ORIGINAL RESEARCH PAPER

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P16 TUMOUR SUPPRESSOR PROTEIN EXPRESSION IN ORAL SUBMUCOUS FIBROSIS USING IMMUNOHISTOCHEMISTRY – A CLINICOPATHOLOGICAL STUDY

KEY WORDS: Oral cancer, oral submucous fibrosis, p16, immunuhistochemistry.

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ABSTRACT

Introduction: A lot of clinical and experimental research and epidemiological studies have been carried out in the field of oncology so far. Based on the current state of knowledge, these factors involved in carcinogenesis include a number of endogenous host factors and environments all malignancies. Epidemiological, molecular and statistical studies have suggested that between six and ten genetic events are required for head and neck cancer. These events are mainly genetic damage of proto-oncogenes, tumour suppressor genes, apoptotic genes and of those genes involved in DNA repair. Genetic abnormalities affecting Rb gene, p53 and p16 genes have been implicated in the early stages of head & neck squamous cell carcinoma. Hence this study was conducted to assess the p16 expression in oral submucous fibrosis ,a common potentially malignant disease in our population.

Methods: This study was conducted in the Department of Oral Medicine and Radiology, Dental College, Trivandrum, with the collaboration of the Division of Cancer Research, Regional Cancer Centre, Trivandrum, India .Patients were screened and those with clinical features suggestive of oral submucuous fibrosis were selected for the study and Informed consent was obtained from all the patients. Instituitional ethics committee clearance was also obtained for the study. An incision biopsy of sufficient width and depth to ensure inclusion of connective tissue was taken from the buccal mucosa. Of the sections cut from formalin fixed, paraffin embedded specimens, one was used for routine hematoxylin and eosin staining and the other was used for immunohistochemistry staining by Avidin and Biotin methods. The immunoreactivity was evaluated and the percentage of cells showing positive nucleus was determined.

Results: The immunoreactivity of OSF was high in all the specimens with a mean expression of 37.24+- 1.1. The expression of P16 protein did not show any statistically significant correlation with regard to age,and gender. When the habits of the patients were correlated with mean expression of p16,it was noted that the patients who practiced paan chewing either alone or in combination with smoking or alcoholism had a higher expression of p16 (34+_ 3.31).An increasing p16 expression was noted with the histologic grading in OSF , with the early lesions showing a p16 expression of 31.6+_2.54, moderately advanced lesion with a value of 37.77+_1.34 and advanced lesions giving a high p16 expression ,39+_2.However the results were not statistically significant.

Conclusion: Further study of multiple biomarkers would aid the screening and identifying patients with premalignant lesion who are at a higher risk of transition to malignancy and could be selected for chemoprevention and intervention strategies aimed at reducing the progression of premalignant disease and will provide a target for designing novel therapeutics in oral cancer.

INTRODUCTION

A lot of clinical and experimental research and epidemiological studies have been carried out in the field of oncology so far. All these have improved our understanding of the cause of cancer and mechanisms involved in transformation of a normal cell into a neoplastic cell.

It is widely known that no single factor is responsible for development of tumours. The role of some factors in production of neoplasia is established while that of others is still unknown. Based on the current state of knowledge, these factors involved in carcinogenesis include a number of endogenous host factors and environments all malignancies. It is generally accepted that carcinogenesis involves the progressive accumulation of genetic abnormalities. Epidemiological, molecular and statistical studies have suggested that between six and ten genetic events are required for head and neck cancer [1,2]. These events are mainly genetic damage of proto-oncogenes, tumour suppressor genes, apoptotic genes and of those genes involved in DNA repair.

Tumour suppressor gene is a gene whose protein product can inhibit the transformation of a normal cell, to a tumour cell and therefore, whose loss of function contribute to the malignant transformation of the cell. Genetic abnormalities affecting TSG like Rb gene, p53 and p16 genes have been implicated in the early stages of head & neck squamous cell carcinoma.

P16 is a G_1 – specific cell cycle regulatory gene. It is located on chromosome 9p21 which is a frequent site of allelic loss in many human malignancies [3]. The major biochemical effect of p16 is to halt cell-cycle progression. Loss of p16 function may lead to cancer progression by allowing unregulated cellular proliferation.p16 inactivation has been reported in many tumour types including oral cancer and oral premalignancies.

In India there is an unequivocal relationship between oral cancer and chewing tobacco. The chronic habit of betel quid chewing induces physical aberrations in oral mucosa, which may create unfavourable environment during wound healing. Exposure to the oncogenic constituents in tobacco and arecanut causes genotoxic insults at the site of application.

