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Expression OF p16INK4a Gene in Premalignant and Malignant Lesions of Oral Cavity

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Squamous cell carcinoma is the summits malignant neoplasm of the oral cavity. Tobacco and alcohol is identified as risk factors, but squamous cell carcinoma can occur in patients with no known risk factors. Oral cancer is the sixth most common malignancy and is one of the major causes of cancer morbidity and mortality worldwide. Cancer is caused due to a series of alteration in genetic and epigenetic factors that occur in multiple steps and is influenced by the genetic predisposition of the individual and by exogenous environmental factors. These factors result in a series of molecular alteration, including inactivation of tumor suppressor genes expression of p16 has been proposed as a marker for malignant transformation. The p16 staining was correlated between the control and study groups and p 16 was shown to be increasing expressed in premalignant and less expressed in malignant category and was found to be statistically significant by Fischer's exact test. This study concluded that p16 was increasingly expressed in premalignant lesions and less expressed in malignant lesion. In the present study 9 of the control cases were p16 negative and one case showed sporadic staining. The study group I showed 1 case of sporadic staining, 6 cases of focal staining and 8 cases of diffuse staining. The study II showed 14 cases of sporadic staining, 6 cases of focal staining and 5 cases of diffuse staining. Hence variations cannot be accurately assessed, but it plays a crucial role in assessing pre-malignant lesions progressing to malignancy. To confirm this, a larger sample study is required. As advances in research have leads to greater understanding of potentially malignant lesions in the oral cavity.

Keywords: Lesions; malignant; tobacco; chemical carcinogens; and genetic.

1. INTRODUCTION

Oral cancer ranks 6th among the most common cancer worldwide. It is constitut 5.5% of all malignancies. In India [1], 65,000 cases are diagnosed annually and 500, 000 new oral cancers are recorded worldwide [2]. Etiological tobacco usage and alcohol consumption as a major source of intra-oral carcinogens globally leading to oral & oropharyngeal cancers [3,4]. In India, the increased prevalence of oral cancer seems to be due to high exposure to sunlight due to farming, smoking, alcohol, spicy food, paan -masala and neglect overall oral hygiene [2]. Tobacco usage can also cause two clinically recognized precancerous lesion like leukoplakia and erythrop lakia. In India & several other South-East Asian countries, oral sub- mucous fibrosis associated with areca- nut chewing as precancerous conditions [5].

In India 19% of tobacco consumed in the form of cigarettes and other forms of tobacco usage is as bidi and chewable tobacco. Areca nut is used as naturally crude areca nut, betel quid or paan - parag. Other formulations are as supari, guthka (with tobacco) and paan masala [6]. Tobacco has been demonstrated in many states as carcinogenic, teratogenic and genotoxic [4]. Although oral cancers do occur in people who do not use tobacco too. The possibility thus existing is genetic susceptibility and inherent genetic alteration. Genetic susceptibility can be done to chemical carcinogens, oncogenic viruses and radiant cosmic energy [7].

Genetic susceptibility is multistep, complex process that includes initiation, promotion and progression. The most important and decisive event of the chemical carcinogenesis is between presumed carcinogens and macromolecules such as DNA, proteins and lipids [8]. These genetic damages result in tumors by disrupting the normal regulatory pathways that control basic cellular functions. Recently, i t is found that malignancy arises from accumulation of mutation in two major classes of genes, which are proto - oncogenes and tumour suppressor genes [9]. Tumor suppressor genes stop the growth of tumors by arresting cells in cell cycles [10]. The expressed product suppresses the expression or function of the other genes involved with cell growth and proliferation [11].

2. MATERIALS AND METHODS

Study Place: Department of Pathology, Sree Balaji Medical College and Hospital.

Study Design: The present cross-sectional study was a prospective study conducted in the Department of Pathology during the period of October 2015- August 2017. A total number of 50 cases of formalin fixed, paraffin embedded tissue samples from the oral cavity were collected.

