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Assessment of p16 Tumour Suppressor Protein Expression In Oral Mucosa of Smokers And Paan Chewers using Immunohistochemistry.

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Introduction:*Environmental factors such as diet, alcohol use, stress, and environmental chemicals are known to elicit epigenetic changes, leading to increased rates of cancers and other diseases. Squamous cell carcinoma of the upper aero digestive tract typically occurs in older patients in their fifth to seventh decade of life and older. Commonly, there is a prolonged history of tobacco exposure and alcohol abuse. The risk of cancer development in this population is both dose‐ and time‐dependent. Less frequently, patients with little or no exposure to known risk factors develop squamous cell carcinoma of the oral cavity, pharynx, and larynx. The alteration of p16 occurs frequently in human oral cancers. 83% of oral cancer and 60% of the premalignant lesion shows the loss of p16 expression, suggesting that p16 alteration is an early event in oral cancer[1]Hence an attempt was made to determine a possible correlation between the p 16 expression and habits of smoking and chewing tobacco in patients attending a tertiary health care centre in south India.*

Methods: *This study was conducted in the Department of Oral Medicine and Radiology, Dental College, Trivandrum, for a period of one year with the collaboration of the Division of Cancer Research, Regional Cancer Centre, Trivandrum, India. Patientswith history of various deleterious habits were screened and details regarding the type, duration and frequency were noted.Patients with clinical features suggestive of oral submucuous fibrosis, and associated oral cancer were selected for the study. Normal subjects were selected from patients reporting for removal of third molars. 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 in OSF patients and from the respective sites in patients with concomitant malignancy. 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. The habit patterns practiced by the patients and the expression of p 16 in all the three groups were analysed..*

Results:*A total of 61 patients were selected for the study.There were 29 patients diagnosed clinically and histopathologically as having oral submucous fibrosis. Seven patients had oral squamous cell carcinoma.25 normalwere selected for the study of which 20 subjects did not have any habits and 5 were smokers. None of them were alcoholics or paan chewers. In the OSF group all patients had the habit of paanchewing ,either alone or in combination with smoking and alcoholism.When the habits of patients were analyzed, a higher p16 expression was found in patients who practiced betel quid chewing either alone or in combination with smoking and alcoholism, when compared to those without habits.*

Key words: *smoking,paan chewing, p16 tumour suppressor protein, immunohistochemistry.*

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I. Introduction

Environmental factors such as diet, alcohol use, stress, and environmental chemicals are known to elicit epigenetic changes, leading to increased rates of cancers and other diseases. Squamous cell carcinoma of the upper aero digestive tract typically occurs in older patients in their fifth to seventh decade of life and older. Commonly, there is a prolonged history of tobacco exposure and alcohol abuse. The risk of cancer development in this population is both dose and timedependent. Less frequently, patients with little or no exposure to known risk factors develop squamous cell carcinoma of the oral cavity, pharynx, and larynx. The alteration of p16 occurs frequently in human oral cancers. The loss of expression of p16 has been observed in oral premalignant lesions and primary tumors of the oralcavity . Mechanisms of inactivation include homozygous gene deletion

gene mutation, and hypermethylation of upstream CpG island regions. In many human cancers including oral cancers, loss of p16 is frequently observed. [2] 83% of oral cancer and 60% of the premalignant lesion shows the loss of p16 expression, suggesting that p16 alteration is an early event in oral cancer.Hence an attempt was made to determine a possible correlation between the p 16 expression and habits of smoking and chewing tobacco in patients attending a tertiary health care centre in south India.

II. Materials and methods.

This study was conducted in the Department of Oral Medicine and Radiology, Govt.Dental College, Trivandrum, with the collaboration of the Division of Cancer Research, Regional Cancer Centre, Trivandrum, India .Patients with history of various deleterious habits were screened and details regarding the type ,duration and frequency were noted. Patients who reported to the outpatient clinic of the Department of Oral Medicine and Radiology, Govt Dental College, Trivandrum were screened for a period of 1 year from March 2004 to March 2005, for features suggestive of Oral Submucous Fibrosis, and their clinical data were recorded in the specially prepared proforma. A total of 61 patients were selected for the study and grouped into three namely, Normal, OSF patients and OSF patients with malignancy. The patients with history of any systemic illnesses like hypertension and diabetes mellitus were excluded from the study. Informed consent was obtained from all the patients. The diagnosis of OSF and associated malignancy was based on clinical examination and histopathological analysis of tissue specimens.