Oral submucous fibrosis, is a chronic debilitating disease, characterized by a generalized submucosal fibrosis. In an epidemiological study on oral cancer and precancerous lesions is a rural Indian population, the malignant transformation rate of OSF was 7.6% over a 17 year period [4,5,6]. The high incidence of oral cancer in Kerala is related to smoking, pan chewing and alcohol use [7]. The age adjusted incidence rates of 24.2 /100,000 for males and 11.2 /100,000 for females reported from Trivandrum city, are probably the_highest in the world[8]. Many studies have been reported on the expression of p53 tumour suppressor gene in oral submucous fibrosis. But little is known about the clinical and biological implications of the inactivation of p16 pathway in oral submucous fibrosis, a common premalignant condition.Hence this study was taken up to assess the expression of p16 protein in Oral submucuous fibrosis,its correlation with clinicopathologic parameters like paan chewing . The degree of p16 expression in the various histologic grading of OSF was also assessed .

MATERIALS AND METHODS.

This study was conducted in the Department of Oral Medicine and Radiology, Dental College, Trivandrum, with the collaboration of the Division of Cancer Research, Regional Cancer Centre, Trivandrum, India .Patients were screened and those with clinical features suggestive of oral submucuous fibrosis (Fig 1) and expressed their willingness to participate were selected for the study. Informed consent was obtained from all the patients. Instituitional ethics committee clearance was also obtained for the study.

Clinicopatholgic characteristics of patients
A total of 29 subjects were selected for the study and following assessment of routine blood and urine investigations, an incision biopsy of sufficient width and depth to ensure inclusion of connective tissue was taken from the buccal mucosa in each case under local anaesthesia.

The clinicopathologic characteristics of patients such as data on age, sex, occupation,deleterious habits like smoking, pan chewing and alcohol consumption were documented. The age of OSF patients included in the study ranged from 23-60 years, of which,13 patients were between 21-40years of age and 16 patients were above 40 years .A male predominance is noted in OSF group with 18(62.06%) males and 11(37.91%) females (M: F 1:0.61).Most of the patients were manual labourers A thorough history regarding the habits were taken and documented. The type, duration and frequency of habits were also noted. All the subjects had the habit of panchewing either alone or in combination with smoking and alcoholism. Considering the duration of chewing in patients with OSF, 27 patients had chewed for more than 2 years and used greater than five times per day. Two patients had developed OSF with less than 2 years of chewing A histopathological grading in Oral Submucous Fibrosis cases into three clearly definable stages –early, moderately advanced and advanced stages according to the criteria of Sirsat and Pindborg (1967) was done(Fig 2).Of the 29 cases of OSF ,5 cases were early ,13 moderately advanced and 11 were advanced cases

Immunohistochemistry
Of the sections cut from formalin fixed, paraffin embedded specimens, one was used for routine hematoxylin and eosin staining and the other was used for immunohistochemistry staining by Avidin and Biotin methods. For antigen unmasking, the deparaffinized slides were placed in a rack in boiling citrate buffer in a pressure cooker containing 10 mM citrate buffer (pH-6) for at least 5 min. The slides were allowed to cool in buffer for 20 min and were washed twice in phosphate buffered saline (PBS) and used for the standard staining procedure. Endogenous peroxidase activity was blocked by incubating slides for 30 min in 0.3% H2O2 in methanol, washed with excess water and stabilized with PBS. The sections were incubated with 3% bovine serum albumin in humidifi ed chamber for 30 min and removed. The sections were incubated with primary antibody p16 (F-12 Santa Cruz Biotechnology,CA) overnight at 4°C in the humid chamber. Washed extensively with PBS and then incubated with secondary biotinylated antibody for 30 min at 37°C. Washed in PBS thrice and incubated with Streptavidin Horse Radish Peroxidase reagent (Dako AS, Denmark) for 30 min. Washed extensively with PBS and substrate diaminobenzidine was added and incubated for 10 min in the dark, and a brown precipitate was formed. Washed with distilled water, and counter stained with hematoxylin for 1 min.The slides were hydrated in ascending order of alcohol ,cleared in xylene and permanently mounted in DPX. Utmost care was taken to avoid drying of slides at any point of time during thr entire staining procedure.

Evaluation of slides
The immunoreactivity was examined under light microscope (Leica-DMLB, Germany) (×400 magnifi cation). The percentage of cells showing positive nucleus was counted by 2 pathologists who were equally experienced and both were blinded.the sections were examined for the presence of a brown coloured end product at the site of target antigen.The immunoreactivity was evaluated and the percentage of cells showing positive nucleus was determined by scoring 100 cells in the field from 10 randomly selected fields. Percentage of p16 protein expression of less than 5% was considered as negative and more than 5% was considered positive.

