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**RESEARCH ARTICLE**

# THE ROLE AND SIGNIFICANCE OF IMMUNOHISTOCHEMICAL METHOD IN ORAL NEOPLASIA

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**ABSTRACT**

Oral cancer has been reported as the sixth most common malignancy in India. Survey conducted by National Institute of Public Health in 2011 has indicated 86 % of total oral cancers in world are contributed from India. Among many diagnostic methods of oral cancer detection, histological diagnosis acts as a powerful investigative tool providing important prognostic and predictive information relative to the disease status and biology. Malignant growth is acquired by the stepwise accumulation of defects in specific genes regulating cell growth. Two major pathways p53 and pRb deregulation is believed to result in tumor progression. Both pathways are mediated by $p16^{INK4A}$ .Owing to the higher predominance of cases observed at Basvatarakam Indo-American Cancer Hospital and Research Institute (BIACH & RI), Hyderabad present study focused on Immune histochemistry (IHC) method to identify importance and contribution of p16 as a molecular marker on clinical samples of Oral neoplasia. Grade of cancer can be useful for treatment plans and so the study was conducted to correlate levels of p16 expression in different grades of tumor. It can be inferred from our study that high p16 expression levels is associated with well differentiated OSCC (low grade), but however there existed no significant difference in the expression range for the different grades of tumors in regard to the percentage of expression with respect to the portions of tissue involved, hence suggesting that $p16^{INK4A}$ cannot be regarded as a significant prognostic tumor marker on the stand alone results of IHC.



## INTRODUCTION

Immunohistochemistry combines histological, immunological and biochemical techniques for the identification of specific tissue components. Cellular markers can be identified by means of specific antigen antibody reaction tagged with a visible label. The visualization is either by chromogenic detection (in which enzyme conjugated to antibody cleaves a substrate to provide a colored precipitate at the location of protein) or by fluorescent detection (in which a fluorophore is conjugated to antibody and can be visualized by fluorescence microscopy).

In present study we adapted chromogenic detection by indirect IHC, to know how $p16^{INK4A}$ expresses in different grades of Oral Squamous Cell Carcinoma (OSCC). Malignant growth is acquired by the stepwise accumulation of defects in specific genes regulating cell growth. p53 and pRb pathway deregulation is believed to result in tumor progression. Both pathways are mediated by $p16^{INK4A}$ (Pande P, *et al*,1998; Robles S, & Adami GR ,1998; Schloech ML ,1999. p16 primarily functions as a negative regulator of the prominent pRb-E2F pathway in cell cycle control. Binding of p16 gene product directly down regulates the activities of CDK4 and CDK6. This ensures pRb in a hypophosphorylated state consequently blocking cell cycle progression thus acting as a Tumor Suppressor Gene (TSG) ( Nagpal JK & Das BR, 2003). Genetic inactivation of the p16 gene either by deletion, promoter hypermethylation or point mutation has been found in nearly 50 percent of all human cancers .( Papadimatrakopulou V, *et al*, 1997; Reed AL *et al*,1997; Ai L, *et al* 2003) . The over expression of p16 at both m-RNA and protein levels has also been associated with prognosis for cancers. Based on this aspect, study has been conducted to show whether the p16 expression holds any characteristic significance alone to contribute as a marker for different tumor grades of OSCC.

## MATERIALS AND METHODS

### Tissue Specimens

Two hundred cases reported at BIACH & RI during 2011-2012 were considered. The cases selected were within a span of one year and irrespective of sex or age limitation. We had excluded cases which consisted of repeats, cases of No Evidence Malignancy (NEM), premalignant lesions like leukoplakia, erythroplakia and cases of unclear tissue profile. Hematoxylin and Eosin (H&E) stained tumor slides for 110 cases were taken for study. The paraffin embedded tissue blocks were obtained to perform IHC. Thin sections of 5µm were cut for performing IHC staining of $p16^{INK4A}$.

### Immunohistochemistry For $p16^{IN\ KA4A}$

The slides containing the sections were deparaffinised and hydrated by washing with xylene thrice followed by alcohol wash

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and rinsing under tap water .This was followed by antigen retrieval. Antigen retrieval was performed in citrate buffer at pH 6 using an electric pressure cooker for 60 seconds at 125 $^0$ C and followed by 120 seconds at 90$^0$ C with cooling to room temperature for 10-20 minutes before proceeding for immunostaining. The tissues were then incubated with 3% hydrogen peroxide for 5-10 minutes followed by washing the slides thrice with Tris Buffered Saline (TBS) for 3 minutes each. Appropriately characterized primary antibody p16 (mouse monoclonal IgG2a,Santa Cruz Biotechnology) was applied followed by incubation for one hour at room temperature .The slides were then washed thrice with TBS. Horse radish Peroxidase (HRP) conjugated anti rapid antibody was applied to each section .Then freshly prepared DAB (diamino benzidine) substrate was added and incubated until stain developed (DAKO Immunohistochemistry - manual) . Sections rinsed the with distilled water were stained with Hematoxylin for 30 seconds. The sections were washed with water followed by dehydration. Cover slips were mounted using permount mounting medium (DPX –Dibutyl Thalate Xylene) and the cases were then evaluated.

correlating with the percentage expression obtained during the first run of IHC.