Tissue specimens

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 representative area in each case under local anaesthesia. In patients with OSF, the biopsy was taken from the buccal mucosa and in those with concomitant malignant changes biopsies from both buccal mucosa and the lesion was taken. Biopsies included 25 cases of apparently normal oral mucosa, 29 Oral Submucous Fibrosis and 7 cases of malignancies arising in Oral Submucous Fibrosis. 8 OSF patients had associated leukoplakic lesions. The normal tissue was taken from the buccal mucosa of patients who reported for extraction of impacted third molar, after taking an informed consent. The clinicopathologic characteristics of patients such as data on age, sex, occupation, habits like smoking, pan chewing and alcohol consumption and family history were documented

Immunohistochemical analysis

Of the sections cut from formalin fixed paraffin embedded specimens, one was used for routine H and E staining and other was used for immunohistochemistry staining by Avidin and Biotin methods. Immunohistochemical (IHC) profile was assessed by subjecting one representative section from tumor block to p16 (F-12 Santa Cruz Biotechnology, CA). Immunohistochemistry was performed on 4 µm thick sections from 10% formalin‑fixed paraffin‑embedded specimens, according to the streptoavidin‑biotin immunoperoxidase technique. Positive and negative controls were run simultaneously. Strong brown nuclear immunoreactivity was considered as positive staining.

Evaluation of immunohistochemistry results

The staining intensity of tissue sections were evaluated under a microscope (Leica, Germany) under 400X magnification by two observers who were unaware of the clinical data and the mean of the two observations were recorded in each case. 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 as positive .

Statistical analysis

The expression of p16 protein in each of the various tissue samples were statistically analyzed using chi-square test . The results were expressed as mean +/- SE for each group and a p value of 0.05 or less was considered significant. Association between the different variables was analyzed by bivariate correlation analysis.

Observations

The age of subjects in the normal group ranged from 13-62years, of which 6 were below 20 years and 14 between 21-40 years and the rest above 40 years. 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. Of the seven OSF patients with malignancy four belonged to group greater than 40.

A thorough history regarding the habits were taken and documented. The type, duration and frequency of habits were also noted. The distribution of patients according to their habits are given in table 1. 21 (34%) patients in the study group had no deleterious habits. A greater percentage of patients had the habit of chewing only (36%), 57% patients had the habit of chewing either alone or in combination with smoking or alcoholism

Of the 27 females, 12 had no habits and 15 patients had the habit of chewing. None of them resorted to other deleterious habits like alcoholism and smoking. Of the 34 males, 9 had no habits and of the remaining 25 males, there was an almost equal distribution among the various habit groups with none of them resorting to alcoholism alone.Table 2shows the various habits with respect to the occupation.The tendencies to resort to various deleterious habits are seen to be more among men and most of them are manual labourers. Among the females, those who chewed were either labourers or homemakers. Table 3 classifies the patients within the three groups (Normal, OSF, OSF+Ca) according to the pattern of habits.Considering the normal group 20 (80%) subjects had no habits like smoking and paan chewing and 5 (20%) were smokers. None of them had the habit of alcoholism and chewing. In the OSF group, all except had the habit of chewing either alone or in combination. One patient with no habits exhibited features of OSF, which was confirmed by biopsy. His medical history revealed long-term use of ayurvedic medicine, which probably contained areca, the well-proven etiologic agent in Oral Sub Mucous Fibrosis.Table 4 shows the distribution of patients with OSF and OSFwith Ca according to their duration and frequency of chewing. Considering the duration of chewing in patients with OSF, majority chewed for more than 2 years and used greater than five timesperday. Two patients had developed OSF with less than 2 years of chewing. In the OSF with cancer group all the patients had chewed for more than 2 years and used more than 5 times per day. Table 5 shows the difference in chewing habits among the conventional and commercial pan chewers.

Evaluation of p 16 expression

Table 6 shows the p16 expression in the study samples with respect to the habit patterns practiced.When the habits of the patients were correlated with mean expression of p16, it was noted that the patients who practiced pan chewing either alone or in combination with smoking or alcohol had a higher expression of p16 (41.0+/-1.34) compared to those who did not have any deleterious habits(6.28+/-1.56). 5 patients who had only smoking habits showed a decreased p16 expression (2.80+/-0.96) when compared to those who practiced only chewing.