Study Population: They were divided into control group and study group.

The control group consisted of 10 formalin fixed paraffin embedded samples of oral cavity, histopathologically diagnosed as hyperkeratosis/ chronic inflammatory pathology with age group from 26- 66 years of age. The study group consisted of 40 formalin fixed paraffin embedded samples which was histopathologically diagnosed as oral leukoplakia and oral squamous cell carcinoma with age group 30 -80 years of age. Relative clinical history was obtained.

The study group was further sub- grouped as

Group 1: 15 samples of histopathologically diagnosed, formalin - fixed, paraffin embedded tissues of oral leukoplakia.

Group 2: 25 samples of histopathologically diagnosed, formalin - fixed, paraffin embedded tissues of oral squamous cell carcinoma.

2.1 Inclusion Criteria

For control group: 10 formalin fixed paraffin embedded samples of oral cavity, histopathologically diagnosed as normal.For study group: 40 formalin fixed paraffin embedded samples which was histopathologically diagnosed as oral leukoplakia an d oral squamous cell carcinoma.

2.2 Exclusion Criteria

Oral biopsy specimen who is not received in formalin Biopsy specimen from patients who underwent chemotherapy or radiotherapy. Specimen with histopathological diagnosis other that premalignant and malignant conditions such as Lichen planus, Pemphigus, Pemphigoid etcetera.

Materials used:

1. Tissue sections prepared from paraffin embedded formalin fixed tissues
2. Haematoxylin and eosin staining kit
3. Pathnsitu p16(G175- 405) mouse monoclonal antibody kit
4. Positive control-Carcinoma cervix specimen
5. Negative control- Lipoma specimen

3. RESULTS

The present study was designed to determine the expression of p16 in oral premalignant and malignant lesions. A group of 40 histologically diagnosed formalin fixed, paraffin embedded oral biopsy/ surgical resection tissue samples were collected and were group into control group and study group. The control group consisted of 10 formalin fixed paraffin embedded tissue samples diagnosed as hyperkeratosis/ chronic inflammatory pathology. The study group included histologically diagnosed formalin fixed, paraffin embedded tissue samples oral biopsy/ surgical resection and are sub-divided into

- Oral Leukoplakia as Group I
- Oral Squamous Cell Carcinoma as Group II

Control group: Out of 10 samples, 5 were males and 5 were females.

Study group: In group I: Out of 15 samples, 12 were males and 3 were females. In group II: Out of 25 samples, 18 were males and 7 were females.

Table 1. Gender distribution among both Control & Study group.

Group	Sex		Total
	Male	Female	
Control	5 (50%)	5 (50%)	10
Study Group I	12 (80%)	3 (20%)	15
Study Group II	18 (72%)	7 (28%)	25
Total	35 (70%)	15 (30%)	50

Control group: The mean age among the control group is 44.8 years with a standard deviation of 12. 27 with a minimum age of 26 years and maximum age of 66 years. Study group: The mean age among the study group I (premalignant) is mean age was 40 .4 years with standard deviation of 6.89 with minimum age of 30 years and maximum age of 55 years. The mean age among the study group II (malignant) is mean age was 60.6 years with standard deviation of 9. 94 with minimum age of 40 years and maximum age of 80 years.

In the control group, 9 cases were negative for p16 whereas 1 case showed sporadic staining and in the study group I which included leukoplakia had no cases of negative staining, 1 case staining sporadic, 6 cases staining focally and 8 cases staining diffusely. The data are tabulated as Table 3.

In the control group, 9 cases were negative for p16 whereas 1 case showed sporadic staining and in the study group II which included oral squamous cell carcinoma had no cases of negative staining, 14 cases staining sporadic, 6 cases staining focally and 5 cases staining diffusely. The data are tabulated as Table 4.