Statistical analysis
The expression of p16 protein in the tissue samples was statistically analyzed using the Student's t-test. The results were expressed as mean ± standard error and $P < 0.05$ was considered to be statistically significant. The association between different variables was analyzed using Bivariate analysis (Spearman Rank Sum Test).

Results
In this study the mean expression of p16 was evaluated using immunhistochemistry. The percentage of p16 protein expression of less than 5 was considered as negative and more as positive.. The immunoreactivity of OSF was high in all the specimens with a mean expression of 37.24+- 1.1 (Fig 3).The expression of P16 protein did not show any statistically significant correlation with regard to age,and gender. When the habits of the patients were correlated with mean expression of p16,it was noted that the patients who practiced paan chewing either alone or in combination with smoking or alcoholism had a higher expression of p16 (34+_ 3.31).An increasing p16 expression was noted with the histologic grading in OSF , with the early lesions showing a p16 expression of 31.6+_2.54, moderately advanced lesion with a value of 37.77+_1.34 and advanced lesions giving a high p16 expression ,39+_2.However the results were not statistically significant.

DISCUSSION
Oral Submucous Fibrosis (OSF) is one of the most common oral precancerous condition. Pindborg et al and Maher et al , are of the opinion that, it has reached an epidemic level in India [5,9]. The exact etiology of Oral Submucous Fibrosis is still uncertain, though many factors have been thought to be causative agents. The various hypotheses put forward so far suggest a multi -factorial origin. Alongside the role of local irritants, such as capsaicin, tobacco, areca nut, systemic factors and immunological factors have been suggested as etiologic factors. Recent reports suggest an abnormal p53 tumour suppressor protein expression in Oral Submucous Fibrosis [10,11,12].

Reports suggest alterations of the p16 tumour suppressor gene to play a critical role in oral carcinogenesis .Only a study by Prishla and Balaram was obtained by extensive review of literature on p16 expression in OSF[13].

Hence, an attempt was made to assess the p16 protein expression in oral submucous fibrosis by immunohistochemistry.

A total of 29 OSF patients were included and their age ranged from 23 to 60 years with a mean age of 46.13 years. This is an agreement with that of Canniff Zet al and Bhonsle et al who reported an age range of 18-72 years[14,15].

The expression of p16 protein was analyzed by immunohisto chemistry (IHC) using mouse monoclonal antibody against amino acid 1 – 167 representing full length of p16 of human origin (F-12 Santa Cruz Biotechnology, CA). IHC has been identified as a straightforward method to detect p16 inactivation .In another study evaluating the immunohistochemical properties of commercially available antibodies ,Geradts et al found antibody G 175-405 to be the most specific[16]. Unfortunately, no comparative study on the specificity of F-12 antibody used in or study is available. The mean expression of p16 protein was evaluated .

All the 29 OSF cases included in the study were p16 positive, the mean expression being 37.24+/-1.1.Chen et al on an immunohistochemical study on oral premalignant lesion reported a progressively increasing degree of p16 positive nuclei in hyperkeratotic and dysplastic lesion when compared to normal tissues[17]. There is a dearth of studies on p16 expression in oral precancers especially in oral submucous fibrosis.

A positive correlation between the over expression of p16 in oral cancer and high-risk HPV types. Viral integration was suggested as a cause for the malfunctioning of this tumour suppressor protein leading to its over expression. Studies in tonsillar carcinoma by Klussman et al revealed positive correlation between increased p16 expression and HPV-DNA. Similar positive correlation was obtained in studies on genital and cervical cancers [18,19].

Zur Haussen have found HPV oncoproteins as a causative factor in p16 gene alterations [20]. Mechanism of inactivation of p16 tumour suppressor gene by HPV as suggested by Yamato et al and Llewellyn et al is due to binding of E7 protein of high risk HPV-DNA to Rb, leading to disruption of binding between Rb and E2F, allowing E2F to activate expression of products encoded by INK4a

gene[21,22]. The presence of HPV-DNA was not analyzed in this study. Further studies on the detection of HPV-DNA by PCR in these patients may explain the over expression of p16 observed in this study.

The up regulation of the INK4a gene, resulting in the over expression of p16 protein may develop through different mechanisms. Studies by Serrano et al , found an increased p16 protein levels in tumour cells lines and primary neoplasms that lack functional Rb[23]. Moreover p16 mediated inhibition of cell cycle progression seems to be dependent on functional Rb [24].

Pande et al evaluated the expression of p16 and pRb to study its role in oral tumourigenesis in Indian cohort and found accumulation of p16 in nearly all the pRb negative cases[25]. Thomas and Balan et al found a decreased expression of pRb in Oral Submucous Fibrosis compared to leukoplakia [26]. The loss of pRb may be a cause for the over expression of p16 noticed in this study. Further studies on immunohistochemistry to assess the pRb level in this sample to identify the functional pRb may explain the over expression in this study.