III. Discussion

A total of 61 cases were taken up for the study, of which 25(41%) were apparently normal cases, 29 (47.5%) OSF patients and 7 (11.5%) OSF with malignancy. The age of the OSF patients included in the study ranged from 23 to 60 years with a mean age of 46.13 years. This is an agreement with that of Canniffet al (1986) and Bhonsleet al (1987) who reported an age range of 18-72 years[3,4]. Regarding the occupation, 72% of the OSF patients were manual labourers belonging to low socioeconomic group. This finding is in conformity with that of Wahiet al (1966a) and that of Hashibeet al (2003) who reported a higher incidence of chewing tobacco, and other habits in individuals with low income in Kerala[5,6]The higher frequency of betel quid chewing with areca nut and tobacco as its components seen in this study is in concurrence with findings of Lal (1953) and Bhonsleet al (1987) in a study in Ernakulam district in Kerala[7,8]. Tobacco smoking and chewing are proven risk factors in oral carcinogenesis. Exposure to these risk factors may increase the predisposition for genetic and epigenetic alterations. The expression of p16 protein in the three groups were analyzed by immunohistochemistry (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 inactivation9.In another study evaluating the immunohistochemical properties of commercially available antibodies ,Geradtset al found antibody G 175-405 to be the most specific[10]. Unfortunately, no comparative study on the specificity of F-12 antibody used in or study is available. In a previous study on p16 expression in normal oral tissues, Prishla and PrabhaBalaram (2002) found that the expression of p16 ranged from 0-10 % with a mean expression of 4.6%. Taking this as the reference for this study, slides showing nuclear staining less than 5% was considered as negative and more than 5% was considered as p16 positive.

The mean expression of p16 protein in various groups were evaluated and various tissue samples were analyzed using the chi square test and a p value of 0.05 or less was considered statistically significant.

All the normal cases in this study were found to be negative for the expression of p16 protein.(fig.1) Contradicting this is the reports on a study in Indian population by Pandeet al (1998) which showed 93% of normal tissue to be p16 positive[11]. However, in their study, tissues showing 0% nuclear staining were considered as negative and the range of positivity being 1-10 %. However, they do report a weak immunoreactivity in normal oral epithelia, and in normal epithelia adjacent to cancers. The difference in the results may be due to different criteria for positivity selected in the two studies.

Shintianiet al have also reported a high p16expression in the studies on normal oral mucosa[12]. Low to undetectable p16 expression has been reported in many normal human tissues analyzed .Gonzalezet al, 1999; Yao et al, 1998 and Shapiro et al, 1995) have reported undetectable levels of p16 in normal human prostate tissue[13-15]. Based on these data, it can be assumed that these negative p16 expressions reflect basal physiological levels of p16 in human tissues.When the habits of patients were analyzed, a higher p16 expression was found in patients who practiced betel quid chewing either alone or in combination with smoking and alcoholism, when compared to those without habits (fig 2). This finding is in conformity to the findings of Lazariuset al (1998) who found an increase in p16 expression with betel quid chewing[16]. However, Pandeet al (1988) and Heizelet al (1996) reported a decreasing p16 expression in betel quid associated carcinogenesis[17,18] .It was of interest to note that a patient who had no habits exhibited features of OSF, which was confirmed by biopsy and had given history of prolonged ayurvedic medication containing areca nut products. His p16 expression was also high and comparable to those of OSF patients with chewing. (35+/-0). These indicate a positive correlation between areca nut usage and p16 alterations.

In the normal group 5 patients were smokers and there was a down regulation of p16 expression (2.80+/-0.96). However in the group with combination of smoking, chewing and/ or alcoholism, there was a considerable increase in p16 expression. (Chewing Smoking- 40+/-5,Chewing+ Smoking+ Alcoholism-34+\-3.31) suggesting a synergistic role of these habits in the expression of p16 protein. No statistical significance was observed between occupation and p16 status. Even though p16 expression in housewives and manual labourers were high (39+/-7.39 and 30.29+/-2.72 respectively), majority of them were chewers. Probably this high p16 expression reflected their habits and does not seem to have an association to their occupation. However further studies in a larger cohort are needed to determine the p16 alterations associated with various habits. Further long-term follow-up studies on p16 may provide unique opportunities to gain insight into the molecular events that drive the carcinogenic process.

IV. Conclusion

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.

