An immunohistochemical study of p53 expression in oral submucous fibrosis

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ABSTRACT

Oral Submucous fibrosis (OSMF), an important precancerous condition occuring almost exclusively in people from the Indian subcontinent, but cases have been reported from several countries throughout the world. The causes of OSMF are unknown and there is no known treatment for it. Chilies, tobacco use, vitamin deficiencies and betel quid chewing have been implicated. Reports of malignant transformation rate of surface epithelium in OSMF ranges from 3% to 19%, among patients attending hospitals. Genetic changes leading to alterations in structure, function or expression level of proteins involved in cell cycle regulation are known to be one of the key events in the malignant transformation of the tissue. Mutations of the tumor suppressor gene, particularly p53, are the most commonly identified events in various human cancers. Extensive data for molecular markers, such as p53, proliferation associated antigens, cytokeratins, BCL2 exist for other premalignant lesions such as leukoplakia, data for OSMF is limited and much work needs to be done. The aim of our study was to determine p53 expression by immunohistochemistry and evaluate their potential as surrogate biomarkers of malignant transformation in OSMF. The result of the study shows that 73% cases of OSMF were positive for p53 marker and thus p53 marker can be used as a diagnostic and prognostic marker for early and prompt treatment planning.

Keywords: Premalignant conditions, tumor suppressor genes, immunohistochemistry,

INTRODUCTION:

Oral Sub mucous Fibrosis (OSMF) is a well-recognized pre-malignant condition having a malignant transformation rate as high as 7.6%. OSMF occurs predominantly among Indians and to a lesser extent among other Asiatic people[1]. The majority of case reports and epidemiological studies have been reported in the subjects with birth in the Indian subcontinent and many of them were related to the families which had migrated from this region.[1] Prevalence by sex varies widely in the different published studies. p53 is a tumor suppressor gene that encodes a Phosphoprotein of 339 amino acids with a molecular mass of 53 kDa. Alteration in tumor suppressor gene like p53 are frequently found in various types of cancers and precancerous conditions or lesions, and have been considered as molecular markers.[2] p53 inhibits cell cycle progression and induces apoptosis. Loss of p53 function causes loss of cell cycle control and presumably accumulates damage-induced mutations that result in malignant transformation of cells.[3] p53 aberration – both non-functional protein as well as gene mutation have been observed in oral submucous fibrosis. Detection of p53 protein by immunohistochemistry has shown to be a reliable tool as indicative of alterations at gene level. The degree of p53 staining has been found to be increased with the morphologic transformation of normal-appearing epithelial cells into dysplastic epithelial cells.[4]Till date data on p53 involvement in OSMF studies is meagre and requires further investigations, henceforth, this study is conducted to detect expression of tumor suppressor gene p53 in OSMF. This study aimed at identifying the expression of tumor suppressor gene p53 in oral submucous fibrosis and to compare with normal oral mucosa and to determine difference of expression in male and female.

MATERIAL AND METHOD:

A total of 40 histopathologically diagnosed, formalin fixed, paraffin embedded tissue samples were collected and categorized into 2 groups:

Control group
Study group.

Control group consisted of 10 healthy individuals without any habits and with no clinical changes in oral mucosa with normal oral mucous membrane tissue samples obtained from healthy volunteers who visited the Department of Oral Surgery for minor oral surgical procedures at D.J. College of Dental Sciences & Research, Modinagar.

Study group consisted of 30 histologically identified paraffin embedded specimen of Oral Submucous Fibrosis selected from tissue archives of Department of Oral & Maxillofacial Pathology and Microbiology, D.J. College of Dental Sciences & Research, Modinagar.

Preparation of the slides:

First, the wax blocks of histopathologically diagnosed lesions of Oral Submucous Fibrosis were selected. Then, 5mm of tissue sections were made using a microtome. The sections were placed on silane-coated slides. The silane-coated slides were used in order to prevent floating of the samples during incubation in the microwave oven for antigen retrieval.

