Original Article

An immunohistochemical study of p53 expressions in oral submucous fibrosis

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Abstract

Background: The objective of this histopathological study was to identify the expression of tumor suppressor gene p53 and to detect the correlation between p53 expression and the degree of dysplasia in oral submucous fibrosis (OSMF).

Methods: A sample size of 30 OSMF patients irrespective of sex was taken up for the study. The tissue samples obtained were subjected to immunohistochemical method to detect p53 protein. The technique used was indirect super sensitive Polymer-HRP IHC detection system. The p53 positive samples were evaluated on a 4-point scale, which ranged from 3+ to negative.

Results: Out of 30 cases 3(10%) cases were negative for p53 expression and 13(43.3%) showed + expression, and 14(46.6%) showed ++ expression. On application of Mantel-Haenszel Chi-Square test a statistically significant $P <= 0.05$ i.e. ($P = 0.012$) was obtained and there was Linear-by-Linear association between p53 expressions and dysplasia that showed the point probability of 0.006.

Conclusion: Immunohistochemistry is a powerful tool to identify distinct patterns of gene expression in premalignant disorders and also Oral Squamous Cell Carcinomas (OSCC) from different populations. In the present study, a significant number of samples of OSMF were positive for p53 protein.

Keywords: Oral squamous cell carcinomas, oral submucous fibrosis, p53

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INTRODUCTION

Oral submucous fibrosis (OSMF) is a chronic oral mucosal condition that occurs predominantly among Indians and people of Indian origin living outside India, occasionally in other Asians and sporadically in Europeans.[1] The prevalence rates in India are about 0.2%–0.5% and prevalence by gender varies from 0.2% to 2.3% in males and 1.2%–4.57% in females.[2]

Evidence has shown that it is a precancerous disorder related to the habit of chewing areca nut, either alone or as a component of betel quid. There are approximately 600 million people worldwide amounting to 10%–20% of the

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world's population who chew raw areca nut or in a processed form. Several case–control studies provide overwhelming evidence that areca nut is the main risk factor for OSMF. Thus, the disease is now a public health issue in many parts of the world. OSMF carries a high risk of transition to oral cancer.[3] Reports of malignant transformation rate of the surface epithelium in OSMF ranges from 3% to 19% among patients attending the hospitals in community.[4,5]

OSMF may be defined as an insidious, chronic disease affecting any part of the oral cavity and sometimes the pharynx; although occasionally preceded by and/or associated with vesicle formation, it is always associated with juxta-epithelial inflammatory reaction followed by fibroelastic change of the lamina propria, with epithelial atrophy leading to stiffness of the oral mucosa and causing trismus and inability to eat.[6] Capsaicin and spicy food have also been suggested as etiological agents for OSMF, areca nut being the fourth most commonly abused substance in the world (following nicotine, ethanol and caffeine).[4] The WHO has defined precancerous condition as a generalized pathological state of oral mucosa associated with significantly increased risk of cancer. OSMF has all the characteristics to be called precancerous condition.[7]

The possible precancerous nature of OSMF was first described by Paymaster who observed the onset of slowly growing squamous cell carcinoma in one-third of such patients. These observations were eventually confirmed by Paymaster.[8] Histologically, OSMF is characterized by juxtaepithelial fibrosis, along with atrophy or hyperplasia of the overlying epithelium, keratinizing metaplasia and accumulation of collagen beneath the basement membrane with the progressive loss of vascularity.[1] Immunohistochemistry (IHC), since first reported in the 1940s, has become a crucial technique and has been used as a critical diagnostic tool in research as well as clinical investigations. It has apparent advantage over traditionally used special enzyme staining techniques that identify only a limited number of proteins, enzymes and tissue structures. Histopathology was the gold standard in quick determination of the tissue status and fast delivery of reports, but this fails to provide information about the severity and changes at the molecular level particularly in neoplastic conditions.[9,10]

p53 protein was first identified in 1979 as a transformation-related protein. Recent research with wild type (wt) p53 clearly demonstrated that p53 was a major "guardian of the genome." At present, p53 is known to play a key role in practically all types of human cancers, and the mutation (mt) or loss of the p53 gene can be identified in more than 50% of all human cancer cases worldwide.[11] mt in the p53 gene can result in abolition of protein function, and this loss of function may be linked to tumor progression and genetic instability. Clearly, inactivation of p53 is a key event in carcinogenesis. The presence of mt p53 protein rather than the complete lack of wt p53 activity may confer a selective advantage to evolution of tumor cells. It has been demonstrated that mtp53 can be a causative factor in tumor progression because expression of some mtp53 proteins in tumor cells with a p53-null background leads to increase of their tumorigenic potential.[11]