Table 2. Age distribution among the Control and Study groups

Group	Observed	Mean (in years)	Standard Deviation	Minimum (in years)	Maximum (in years)
Control	10	44.8	12.27844	26	66
Study Group I	15	40.4	6.89734	30	55
Study Group II	25	60.6	9.947864	40	80

Table 3. Comparison of p16 between control group and study group I

Group	p16 expression				Total
	Negative	Sporadic	Focal	Diffuse	
Control	9	1	0	0	10
Study Group I	0	1	6	8	15
Total	9	2	6	8	25

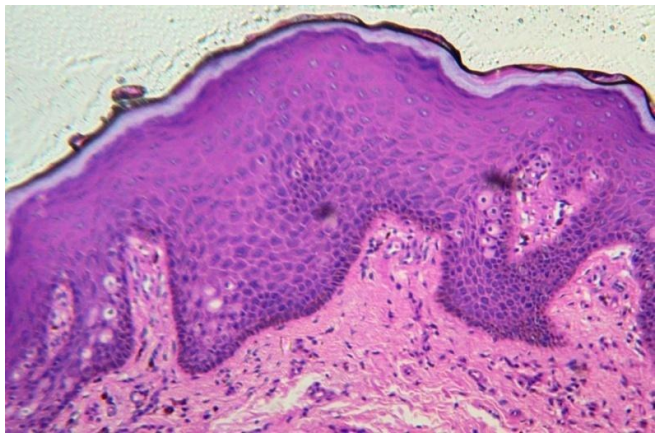

Fig. 1. Normal Oral Mucosa H&E stain 10x, magnification

Table 4. Comparison of p16 between control group and study group II

Group	p16 expression				Total
	Negative	Sporadic	Focal	Diffuse	
Control	9	1	0	0	10
Study Group II	0	14	6	5	25
Total	9	15	6	5	35

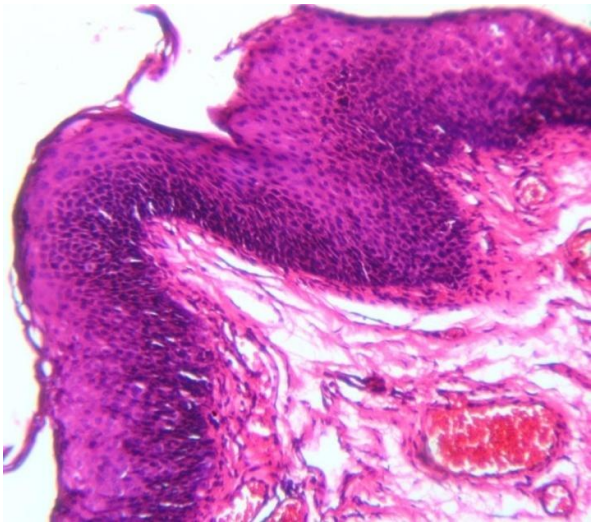

Fig. 2. Leukoplakia with severe dysplasia, H&E stain, 10x magnification

Using Fischer's exact test, the test of significance for both the study and control group were calculated and by using SPSS software version 23, the test of significance for two sided showed .002 which is statistically significant.

p16 immunohistochemistry stains both the nucleus and cytoplasm of the cells. It is seen as a brown stain in nuclear and cytoplasm involving all the layers of epithelium in study group I and sporadic staining was observed in most of the malignant lesions.

4. DISCUSSION

The gene expression of p16 INK4A is prominent in premalignant lesions where it acts as a cell cycle inhibitor by binding to CDK4/6 and prevents its interaction with cyclin D and thereby arresting cell proliferation and arresting carcinogenesis. In

my current study of expression of p16 in premalignant and malignant lesions of oral cavity, there is strong expression in premalignant category and weak expression in malignant category [12].

