Research Article

P53 Expressions in Oral Submucous Fibrosis and Oral Squamous Cell Carcinoma.

Nishat Sultana, Shambulingappa Pallagatti, Ali Imam Mohamed.

Abstract

Background and objectives: Expression of p53 tumor suppressor gene is one of the common findings in human cancers including the oral cancer. The product of p53 gene, p53 protein is known to be expressed in pre-malignancies and Oral Squamous Cell Carcinoma (OSCC), but only few literatures is available regarding p53 expression in oral sub mucous fibrosis. Hence this study was carried out (i) to determine the expression of aberrant p53 in Oral Submucous Fibrosis (OSMF) and OSCC (ii) to study to study correlation if any between p53 expression and degree of dysplasia in OSMF and OSCC patients (iii) to study correlation if any between p53 expression and habits in OSMF and OSCC patients.

Methods: A 30 biopsy specimens from OSMF cases and 30 specimens from OSCC cases were subjected for staining by immunohistochemistry for p53 protein using monoclonal primary antibody DO-7 by LSAB visualization system kit. Clinical details along with habits were recorded and the data was analyzed with chi-square and fisher exact test.

Result: The result of the study revealed 15 cases of OSMF and 24 cases of OSCC being positive for p53 protein. Among which only 3 cases of OSCC showed (+ +) moderate degree of dysplasia and the rest all had (+) mild dysplasia and in OSMF all 15 cases showed (+) mild degree of dysplasia. 19 out of 22 patients of OSCC and 15 of 25 OSMF patients with chewing habit showed positivity for p53 staining.

Interpretation and conclusion: This study demonstrates a high incidence of p53 over expression in OSMF and OSCC. The results indicate that p53 over expression may play a role in the development of Oral squamous cell carcinoma and in pathogenesis of OSMF. Areca nut chewing and/or smoking in OSMF and OSCC cases may play a role in the p53 over expression.

Key words: Oral Submucous Fibrosis; Squamous Cell; Carcinoma; p53; Tumor Suppressor Protein; Gene.


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Introduction

Oral submucous fibrosis (OSMF) is a well recognized potentially malignant condition, which is an insidious chronic disease affecting any part of the oral cavity and sometimes pharynx. Although occasionally preceded by and/or associated with vesicle formation, it is always associated with juxta-epithelial inflammatory reaction followed by fibro elastic change of the lamina propria, with epithelial atrophy leading to stiffness of the oral mucosa and causing trismus and inabiity to eat.1

To date, no conclusive etiologic agent has been identified and development of cancer is now considered to be a multi-hit process that involves a number of aberrant genetic defects culminating to malignant transformation. Plenty of data have also been generated on the various aspects of this disease. In Southeast Asia where Oral cancer is a major public health problem, over 90% of oral malignancies are known to arise from preexisting potentially malignant lesions and conditions.2

So far the gene frequently mutated in human cancer is p53. Normal p53 protein has a very short half-life 6-20 minute making it hard to be detected in normal tissues. But an altered protein has a half-life of about 6 hours so that it can be detected in premalignant and malignant tissue through immunohistochemistry.3 Till date, only few literatures are available regarding p53 expression in OSMF. Hence it would be worthwhile to explore the possible contribution of this tumor suppressor gene and its protein to the precancerous aspects of OSMF.

Therefore this study was taken up to evaluate
Materials and Methods
The aim of this study was to (i) determine the expression of aberrant p53 in OSMF and OSCC (ii) to study to study correlation if any between p53 expression and degree of dysplasia in OSMF and OSCC patients and (iii) to study correlation if any between p53 expression and habits in OSMF and OSCC patients

Sample size: The subjects included for the study were 30 patients with OSMF and 30 Oral Cancer patients irrespective of age and sex who visited to the Department of Oral Medicine and Radiology.

Collection of data: Data was collected regarding the habit history (chewing and non chewing habit). Diagnosis of OSMF and OSCC was made on the basis of characteristic clinical features.

Method: All subjects were subjected to biopsy procedure and specimens were fixed in 10% of neutral buffered formalin for 24-48 hours and embedded in paraffin wax and immunohistochemistry staining was performed on the collected tissue specimens.

LSAB plus visualization system (DAKO Corporation) was used for the immunohistochemistry. Formalin fixed paraffin embedded tissues were sectioned at 4μm and mounted on 3-Aminopropyltriethoxysilane (APES) coated slides. The slides were deparaffinized by heating on a slide warmer at 60°C for one hour and treated with two changes of xylene for 5 minutes each.

All slides were treated with change of 100% alcohol followed by graded alcohol 90%, 80% 70% for 5 minutes each. The slides were rinsed with distilled water followed by 2 changes of wash buffer in Phosphate buffer saline (PBS) (pH 7.2-7.67). Endogenous peroxidase activity was blocked by incubating the slides with 3% H2O2 for 5 minutes. Excess of H2O2 was wiped off and sections were rinsed with two changes of PBS for 5 minutes each.