Immunohistochemistry:

For immunohistochemistry, the tissue sections were first deparaffinized with xylene, then rehydrated with grades of alcohol, and treated with 3% Hydrogen Peroxide (H_2O_2) for 15 minutes to quench endogenous peroxidase activity. Wash slides with Phosphate Buffer Saline (PBS) for 5 minutes. Heat Induced Antigen Retrieval was used by inserting slides in petridish containing citrate buffer and placing in a microwave for 10 minutes. Slides were incubated in primary antibody DO-7 at room temperature for 1 hour and wash with PBS for 5 minute. Biotin labelled secondary antibody was applied and incubated for 30 minutes and washed with PBS. Diaminobenzidine solution was applied and incubated for 5 minutes. Counter stain with Hematoxylin for 1 minute. Dehydration of slide was done by increasing grades of alcohol and cleared by xylene. Slides were then dried and mounted by DPX. A brown precipitate in the Nucleus confirmed the presence of p53 protein. The p53 positive samples were then evaluated semi quantitatively on a 4- point scale based on the percentage of cells showing p53 staining:

Negative or – if less than 10% of the cells show positive staining

1+ if 10-30% cells show positive staining

2+ if 30-50% cells show positive staining

+ if more than 50% of the cells show positive staining

RESULT:

Out of 30 cases of Oral Submucous Fibrosis, 22 (73.3%) were male and 08 (26.7%) were female. Among 10 cases of control group 07 (70%) were male and 03 (30%) were female. Mean age among control group was 40.33 years and in case group was 36 years. Expression of p53 was positive in 22 (73.3%) cases and negative in 8 (26.7%) cases. Among control group all the cases shows negative p53 expression (Table 1) (Figure 1).

Table 1: P53 Status among Control Group

Sample	Age/Sex	Count	Grade
Sample no 1	29/M	0%	-
Sample no 2	42/M	0%	-
Sample no 3	53/F	0%	-
Sample no 4	44/M	5%	-
Sample no 5	32/F	0%	-
Sample no 6	54/M	0%	-
Sample no 7	23/M	0%	-
Sample no 8	38/M	3%	-
Sample no 9	18/F	0%	-
Sample no 10	27/M	0%	-

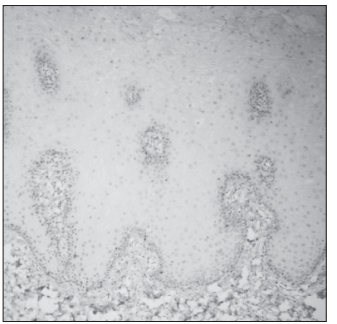

Fig. 1: p53 Expression in Normal Oral Mucosa

From the total,14 (46.6%) out of 30 cases from case group shows 1+ grading (Figure 2), 4 (13.3%) shows 2+ (Figure 3) and 4 (13.3%) shows 3+ grading (Figure 4), (Table 2).