Papadimitrakopoulou et al. [13] in the year 1997, observed in a study of frequent inactivation of p16 in oral premalignant lesions which analysed 36 patients oral biopsies for p16 by immunohistochemistry. There is high expression of p16 in 19 cases and loss of p16 expression was found in 17 cases. Chen QM et al in the year 1999, studied p16 in oral premalignant lesion and oral squamous cell carcinoma. 74 samples were obtained with 10 hyperkeratosis, oral premalignant lesion (10 mild dysplasia, 10 moderate dysplasia and 10 severe dysplastic lesions) and 15 cases of oral squamous cell carcinoma. Hyperkeratosis and oral premalignant lesions showed high p16 compared to oral squamous cell carcinoma, which supports my case study.

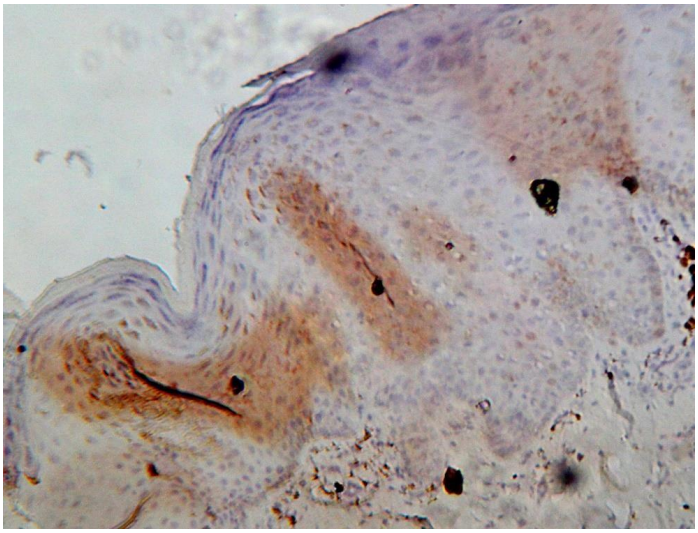

Fig. 3. Leukoplakia with mild dysplasia, p16 staining showing focal positivity, 10x magnification