The gene most frequently mutated in human cancer is p53. Evidences say that up to 80% of human squamous cell carcinomas in the oral cavity harbor mutated p53 tumor suppressor genes. Such a high frequency of mt suggests an important role for this gene in carcinogenesis, which may be represented by the so-called "premalignant" lesions and conditions.[12] Several studies have reported a high incidence of p53 protein expression in betel quid-associated potentially malignant lesions and oral cancers from India, Taiwan, Sri Lanka, Thailand and China.[6]

To date, data on p53 involvement in OSMF are meager. Hence, it would be worthwhile to explore the possible contribution of this tumor suppressor gene and its protein to the precancerous aspect of OSMF. The potentially malignant oral lesions at high risk of transition to malignancy, if identified, can be effectively managed by early intervention. Treatment options include iron and multivitamin supplements including lycopene – an extract of tomato and a range of medicines (e.g., intralesional injection of steroids, hyaluronidase, human placenta extracts, chymotrypsin, pentoxifylline and collagenase). Laser ablation and surgery, including cutting of the fibrous bands of the jaw muscles and temporomandibular joint, has been used for more extreme cases.[1,13-16] In this study, histopathological staining was used to identify the expression of tumor suppressor gene p53 and its correlation with degree of dysplasia in OSMF.

METHODS

This study was designed and approved by institutional review board of Raja Rajeswari (RRDCH) Dental College and Hospital, Bengaluru, Karnataka, India. The consent was also obtained from all the participating patients during the study.

Sample

A total number of 30 histopathologically diagnosed, formalin-fixed and paraffin-embedded tissue samples were collected.

H and E staining

Representative tissue sections of 3 μm thickness were cut on a microtome, and the slides were kept at 60°C for 30 min. Sections were dewaxed in two changes of xylene for 5 min each. Sections were rehydrated in 100% alcohol, 95% alcohol, 80% alcohol and 70% alcohol for 2 min each, and then, the slides were plunged in running tap water for 1 min. Delafield's hematoxylin for 1–2 min. Excess stain was rinsed off by washing the sections in running tap water for 1 min. Sections were later stained with eosin for few seconds and excess was removed by alcohol changes from 95% to 100%, and xylene for 2 min each and sections were then mounted in diestrene dibutyl phthalate xylene.

Preparation of the slides for immunohistochemistry

Wax blocks of histologically diagnosed cases of OSMF were retrieved from the archives of the Department of Oral Pathology and Microbiology, RRDCH; 3 μm thick sections were obtained using a semi-automatic microtome. The sections were placed on silane-coated slides. The silane-coated slides were used to prevent floating of the samples during incubation in the microwave oven for antigen retrieval. The slides were preserved in a slide-holding box until they were stained for p53.

Methodology of p53 immunohistochemistry

3–4 μm thick paraffin-embedded tissue section was cut and mounted on poly-l-lysine-coated slides and dried at 56°C for 30 min. Later, the specimens were deparaffinized with xylene, rehydrated in serial-graded (100%, 90%, 70% and 50%) alcohol and water-ethanol solutions and rinsed in deionized water. Target retrieval was performed with target retrieval solution in preheated (115ºC) pressure cooker for 20 min as per vendor instruction. After antigen retrieval, endogenous peroxidase activity was blocked by immersion of slides in peroxidase block solution (3% hydrogen peroxide in water, provided by Biogenex, Fremont USA) 15 min at room temperature. The slides were washed with tris-buffered saline (TBS) for 5 min, and the excess was wiped off using a blotting paper. After washing with TBS then treated with a highly effective universal protein-blocking reagent (contains casein and proprietary additives in PBS with 15 mM sodium azide, Levamisole, provided by Biogenex, Fremont USA).