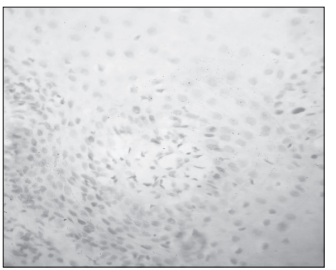

Fig. 2: p53 Expression (+) in Osmf

Table 2: p53 status among case group

Sample	Age/sex	Count	Grading
Sample no 1	34/M	16%	1+
Sample no 2	29/M	8%	-
Sample no 3	45/M	28%	1+
Sample no 4	48/F	18%	1+
Sample no 5	56/M	68%	3+
Sample no 6	25/M	26%	1+
Sample no 7	29/F	20%	1+
Sample no 8	34/M	24%	1+
Sample no 9	38/M	37%	2+
Sample no 10	43/M	42%	2+
Sample no 11	44/F	22	1+
Sample no 12	39/F	5%	-
Sample no 13	25/M	3%	-
Sample no 14	28/M	14%	1+
Sample no 15	31/F	6%	-
Sample no 16	52/M	74%	3+
Sample no 17	42/M	78%	3+
Sample no 18	58/M	34%	2+
Sample no 19	54/F	8%	1+
Sample no 20	45/F	27%	1+
Sample no 21	56/M	43%	2+
Sample no 22	35/M	26%	1+
Sample no 23	43/M	14%	1+
Sample no 24	34/M	4%	-
Sample no 25	46/M	77%	3+
Sample no 26	43/M	13%	1+
Sample no 27	34/F	6%	-
Sample no 28	45/M	3%	-
Sample no 29	32/M	8%	-
Sample no 30	43/M	24%	1+

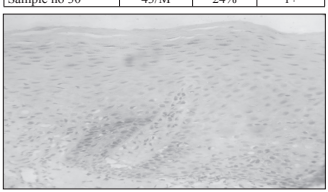

Fig. 3: p53 expression (++) in Osmf

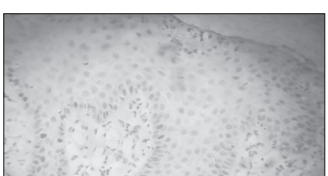

Fig. 4: p53 expression (+++) in Osmf

It was observed that maximum average counting were maximum in cases for males while it was least in for females in cases and control. (Table 3).

Table 3: the mean & S.D. abd S.E.M. in cases & controls for the countings in males and females

S.NO.	Gender	Cases (30) (Mean±S.D.)	S.E.M.	Control (10) (Mean±S.D.)	S.E.M.
1	Males	30.18±24.38	4.45	1.14±2.04	0.65
2	Females	14±8.70	1.59	0±0	0

S.E.M : Standard Error of Mean

No significant difference was present between cases and controls for age in males and females respectively (Figure 5).

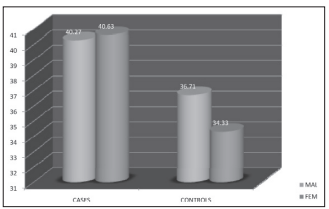

Fig. 5: Bar diagram of average age (in years) of males and females in cases and control

DISCUSSION

In the present study, p53 expression was positive in 22 (73.3%) case s and negative in 8 (26.7%) cases. Among the control group all the cases showed negative p53 expression. These results are in accordance with the studies conducted by Warnakulasuriya *et al* (1998) and Murti *et al* (1998) Murti *et al*. (1998) observed that 21/68 (31%) samples of precancerous lesions that did not progress to cancer and 6/22 samples of precancerous lesions and conditions that progressed to cancer were positive for p53.[5,6] In the present study 73% of the Oral Submucous Fibrosis cases showed positive staining with p53 expression which is in accordance with the observation made by Kerdpon D *et al* (1997), who showed in 75% of OSMF cases with p53 positive staining. However lower percentage was observed by Sultana *et al* (2011), and Kaur J *et al* (2004), being 48%. Variation in techniques employed may account for these discrepancies.[7,8,9]

The previous studies showed that p53 expression in premalignant lesions ranges from 17-67% and in oral cancer the expression ranged anywhere between 11-75%. The reason for this wide range in expressability of p53 proteins have been stated by Ogden G R *et al* (1996) and Rich A M *et al* (1999).[10,11] Reasons are as follows:

Variations in the etiological factors and ethnic background of the patients, with a generally lower prevalence of p53 alterations in oral squamous cell carcinoma in patients from the Indian subcontinent and a higher prevalence in patients from Western societies.

Variations in the immune histo-chemical technique used also explain some of the differences, with the use of microwave antigen retrieval increasing the proportion of positive results.

Tumors may have lost both the alleles of the p53 gene

Tumors may have a level of p53 protein that cannot be detected by immunohistochemistry.