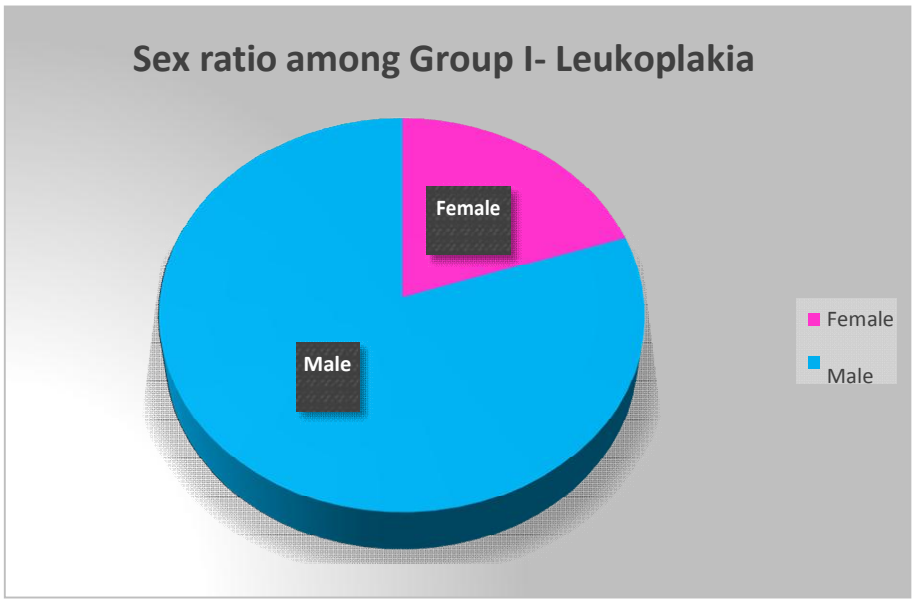

Fig. 4. Sex ratio among study group I- Leukoplakia

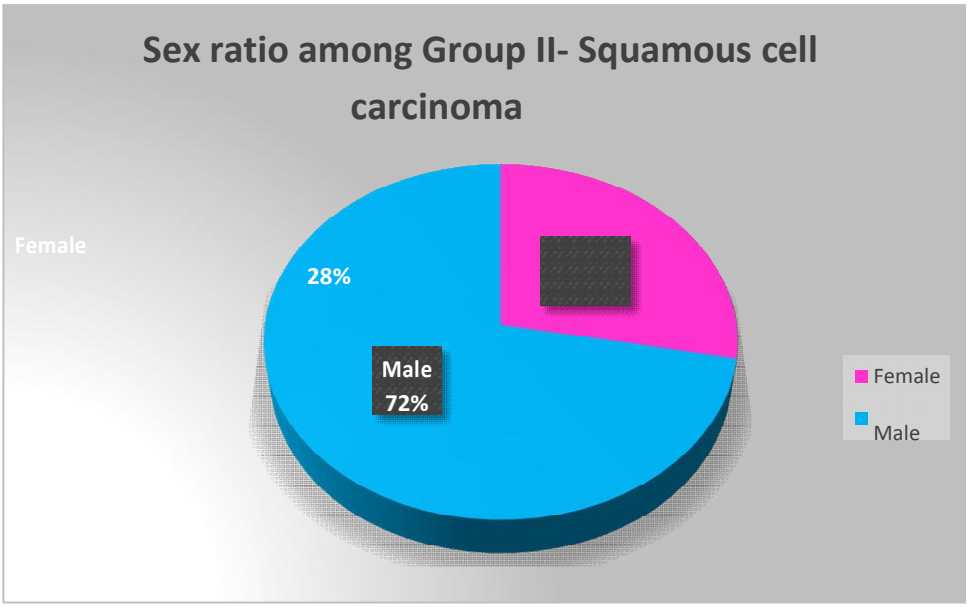

Fig. 5. Sex ratio among study group II- Squamous cell carcinoma

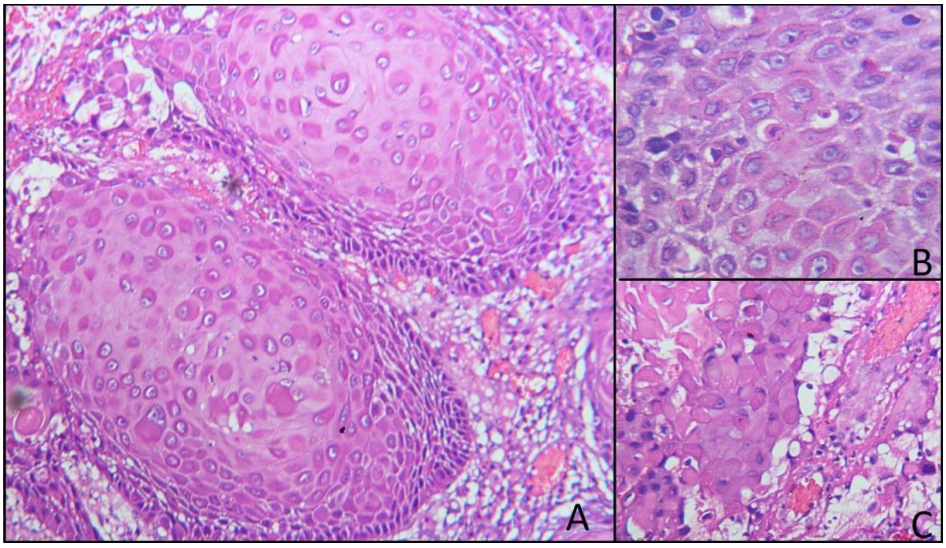

Fig. 6. Oral Squamous cell carcinoma, H&E stain, showing nests of squamous epithelium with inset showing mitotic figures and malignant squamous cells, A-10x magnification, B&C-40x magnification

Nakahara et al. [14] in the year 2000, studied alteration of Rb, P16INK4a and cyclin D1 in tumorigenesis of oral squamous cell carcinoma. In this study, 78 cases of oral squamous cell carcinoma, 46 cases of leukoplakia and 20 normal mucosa was included. p16 and Rb wa s observed in all normal mucosa & most of leukoplakia. Lack of p16 was observed in 67. 9% of squamous cell carcinoma similar to my current study. Kresty et al in the year 2002, studied the alternation of expression of p 16 in patients with oral epithelial dysplasia and normal oral mucosa with increased expression in oral epithelial dysplasia similar to the current study [15].