The IHC expression for p16 was evaluated for different tumor grades of OSCC and classified according to nuclei and cytoplasm positive to negative .The expression was regarded **Negative:** indicating negative p16 expression ( 0-5% of nuclei and cytoplasm positive); **Sporadic:** indicating low expression **(** for 5-10% on N&C with weak and scattered positivity)**; Focal:** strongly positive, spreading in one tissue area indicating moderate expression; **(** for >10-30% of labeled nuclei and cytoplasm); **Diffuse:** indicating high expression( >30-85% of labeled cells with strong positivity spreading in several tissue areas ) (Klaes R, *et al* 2001) .

Out of 105 valid cases of Oral biopsy specimens (Table 2 and Figure 1) including OSCC, hyperplasia, dysplasia, verrucous carcinoma, 73/110 (66.34%) cases showed diffused pattern of expression relating to high p16 expression, 24/110 (21.81%) showed focal pattern indicating moderate expression for p16 and 3/110 (2.7%) showed sporadic pattern implying low expression of p16 and 5/110 (4.5%) showed negative expression.

**Table 1** General distribution of cases under study

| AGE/SITE | TONGUE | BUCCAL MUCOSA | FLOOR OF MOUTH | RMT | GBS | OTHERS |
|---|---|---|---|---|---|---|
| <30 | 05 | 02 | 02 | 00 | 00 | 01 |
| 30-50 | 22 | 25 | 00 | 04 | 03 | 06 |
| >50 | 18 | 10 | 00 | 01 | 00 | 06 |
| TOTAL | 45(42.85%) | 37(35.23%) | 02(1.9%) | 05(4.76%) | 03(2.85%) | 13(12.38%) |

*The study included oral biopsy specimens identified and grouped into three age groups and according to site of occurrence. The table illustrates cases categorized based on age and site of biopsy and shows predominant occurrence of tumor in the tongue (42.85%) and buccal mucosa (35.23%). The age group 30-50 recorded the highest number of cases overall.

These expression patterns measured involved full thickness and basal /suprabasal cell layers**.**

From Table 3 it was clear that 69 cases showed diffuse pattern

**Table 2** P16 $^{INK4A}$ Immunohistochemical expression in Oral biopsy cases.

| Grade/p16 Expression | Diffuse | Focal | Sporadic | Negative | Rejections |
|---|---|---|---|---|---|
| Well differentiated | 49(44.54%) | 14(12.72%) | 02(1%) | 03(2.7%) | 02(1%) |
| Moderately differentiated | 18(16.36%) | 07(6.3%) | 01(0.9%) | 02(1%) | 02(1%) |
| Poorly differentiated | 02(1.0%) | 00 | 00 | 00 | 01(0.9%) |
| Verrucous carcinoma | 02(1.0%) | 02(1.0%) | 00 | 00 | 00 |
| Moderate dysplasia | 01(0.9%) | 00 | 00 | 00 | 00 |
| Hyperplasia | 01(0.9%) | 01(0.9%) | 00 | 00 | 00 |
| Total | 73(66.34%) | 24(21.81%) | 03(2.7%) | 05(4.5%) | 05(4.5%) |

Illustrates the percentage levels of p16 expression for 105 cases including OSCC, premalignant lesions. The general figures depicts a higher percentage of cases with Diffuse positivity for p16 expression involving full thickness and basal suprabasal regions.

of p16 expression while only 21 cases were showing focal pattern irrespective of grade of OSCC.

## RESULTS AND DISCUSSIONS

Oncogenesis in oral cavity is widely believed to result from cumulative genetic alterations that cause a step wise transformation of the mucosa from normal to dysplastic to invasive carcinoma. The tumor suppressor gene p16 is localized on 9p Chromosome 21 locus and its inactivation is considered to be a significant event in development of many tumor types including oral carcinoma (Cairns P, 1995).