Trypsin induced antigen retrieval was done by placing the rehydrated sections vertically in a rack suspended in the solution for 20 minutes and was gently stirred. Excess of trypsin was wiped off and sections were washed in buffer (PBS) two changes each. Slides were incubated with primary antibody for one hour in a humidifying chamber. Excess was wiped off and Slides were incubated with biotinylated link secondary antibody for 20 minutes in a humidifying chamber. Slides were incubated with DAB substrate chromogen for 10 minutes and then rinsed with distilled water. The sections were counterstained with Harri’s haematoxylin for 20 seconds followed by bluing in running tap water, cleared in xylene and mounted using DPX. The mounted sections were studied under research microscope for protein expression by a qualified pathologist. The expression of p53 staining was evaluated on the basis of presence or absence of brown staining within the nuclei and grading was given according to percentage of positive cells.

Fischer’s exact test was applied to analyze the contingency tables when the sample size is small, because the significance of deviation for a null hypothesis cannot be calculated exactly then relying on an approximation.

The protein expression was scored as follows:

1. (+++) When greater than 50% of cells staining positive.
2. (++) When 25% - 50% of cells staining positive.
3. (+) When 5% - 25% of cells staining positive.
4. (-) when < 5% of cells staining positive were considered.

Chi square test of significance was applied to show the association or relationship between two attributes i.e. p53 and OSMF, p53 and oral cancer. The Z test was also used for judging the significance of differences between means of two independent samples.

Results
A total of 30 cases each of OSCC and OSMF with mean age for OSMF patient (20-48 years) and for OSCC (38-70 years), were studied for the expression of p53 oncprotein. The p53 expression was positive in 15 (50%) out of 30 OSMF cases (Fig 1) and 24 (80%) out of 30 OSCC cases (Fig 2) (Table 1). Only 3 cases of OSCC showed ++ (25-50% positive stained cells) staining and 21 cases showed + (5-25% positive stained cells) staining and 6 cases were –ve (<5% positive stained cells), while all positive OSMF cases showed + (5-25% positive stained cells) staining (Table 2).
These results were statistically significant among these two attributes ($X^2 = 4.68 \, P< 0.05$). The comparison of p53 expression among OSMF and OSCC patients were highly significant ($Z$ test, $p1 = 0.4$, $p2 = 0.8$, $Z = 3.46 \, P< 0.01$).

**Figure 1:** Immunohistochemical staining of OSMF case showing positive for mutated p53 which is shown by brown nuclei

<table>
<thead>
<tr>
<th>Patients</th>
<th>p53 Positive</th>
<th>p53 Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSMF</td>
<td>15 (50%)</td>
<td>15 (50%)</td>
<td>30(100%)</td>
</tr>
<tr>
<td>CA</td>
<td>24 (80%)</td>
<td>6 (20%)</td>
<td>30(100%)</td>
</tr>
<tr>
<td>Total</td>
<td>39 (65%)</td>
<td>21 (35%)</td>
<td>60(100%)</td>
</tr>
</tbody>
</table>

**Table 1:** Comparison of P53 expression in OSMF and OSCC patients

<table>
<thead>
<tr>
<th>Cases</th>
<th>Chewing habits</th>
<th>Smoking habits</th>
<th>Alcohol consumption</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSMF</td>
<td>25 (83%)</td>
<td>4(13%)</td>
<td>1(3%)</td>
</tr>
<tr>
<td>OSCC</td>
<td>22 (73%)</td>
<td>6 (37%)</td>
<td>2(7%)</td>
</tr>
</tbody>
</table>

**Table 2:** Comparison of degree of dysplasia and p53 expression

Among 30 cases of OSMF 25 had chewing habit and, among 30 OSCC cases 22 had chewing habit. Four cases of OSMF and 6 of OSCC cases were with smoking habit. Out of each 30, 1 (3%) of OSMF and 2 (7%) of OSCC patients had habit of alcohol consumption. (Table 3)

<table>
<thead>
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<td>2(7%)</td>
</tr>
</tbody>
</table>

**Table 3:** Chewing and smoking habits in OSMF and OSCC cases

On application of Fischer’s exact test a statistically significant ($p<0.05$) relationship was seen between p53 expressions and chewing habit in OSMF, while statistical analysis in oral cancer was not significant ($P>0.05$) with chewing and also with smoking habits. (Table 4 and 5)

<table>
<thead>
<tr>
<th>Cases</th>
<th>P53 Positive</th>
<th>P53 Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chewing habits</td>
<td>15 (60%)</td>
<td>10 (40%)</td>
<td>25(100%)</td>
</tr>
<tr>
<td>Other habits</td>
<td>0</td>
<td>5 (100%)</td>
<td>5(100%)</td>
</tr>
<tr>
<td>Total</td>
<td>15(100%)</td>
<td>15(100%)</td>
<td>30(100%)</td>
</tr>
</tbody>
</table>