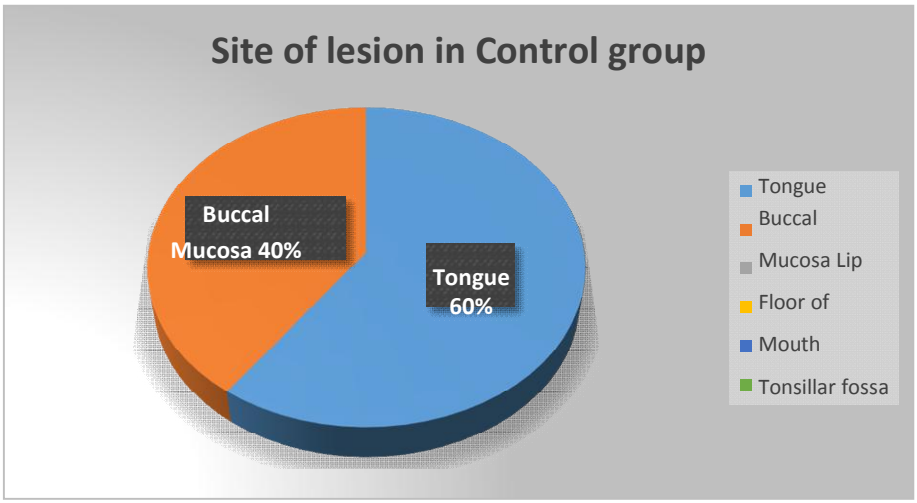

Fig. 7. Site of lesion in control group

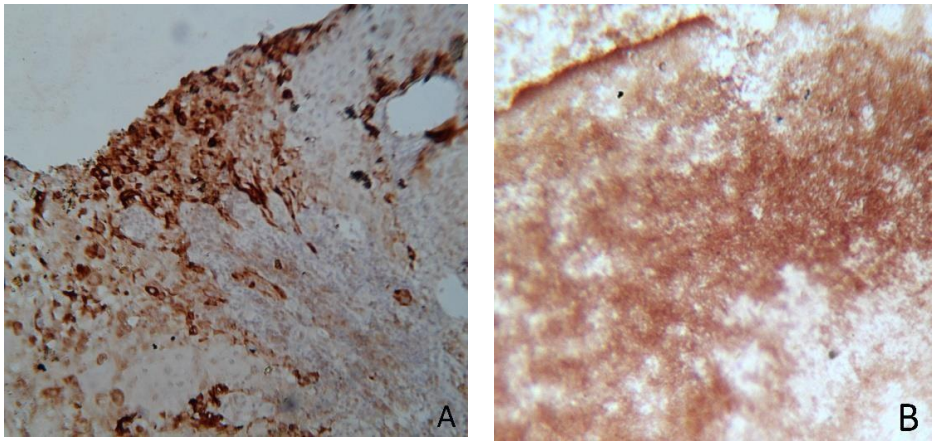

Fig. 8. A&B. Leukoplakia with severe dysplasia, p16 staining showing diffuse positivity, 10x magnification

Elefthenios et al in a study conducted at University of Athens Medical School in the year 2007, made p16 in oral squamous cell carcinoma chemically induced in Syrian Golden hamsters. P 16 was found increased in hyperplasia, sharply decreased in dysplasia and oral squamous cell carcinoma and hence found p16 useful in prognosis or oral cancer Dragomir et al in the year 2012 studied p53, p16, and Ki- 67 in oral squamous cell carcinoma. 34 cases with 11 cases of adjacent epithelial dysplastic lesions with increasing p16 staining intensity towards dysplastic lesions and less expressed in malignant lesions [16].