Later on, the sections were incubated with primary antibody against p53 (mouse monoclonal anti-p53, clone DO-7, diluted 1:50, Biogenex, Fremont USA) for 60–75 min at room temperature. The slides were again washed with TBS for 5 min, and the excess was wiped off using a blotting paper. The secondary antibody conjugated with polymer horseradish peroxidase (polymer-HRP) (HRP, Biogenex, Fremont USA) was applied to cover the tissue samples and was incubated for 30–35 min at room temperature. The slides were washed with TBS for 5 min, and the excess was wiped off using a blotting paper.

Antigen-antibody reaction color development was carried out by diaminobenzidine (DAB), freshly prepared by mixing one drop (approximately 20 ml) of DAB chromogen with the diluting buffer to make up 1 ml of the solution. This DAB solution was then applied to the tissue samples and incubated for 2–10 min at room temperature. Antigen-antibody reaction color development was carried out after applying substrate-chromogen solution (Biogenex, Fremont USA). The slides were then washed with TBS to stop the reaction. The slides were then counter-stained lightly with Delafield's hematoxylin (1 dip). Slides were washed well in running tap water for 10 min. Sections were dehydrated in 70% alcohol, 80% alcohol, 95% alcohol and 100% alcohol for 2 min each. The tissue samples were mounted in dibutyl phthalate xylene, and the coverslip was placed. One positive and one negative control were run with each batch of immunostained sections. Positive controls for p53 protein consisted of breast cancer tissue sections with known positivity. All reactions were performed with negative controls using TBS instead of primary antibody.

Interpretation of the p53 staining

The slides stained for p53 were observed under light microscope with a magnification of ×400. The tissue samples were thoroughly examined, and total numbers of 500 cells (150–175 cells per field) were counted. Then, among these, the number of cells which had taken up the stain was counted. A brown precipitate seen within 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.

Statistical analysis

Statistical analyses were performed using Statistical Package for the Social Sciences, version 22.0 software for Windows (SPSS Inc., 2007, IBM Business analytics, Chicago, USA). The Mantel–Haenszel Chi-square statistic tests the alternative hypothesis that there is a linear association between the row variable and the column variable. On application of Mantel–Haenszel Chi-Square test, a statistically significant $P \leq 0.05$, i.e., $P = 0.012$ was obtained, and this tells us that there is an association between p53 expressions and dysplasia.

RESULTS

The histopathological study was done in an effort to identify the expression of tumor suppressor gene p53 and to detect if any correlation between p53 expression and degree of dysplasia in OSMF. After obtaining the necessary approval from the institutional review board and the written consent, this study was conducted on tissue samples collected from the patients suffering from OSMF who visited the outpatient department of Rajarajeswari Dental College and Hospital. A sample size of 30 OSMF patients irrespective of sex was taken up for the study. The tissue samples obtained were subjected to immunohistochemical method to detect p53 protein. The technique used was indirect super sensitive polymer-HRP IHC detection system. The p53-positive samples were evaluated on a 4-point scale, which ranged from 3 + to negative.

Out of 30 cases, 14 (46.6%) cases did not show dysplasia, 13 (43.3%) cases showed mild dysplasia and 3 (10%) showed moderate dysplasia. Out of 30 cases, 3 (10%) cases were negative for p53 expression, 13 (43.3%) showed + expression and 14 (46.6%) showed ++ expression [Table 1]. On application of Mantel–Haenszel Chi-Square test, a statistically significant $P \leq 0.05$ i.e., $P = 0.012$ was obtained, and there was linear-by-linear association between p53 expressions and dysplasia that showed the point probability of 0.006 [Table 2].

DISCUSSION

OSMF is a chronic progressive disorder prevalent in the Indian subcontinent and migrants from that region. It affects the mouth, pharynx and esophagus. Epithelial atrophy and dysplasia are regularly encountered in the oral lesions, and the incidence of oral squamous cell carcinomas (OSCC) has been estimated to be as high as 7.6% in patients followed over 10 years.[17] Recent reports of malignant transformation rate of the surface epithelium in OSMF range from 3% to 19% among patients attending the hospitals in the community.[4] Growth fraction and p53 expression have been used in evaluating the malignant potential of epithelia in biopsies.[17] According to most studies, p53 is not detected in normal oral mucosa, but it can be demonstrated with immunohistochemical techniques in OSCC and potentially malignant oral mucosal lesions. In general, normal p53 protein lives very short half-life of maximum 20 min which remains undetectable in normal tissues. P53 gene frequently gets mutated in human cancer and/or OSMF, and this altered protein is detectable as now it has increased the life cycle of few days.