**Table 4:** P53 expression in OSMF patients with Chewing habit (Fisher’s Exact test, ($P<0.05$) Significant)

<table>
<thead>
<tr>
<th>Cases</th>
<th>P53 Positive</th>
<th>P53 Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chewing habits</td>
<td>19 (63%)</td>
<td>3 (10%)</td>
<td>22(100%)</td>
</tr>
<tr>
<td>Other habits</td>
<td>5 (17%)</td>
<td>3 (10%)</td>
<td>8(100%)</td>
</tr>
<tr>
<td>Total</td>
<td>24(80%)</td>
<td>6(20%)</td>
<td>30(100%)</td>
</tr>
</tbody>
</table>

**Table 5:** P53 expression in OSCC patients with chewing habits (Fisher’s Exact test, ($P>0.05$) Not Significant)

**Discussion**

OSCC ranks number one in men and number three among women in India. The sex difference could be a direct consequence of tobacco habits. The literature on the etiology of OSCC is voluminous, but few firm conclusions can be drawn except for the role of some form of tobacco usage.
The period of initiation of carcinogenic tobacco habits and the development of invasive Oral cancer, well defined oral precancerous lesions and conditions like Leukoplakia, OSMF and Erythroplakia exist.

OSMF is a condition unique in Indians and Pakistanis. The exact etiology is not known and hypotheses are abundant. The incidence of Oral cancer in OSMF is 7.6% for a medium 10 years follow up period. However, the significant roles played by oncogenes and tumor suppressor genes in the development of OSCC has been explored, little is known to the genetic events involved in the progression of cancer.

The gene most frequently mutated in human cancer is p53. In normal cells, wild type p53 protein has a very short half life (6-20 min) and is present in such small quantities that it cannot be detected by immunohistochemical methods. However, missense mutations in the p53 gene often result in a more stable gene product and prolong the half life of the p53 protein, causing it to accumulate within cell nuclei to the extent that it can be easily detected by means of immunohistochemistry.

To date, data on the involvement of the p53 in the pathogenesis of OSMF is lacking. The present study was thus conducted to determine the part played by p53 aberrations in the pathogenesis of OSMF and Oral cancer.

In present study peroxidase labeled streptavidin biotin technique and DO-7 anti p53 antibodies which recognize both wild and mutant form of p53 was used. A study conducted by Wylander K. et al had showed 64% positive nuclear staining for p53 using the DO-7 antibody which turned out to be most effective antibody for p53 detection.

In the present study 50% of the OSMF cases showed positive staining with p53 expression which is in accordance with the observation made by Prabhat et al and Kaur et al being 48%. However higher percentages was observed by Cox et al and Trivedi et al who showed in 75% of OSMF cases with p53 positive staining. Variation in techniques employed may account for these discrepancies. Delay in the placement of excised tissue into fixative may reduce antigen expression.

In the present study 80% Squamous cell carcinoma cases expressed p53 protein which is in accordance with the report of Langdon et al and Sakai et al (80%), Wong Y.K et al (77%), Kaur et al (75%), and Rajaram et al (70%).

However higher percentage (100%) and (94%) was observed by Allison R.T and Kerdpan et al. This higher percentage may be due to the detection of wild type p53 with the microwave antigen retrieval technique used by the author.

In OSCC out of 30 cases, 21 were graded with (+) positive stained and 3 were graded with (++) positive stained and remaining 6 cases were negative stained. The intensity of staining varied from cell to cell in the layer of epithelium. The p53 expression correlates well with increasing dysplasia or malignant transformation.

Twenty nine cases of OSMF patients were with habit of smoking and chewing, of which 15(60%) with only chewing habit showed positive results for p53 staining. The mechanism by which areca nut may induce fibrosis and perhaps p53 mutation remains unexplained. Difference in population groups and diversity of risk habits may contribute to the difference of p53 expression.

Among 28 cases of OSCC patients with habit of chewing and smoking, 24 (80%) showed positive results for p53 expression. The studies showing the p53 expression in premalignant lesions ranges from 17-67% and in Oral cancer the expression ranges from 11-75%. The reason for this wide range in expressibility of p53 protein stated by Reich et al, Diogene et al. and Ogden G.R are as follows:

- Variation in the etiological factors and ethnic background of the patients. Generally lower prevalence of p53 alteration in OSCC in patients from western societies has been observed.
- Variation in the immunohistochemical technique used also explains some of the differences.
- Tumors may have lost both the alleles of the p53 gene.
- Tumors may have a level of p53 protein that cannot be detected by IHC. Accumulation of p53 protein might reflect a persistent response to DNA-damage agents present in the areca nut and/or tobacco smoke.

Conclusion
In conclusion this study demonstrates a high incidence of p53 over expression in OSMF.
and Oral cancer. The results indicate that p53 over expression may play a role in the development of Oral squamous cell carcinoma and in pathogenesis of OSMF. Areca nut chewing and/or smoking in OSMF and Oral cancer cases may play a role in the p53 over expression.

The p53 protein detected by IHC may be one of the important tumor markers which may help not only in determining the prognosis but also has a key role in gene therapy in future.

However, long term follow-up studies would further help to know the significance of p53 in pathogenesis of OSMF and into its malignant transformation.

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