In another study by Angiero F et al. [17] in a study of expression of p16 in progression of epithelial dysplasia of oral cavity in the year 2008. He found in 54 biopsy specimen, 18 specimen were normal mucosa, 25 specimen were dysplastic, and 11 specimen were invasi ve carcinoma. p16 was negative in normal and no dysplasia and was increased in moderate to severe dysplasia. P16 was expressed in 54.5 % in invasive carcinomas which is also contradictory.

All controls were normal oral mucosa showed negative staining. He concluded telling the existence of a subset of malignant lesions staining p16 positive. Azizi et al. [18] in a study made at Department of Pathology, National University of Malaysia in the year 2016. The study made was expression of p53 and p16 with invasive t umour front of oral squamous cell. 28 cases were analysed, p16 was expressed in

92.8% cases of oral squamous cell carcinoma with 50% showing strong intensity of staining, while 80% of normal oral mucosa were positive with 60% showing strong intensity [19].

Expression of p16 INK4A is not seen in normal cell cycle. In case of carcinogenesis, p 16 INK4 a is expressed to act as a cell cycle inhibitor by binding to CDK4/ 6 and prevents its interaction with cyclin D and thereby arresting cell proliferation and arresting carcinogenesis. In premalignant lesions, p16INK4A is highly expressed limiting cell cycle and inhibiting the cells to turn malignant and so p16 is found to be high in the premalignant slides. In malignant lesion s, there is loss of p16 activity and hence there is a high turnover of cells without any checkpoint and hence is the reason for malignancy [20-25].