By comparing study group with the control group, it was found that 22/30 (73.3%) samples of Group I and none of the normal tissue samples of the control group showed positivity for p53. The p-value was not statistically significant. In the current study, study group showed positivity for p53 in 22 samples.

Trivedy *et al* (1998), in their study from observed 75% (15/20) OSMF and all cases of cancer expressed p53,which is in accordance with present study.[5] It has been shown that there is a step wise increase in p53 positivity in the sequence of progression of normal oral mucosa to OSMF to oral cancer. These results might indicate an involvement of p53 in neoplastic transformation as well as in proliferative events. In a study conducted by Ranganathan and Kavitha (2011) one normal tissue was positive for p53 and this was basally.[12] In the present study none of the normal tissue was positive for p53. The positivity of normal tissue could indicate early alteration in the epithelium due to unknown factors or may be due to stabilization of wild p53 by other influences such as HPV rather than mutation.

The inability to detect p53 protein in the normal tissue samples can be attributed to the short half-life of the normal tissue. The normal or the wild-type p53 has a half-life of 6-20 minutes and is present in such minute quantities that it cannot be detected by IHC. But when the p53 gene is mutated there is excessive production of altered p53 protein which has a long half-life of 6 hours and thus can be detected by immune-histochemical technique.[13]

Comparing the samples of study group it was found that 14 (46.6%) out of 30 cases from control group shows 1+ grading, 4 (13.3%) shows 2+ and 4 (13.3%) shows 3+ grading. This shows that less number of cells contain mutated or stabilized p53 protein in case of OSMF. This supports the hypothesis given by Kaur J *et al* (2004) that a progressive increase in p53 expression occurs in oral squamous cells as they acquire a more malignant phenotype.[9]

Kaur J *et al* (2004) reported increased expression of p53 in the nuclei of epithelial cells was predominantly limited to the basal layer, which is the proliferative invasive layer of the epithelia.[9] The staining was mainly nuclear, while in some cases both nuclear and cytoplasmic immunoreactivity was observed. In present study, p53 staining was almost always limited to the basal layers in the samples of study group with varying intensity.

The result of present study is in accordance to a study done by Warnakulasuriya *et al* (1992) who evaluated 21 cases of OSMF for p53 positivity.[14] They found that 15 out of 21 (75%) cases were positive for p53. They concluded that since results showed high frequency of p53 aberration in Oral Submucosis Fibrosis, p53 can be used as biomarker of DNA damage in OSMF.

Similar study was conducted by Kaur J *et al* (2004), they observed 24 out of 50 cases (48%) of OSMF were positive for p53 staining. [9] In present study out of 8 tissue samples of female in study group, 5 showed (62%) positive p53 staining and 3 cases showed negative staining. Among positive female samples all 5 samples showed 1+ grading. 18 out of 22 samples (81.8%) of male showed positivity for p53 and 4 samples (18.2%) showed negative p53 staining. Among positive samples 9 samples (50%) showed 1+ grading, 5 samples (27.7%) showed 2+ and 4 sample (22.2%) showed 3+ grading. Further study with more number of sample size is needed for getting a more statistically significant result. The finding of the present study indicates that p53 exhibit altered expression in OSMF compared to normal mucosa, and has the potential to be used as surrogate marker.

Loss of TSG function and thus of regulation of cell proliferation function is an important factor in cancer development. Nearly all human cancers are associated with a loss of function of the *p53* TSG, indicating the important role of *p53* in cancer development. Present study demonstrates a high incidence of p53 over expression in OSMF. The results indicate that p53 over expression may play a role in pathogenesis of OSMF and conversion into carcinoma. Thus, it can also be inferred that p53 may be considered as a tumor marker. Expression of p53 protein may help in determining the prognosis as well as play a key role in the gene therapy for the treatment of OSMF. As p53 can detect earliest carcinomatous changes in patients with OSMF, it can be useful in community for early detection of cases and decision can be made from treatment point of view.

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