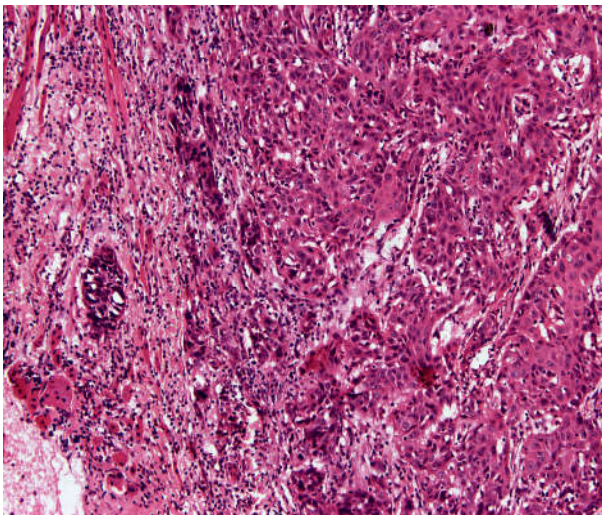

Fig. 3: Invasive tumour front of oral squamous cell carcinoma, Bryne's grade > 8 (Group II). H & E stain. (100x magnification)

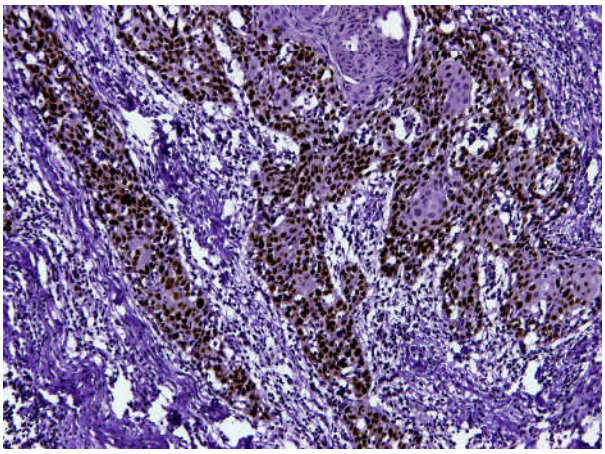

Fig. 4: p53 expression at invasive tumour front of oral squamous cell carcinoma, Bryne's grade > 8 (Group II). (100x magnification)

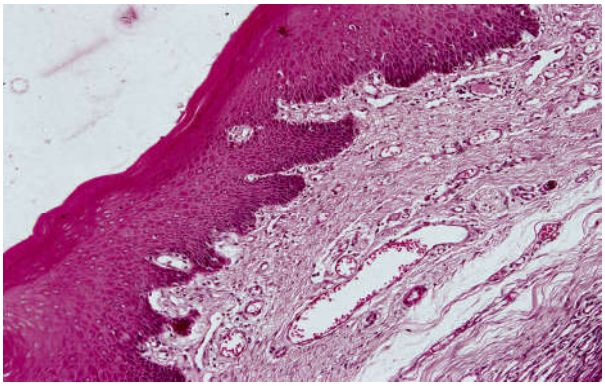

Fig. 5: Normal oral mucosa. (Group III) H & E stain. (100x magnification)

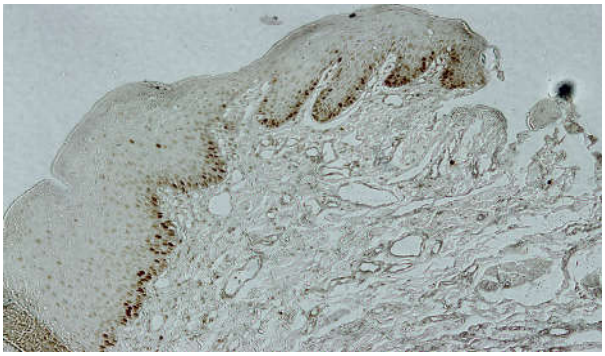

Fig. 6: p53 expression in normal oral mucosa (Group III). Basal cells stained with DO-7. (100x magnification)

Discussion

Oral cancer is now considered to be a multi-hit process which involves a number of aberrant genetic events culminating in malignant transformation [11].

In our study some cases showed immunoreactivity for p53 in normal oral mucosa. Similar results were obtained by Kannan S et al [12], Cruz IB et al[13] and Iamaroon A et al [14]. However, Warnakulasuriya K and Johnson N[15], Langdon JD and Partridge M [16], Kusama K et al [17] Ibrahim SO et al [18] were unable to detect expression of p53 in normal mucosa presumably due to the fact that levels of p53 were too low to be detected by immunohistochemistry. p53 expression is always restricted to the basal cell layer which constitutes the proliferative compartment of normal oral epithelium; it is likely, therefore, that exposure to genotoxic stress will lead to p53 accumulation in the basal cell layer [13].