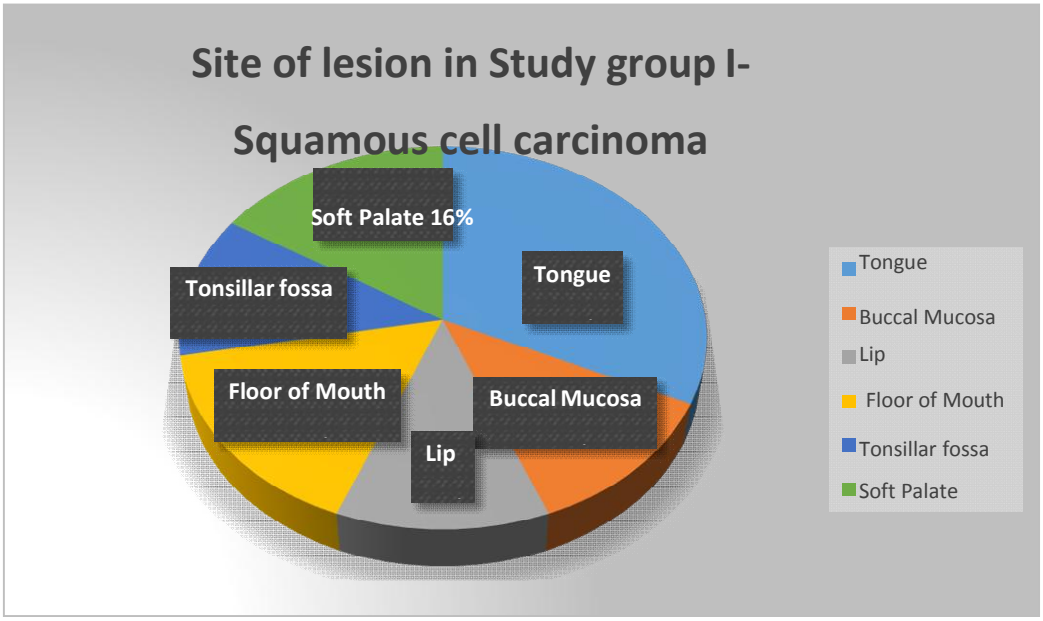

Fig. 9. Site of Lesion in Study group II- Squamous cell carcinoma

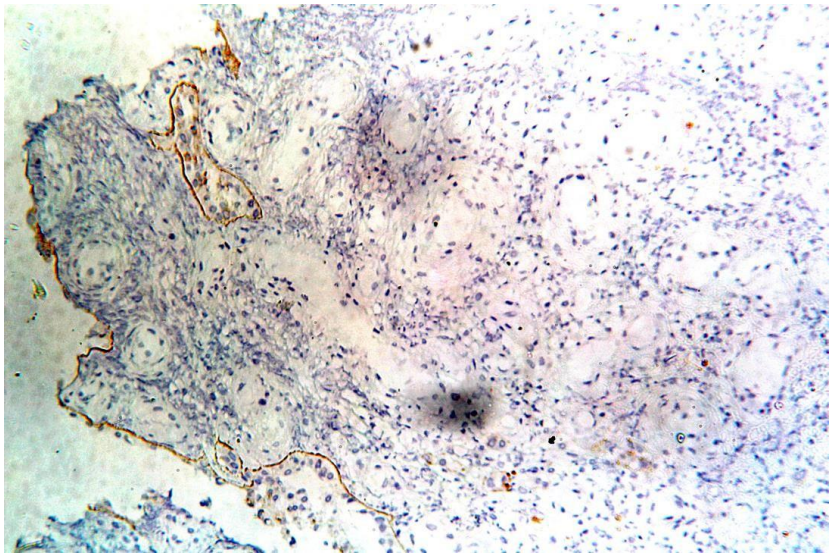

Fig. 10. Oral Squamous cell carcinoma, p16 staining showing sporadic positivity, 10x magnification

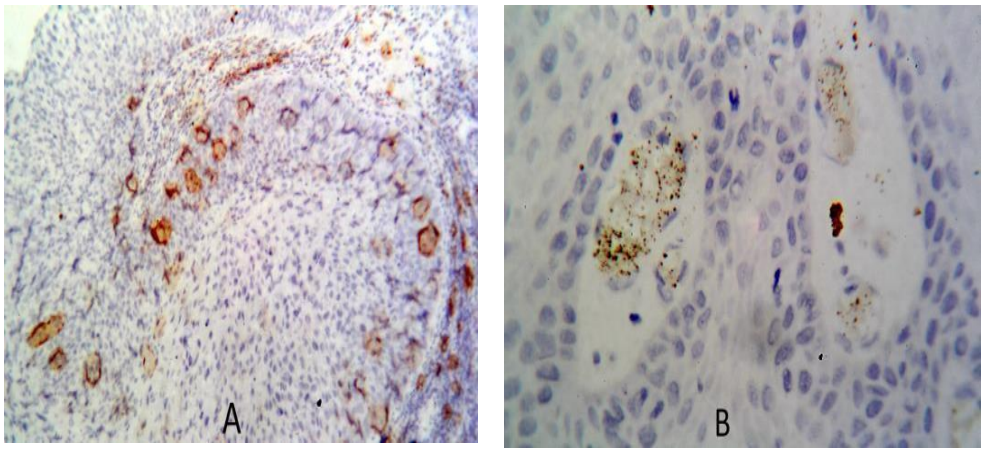

Fig. 11. Oral Squamous cell carcinoma, p16 staining showing sporadic positivity, A-10x magnification, B&C- 40x magnification

5. CONCLUSION

The current study supports this hypothesis by saying whenever an oral biopsy is taken, expression of p16 can help in categorizing malignant or premalignant and can also help with the prognosis of the condition. p16 may be considered as a tumour marker and can be used in conjunction with routine hematoxylin & eosin slides to help in determining the premalignant/ malignant nature and also the prognostic nature. But our sample study is small. Hence variations cannot be accurately assessed, but it plays a crucia l role in assessing pre-malignant lesions progressing to malignancy. To confirm this, a larger sample study is required.

CONSENT

As per international standard or university standard, patients' written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

Ethical clearance for the study was obtained from the Institutional Human Ethical Committee, Sbmch.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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