Alternatively, enhanced activation of the INK4a gene may occur via an indirect effect of E2F1 over expression. Over expression of cyclin D1 and /or of cdk4 have also been reported to influence p16 expression through a compensatory feedback loop[27,28] Further studies on the other proteins on the p16/pRb pathway like the E2F, cyclin D, and cdk4 may help to substantiate the findings of this study.

Another possibility for the difference of p16 expression found in this study could be attributed to the difference in the specificity of the p16 antibody used in this study. The one used in this study detects the whole length of p16. So a positive expression will be obtained if there is a mutation in the exon 1-3 region, which is mostly mutated as demonstrated in previous studies [29,30]. Further studies on detection and methylation analysis may reveal the mutations of p16 in the group studied.

When the p16 expression was assessed in relation to the histologic grading in OSF, it was found to increase from early to advanced stages. (32+/-2.54 and 39.09+/-2 respectively). However it was not statistically significant.

The high expression of p16 in patients with combined habits of Paan chewing, smoking and alcohol consumption indicates that p16 pathway may be altered in habit-related oral malignancies. Further long-term follow-up studies on p16 may provide unique opportunities to gain insight into the molecular events that drive the carcinogenic process

CONCLUSION

p16 alterations and its effect in oral pre malignancies, especially oral submucous fibrosis is a less explored territory in oral oncology. In this study a significantly higher expression of p16 was noted in Oral Submucous Fibrosis and in patients with OSF with cancer compared to normal mucosa. The possible explanation that can be given for this can be attributed to the disruption of binding between Rb and E2F, which is found to activate expression of products encoded by INK4a gene. Yet another mechanism that can be suggested is the increase of Rb gene mutation in oral cancer and precancers and its inverse relationship to p16 expression. Some authors have suggested an enhanced activation of INK4a gene as an indirect effect of E2F over expression. Similarly over expression of cyclinD1 /cdk4 have also been reported to influence p16 over expression through a compensatory feedback loop. It is a well-proven fact that oral carcinogenesis is a result of a plethora of genetic alterations. p16 activation is only one amongst those. Further long-term studies on these genetic alterations in a large cohort may help in arriving at a definite pathway in oral carcinogenesis.

In conclusion, the immunohistochemical study of multiple biomarkers would aid the screening and identifying patients with premalignant lesion who are at a higher risk of transition to malignancy and could be selected for chemoprevention and intervention strategies aimed at reducing the progression of premalignant disease and will provide a target for designing novel therapeutics in oral cancer.

Conflicts of interest

The authors disclose the absence of any conflicts of interest.

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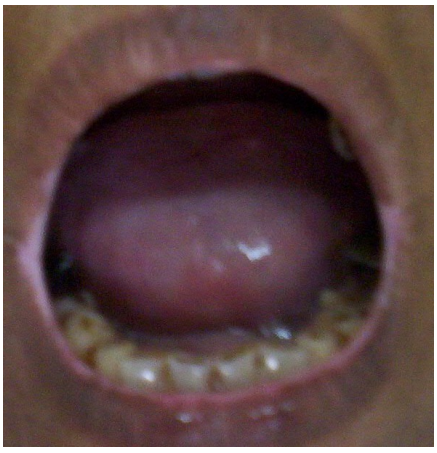

Fig 1- Photograph showing clinical signs of Oral Submucous fibrosis.

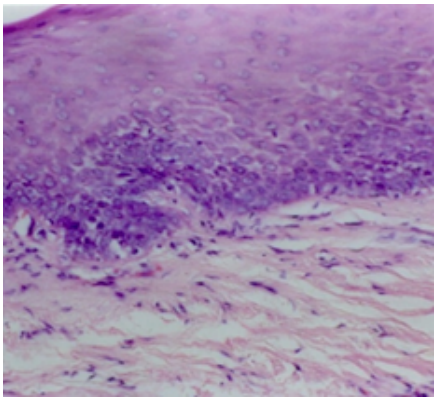

Fig 2 –Oral submucous fibrosis (H&E stain)

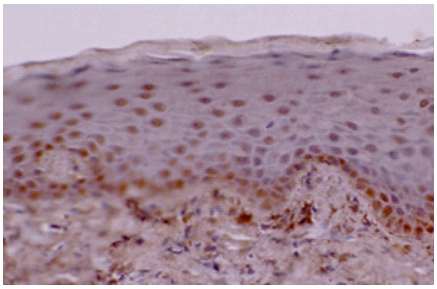

Fig 3.- Showing the increased p16 expression by immunohistochemistry.

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