Most reports have shown that there is an increase in the proportion of cases that show p53 abnormalities as detected by IHC from hyperplasia to dysplasia to neoplasia.[16] In this study, IHC staining of p53 was done, and light microscopic (Brightfield microscopy) analysis was done to a group of 30 histopathologically diagnosed, formalin-fixed and paraffin-embedded tissue samples, which were retrieved from the Department of Oral Maxillofacial pathology, RRDCH. 30 OSMF patients irrespective of their sex were grouped into study group. The histopathological study was done in an effort to identify the expression of tumor suppressor gene p53 and to detect if there is any correlation between p53 expression and degree of dysplasia in OSMF. In a study by Regezi *et al*,[18] it was reported that 6 out of 13 cases of epithelial dysplasia and 4 out of 6 cases of carcinoma-in-situ were p53 positive. The authors concluded that p53 expression appears to be an early event in the development of p53-positive OSCC, and once p53 expression occurs in dysplasia or carcinoma-in-situ, it appears to be persistent, with transition to invasive carcinoma appearing unpredictably months to years later. Our studies also recorded an increased p53 expression when in comparison with that of dysplastic to nondysplastic cases. In 1997, study by Rich *et al*.[16] showed p53 positivity in dysplastic oral lesions ranges from 35/41 (85%), the authors

Table 1: Cross tabulation of p53 and dysplasia

	p53 expression				
	Nil	+	++	+++	Total
Dysplasia					
Nil	3	7	4	0	14
Mild	0	6	7	0	13
Moderate	0	0	3	0	3
Total	3	13	14	0	30

+++: >50% of cells staining positive; ++: 26%–50% of cells staining positive; +: 6%–25% of cells staining positive; −: <5% of cells staining positive were considered

Table 2: Chi-square tests

	Value	df	Asymptotic significant (two-sided)	Exact significant (two-sided)	Exact significant (one-sided)	Point probability
Pearson χ^2	7.851	4	0.097	0.087		
Likelihood ratio	9.984	4	0.041	0.067		
Fisher's exact test	6.606			0.114		
Linear-by-linear association	6.279	1	0.012	0.011	0.008	0.006
Number of valid cases	30					

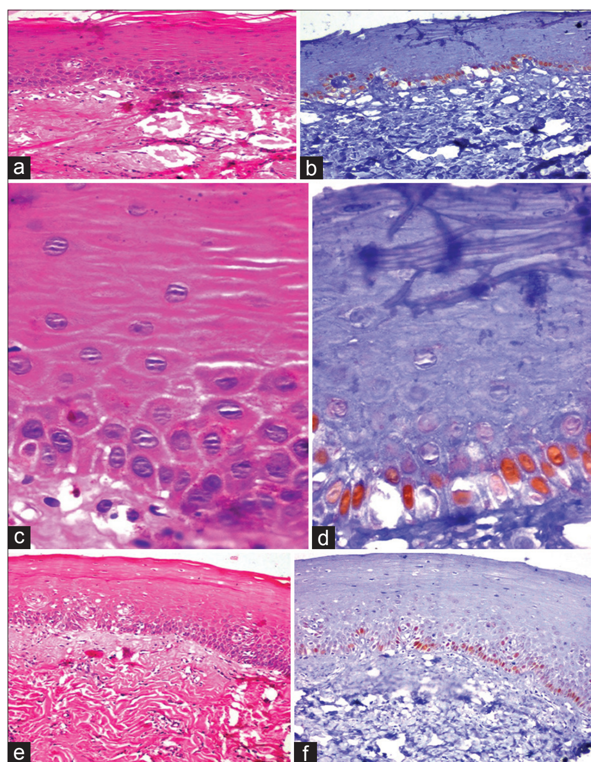

Figure 1: (a) Oral submucous fibrosis, with no dysplastic changes under low power (H&E, ×10), (b) expression of p53 in oral submucous fibrosis, with no dysplastic changes under low power (IHC, ×10), (c) Oral submucous fibrosis, with no dysplastic changes under high power (H&E, ×40), (d) expression of p53 in oral submucous fibrosis, with no dysplastic changes under high power (IHC, ×40), (e) oral submucous fibrosis, with mild dysplastic changes under low power (H&E, ×10), (f) expression of p53 in oral submucous fibrosis, with mild dysplastic changes under low power (IHC, ×10)