In the present study a few cases showed expression of p53 protein in overlying/adjacent mucosa of OSCCs. Piffko J et al observed scattered single or focally aggregated p53 positive cells frequently in the basal and parabasal layers of the epithelium in the adjacent non-tumourous mucosa [19]. Araujo VCD et al found 2 cases (5%) of mucosal epithelium covering the neoplasm showing p53 positivity in basal and parabasal cells [20]. Ibrahim SO et al showed p53 positive immunoreactions in the dysplastic cells of the basal region of the overlying squamous epithelium in OSCCs [18]. The p53 alterations in the overlying/adjacent epithelium may represent an early event in oral carcinogenesis or might simply indicate a normally working p53 system activated in genetically stressed cells which may either be restored, pushed towards apoptosis, or enter the vicious circle of malignant transformation [19].

The expression of p53 in suprabasal cells is likely to reflect the presence of mutant p53 protein which, due to its decreased turnover, persists for longer periods of time. Alternatively, it may indicate the presence of proliferating cells with DNA damage in more superficial compartments of the epithelium, especially in cases showing dysplasia [13].

Our results showed increased expression of p53 protein in OSCCs as compared to control group. This was in concordance with studies showing expression of p53 protein in oral squamous cell carcinoma ranging from 43-80% [14,16,20-22].

Increased expression of p53 protein in OSCCs can be due to its accumulation as a result of mutation of the p53 gene which is more stable than its wild type counterpart and thus can be visualized by immunohistochemistry [12]. Mutation of p53 causes uncontrolled cell growth leading to malignant transformation [14]. Alternatively, increased expression of p53 is thought to be due to stabilization of wild type p53 protein via interactions with other intracellular proteins (e.g., SV40 large T antigen, adenovirus E1B protein, HPV16 and HPV18 E6 oncoprotein, members of the heat-shock protein family, MDM-2 oncoprotein) or transcriptional induction rather than mutation [23].

The percentage of p53 immunoreactivity at the ITF in our study is comparable to the study of Ibrahim SO et al showing expression of p53 in 62% of cases [18]. The immunoreactivity in our study was higher compared to Piffko J et al demonstrating 40% of p53 immunoreactive tumours with a predominant accumulation of p53 positive tumour cells at the invasive front of the carcinomas [19]. 54% of tumours exhibited p53 immunostaining predominantly at the invasive tumour areas in the study of Kurokawa H et al [9].

In the present study, the mean p53 LI at ITF was more as compared to p53 LI at centre in both low grade and high grade carcinomas. This suggests that ITF might have putative biological significance in

OSCC because of the increased expression of tumour suppressor gene p53 as compared to the superficial areas of the tumour.

Few studies have tried to correlate expression of p53 protein in OSCC and various malignancy grading systems [9,19,24]. In the present study the mean p53 LI at ITF increased with an increase in the IFG score within the same group of carcinomas. Though there was increase in p53 expression as we progressed from group I to group II, the difference was statistically non-significant.

Kurokawa H et al showed that overexpression of p53 at the deep ITF is associated with histologic grade of malignancy in OSCC [9]. Piffko J et al found no significant correlation between p53 immunoreactivity and the histopathological grade of tumours [19].

We also analyzed whether any correlation was found between p53 protein expression and individual parameters of Bryne's IFG. In group I there was a positive correlation between p53 expression and degree of keratinisation; and nuclear polymorphism. In group II there was a positive correlation between p53 expression and nuclear polymorphism; and pattern of invasion.

Araujo VCD et al showed a positive correlation between nuclear accumulation of p53 and histological grade of malignancy. This correlation was also detected for degree of keratinization, nuclear polymorphism and number of mitoses [20].

The data from the present study shows that p53 protein at the ITF is related to the tumour cells as well as tumour architecture.

Thus the factors related to biology of tumour cells such as degree of keratinization and nuclear polymorphism and the factor that indicates tumour aggressiveness such as pattern of invasion may be considered to have significant prognostic value in OSCC.

There are certain limitations to our study. A quantitative prognostic relevance of p53 protein in OSCCs could not be established in the present study. This could be due to small sample size and heterogeneous patient population. Histologic malignancy grading systems are complicated by the heterogeneity between subtypes of oral carcinomas and the different behaviour of carcinomas arising in localized but distinct areas of the oral mucosa. Homogeneity with respect to characteristics such as site, size, TNM stage, and treatment of the carcinoma are required for analysis of the relative prognostic value of each of the individual parameters of the grading system.

Conclusion

There is a high incidence of expression of p53 protein in OSCC. The cells present at the ITF of the carcinoma have different molecular characteristics when compared with those in the superficial areas of the tumour as reflected by increased expression of p53 protein at ITF.

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