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Habits	**No**.	**%**
Nil	21	34
Chewing only	22	36
Smoking only	5	8
Alcohol only	-	-
Chewing +Smoking	2	3
Chewing+Alchol	6	10
Smoking+Alchol	-	-
Chewing +Smoking+ Alcohol	5	8
Total	61	100

Table 1 –showing the various habit patterns practiced by the subjects

Sex	**Habits**	**Occupation**					
		Nil	**Labourers**	**Home makers**	**Business**	**Govt employee**	**Total**
Male	**Nil**	5	3	-	-	1	9
	Chewing only	-	5	-	2	-	7
	Smoking only	1	3	-	1	-	5
	Chewing+Smoking	-	1	-	1	-	2
	Chewing +Alcohol	1	4	-	1	-	6
	Chewing+smoking+ Alcohol	-	5	-	0	-	5
	Total	7	21	-	5	1	34
Female		7	2	1	-	2	12
	Chewing only	-	9	5	-	1	15
	Total	7	11	6	-	3	27

Table 2 –showing the occupation and the habit patterns practiced by the subjects.

Habits	Groups			
	Normal	OSF	OSF+Ca	Total
Nil	20	1	-	21
Chewing only	-	16	6	22
Smoking only	5	-	-	5
Chewing+Smoking	-	2	-	2
Chewing+Alcoholism	-	5	1	6
Chewing+Alcoholism+ smoking	-	5	-	5
Total	25	29	7	61

Table 3 -shows the habit patterns in the three study groups

	Duration of Chewing				Frequency of chewing/day			
Group	<2yrs	2-10yrs	>10yrs	Total	<5quids	5-10quids	>10quids	Total
OSF	2(7%)	13(45%)	14(48%)	29	2(7%)	18(62%)	9(31%)	29
OSF+Ca	-	3(43%)	4(57%)	7	-	5(71%)	2(29%)	7

Table 4. showing the duration and frequency of chewing practiced by the subjects in the two groups

	Habits	OSF	Ca	Total
Conventional	Chewing only	15	6	21
	Chewing+alchol	5	1	6
	Smok+chew+alch	4		4
Commercial	Chewing only	1		1
	Chewing+smoking	2		2
	Smok+chew+alchol	1		1

Table 5 –showing the different patterns of chewing practiced by the subjects

Habits	**No. of patients**	**Mean+/-SE (p16)**	**p value**		
			Vs Nil	**Vs Chew**	**Vs Smoking**

Nil **Normal** **OSF**	 20 1	 6.28+/-1.56 35.0+/-0	 NA NA	 NA NA	 NA NA
Chewing only	22	41.0+/-1.34	NA	NA	NA
Smoking only	5	2.80+/-0.96	NA	<0.009	NA
Chew+smok	2	40.0+/-5	<0.009	<0.825	<0.009
Chew+alcohol	6	35+/-1.82	< 0.009	<0.038	<0.009
Chew+smok+alcohol	5	34+/-3.31	< 0.009	<0.038	<0.009

Table 6 showing the mean p16 expression amongst the various

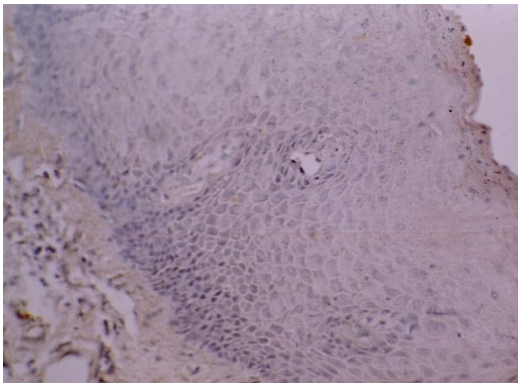

Figure 1 - p16 expression in normal oral mucosa

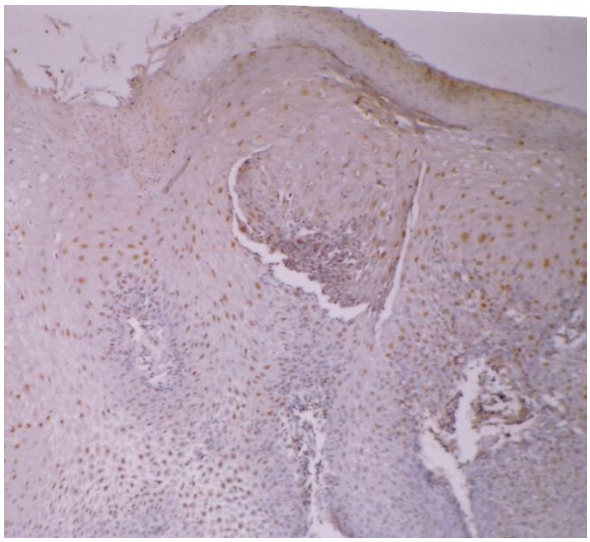

Figure 2-p16 expression in squamous cell carcinoma

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