concluded different pattern of p53 expression increased from Hyperplasia to Dysplasia to OSCC. Different pattern of p53 staining when in comparison with Dysplastic and Nondysplastic OSMF cases. In a study conducted by Cox and Walker (1993–1994),[17] abnormal expression of p53 protein identified by IHC with a panel of antibodies was found in 70% of the OSMF specimen. Our studies recorded (27/30 cases) 90% of p53 positivity which may be attributed to the fact that 16 of the cases were confirmed for epithelial dysplasia.

Alterations in tumor suppressor gene such as expression of p53 protein is one of the most common events in oral carcinogenesis. There is a wide range of expression of p53 protein in premalignancies and squamous cell carcinoma of the oral cavity. In the present study, a significant number of samples of OSMF were positive for p53 protein. There was difference in the pattern of p53 expression with mild type of dysplasia showing p53 expression only in the basal cells and p53 expression seen in both basal and parabasal region of epithelium of moderate dysplasias of OSMF

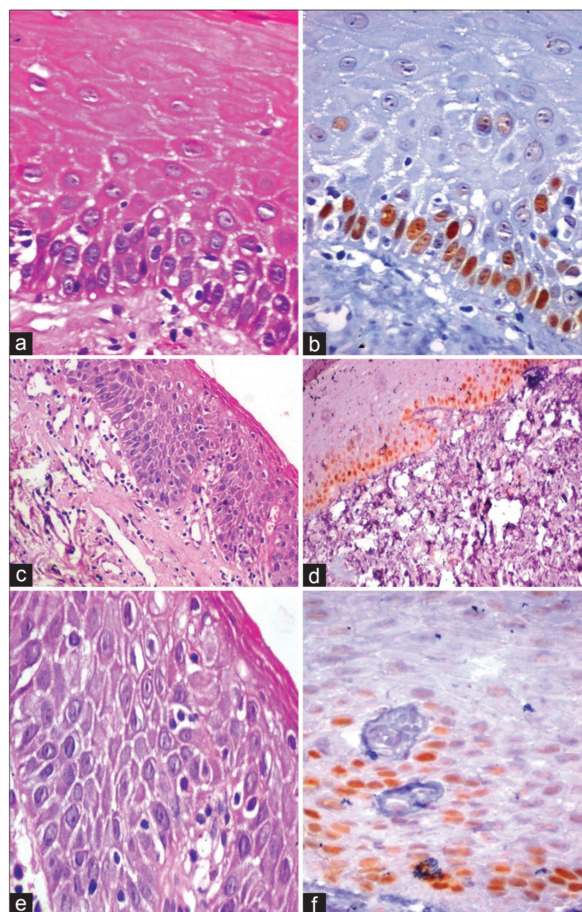

Figure 2: (a) Oral submucous fibrosis, with mild dysplastic changes under high power (H&E, ×40), (b) expression of p53 in oral submucous fibrosis, with mild dysplastic changes under high power (IHC, ×40), (c) E-stained oral submucous fibrosis, with moderate dysplastic changes under low power (H&E, ×10), (d) expression of p53 in oral submucous fibrosis, with moderate dysplastic changes under low power (IHC, ×10), (e) oral submucous fibrosis, with moderate dysplastic changes under low power (H&E, ×10), (f) expression of p53 in oral submucous fibrosis, with moderate dysplastic changes under high power (IHC, ×40)

[Figures 1 and 2]. Thus, it can also be inferred that levels of p53 protein rise as the normal cells change to dysplastic lesions and later to invasive carcinoma.

CONCLUSION

Expression of p53 protein may help in determining the prognosis and plan treatment modalities. However, further studies, long-term follow-ups and staining with other proliferative markers would further help to assess the significance of p53 protein as a prognostic marker in premalignancies.

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Conflicts of interest

There are no conflicts of interest.

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