The cases were categorized based on age as below 30yrs, between 30-50yrs and above 50 yrs and site of carcinoma identified (Table 1). From the table, majority of oral carcinoma cases were located at Tongue (42.85%) and buccal mucosa (35.23%). The present data indicates high occurrence of oral carcinoma between the age group 30 to 50 years. This could be due to the habit of chewing *Ghutka, tambaku/Tobbaco.*

In total of 110 oral biopsy specimen cases were analysed, five cases were rejected because of poor staining, lifting of tissue sections and poor tissue profiles. Ten cases were subjected to repeated IHC to confirm the validity of expression profiles by

**Table 3** Levels of p16 expression with reference to grades of OSCC

| Grade /p16 Expression | Diffuse | Focal | Sporadic | Negative | Rejections |
|---|---|---|---|---|---|
| Well differentiated | 49 | 14 | 2 | 3 | 2 |
| Moderately differentiated | 18 | 7 | 1 | 2 | 2 |
| Poorly differentiated | 2 | 0 | 0 | 0 | 1 |
| Total | 69 | 21 | 3 | 5 | 5 |

*Sample size = 103; Obtained P value = 0.499 for df =9, Statistically significant value P=0.05.

The p16 $^{INK4A}$ IHC expression were analysed by the $\chi^2$ probability test, with P <0.05 being considered statistically significant. This test was used to test the null hypothesis that immunohistochemical expression is unrelated to the grade of OSCC. The results showed negative association between expression of p16 protein and grade of OSCC as the P value is 0.499 which was greater than P value 0.05.

Table illustrates the summary obtained by doing Anova on the

data obtained by categorizing levels of p16 expression into different age groups.

**Table 4** Levels of p16 expression relative to age

| Age/p16 expression | Diffuse | Focal | Sporadic | Negative |
|---|---|---|---|---|
| <30 | 9 | 1 | 0 | 0 |
| 30-50 | 43 | 12 | 0 | 3 |
| >50 | 24 | 8 | 3 | 2 |
| TOTAL | 76 | 21 | 3 | 5 |

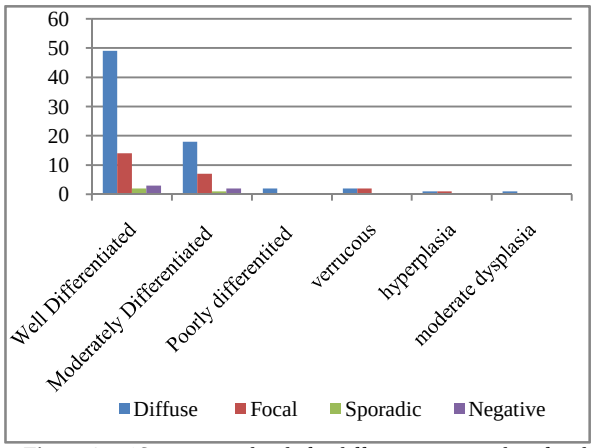


**Figure 1** p16 expression levels for different tumor grades of oral biopsies

Comparative levels of expression and distribution of the cases under study.

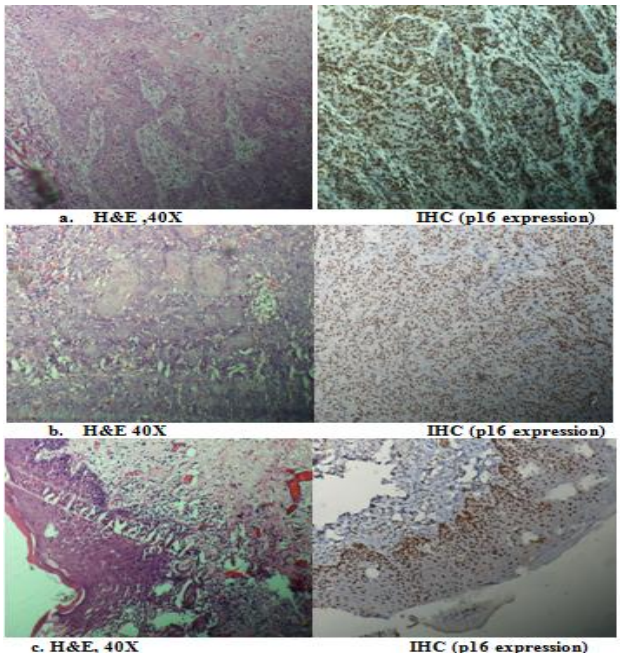


**Figure 2** H&E slides and relative p16 immunostained slides depicting p16 expression .

Figure a. Shows moderately differentiated OSCC and corresponding p16 expression full thickness 90%; Figure b. Depicts moderately differentiated OSCC full thickness 80% (tongue)Figure c. Shows moderately differentiated OSCC sowing 70% positivity in basal suprabasal region (tongue).

ANOVA (Analysis of Variance) test was performed between age and the levels of p16 expression to study the interrelation between them. The results obtained were summarized in Table 4 and 5 . From the ANOVA analysis, we found that for age ( $F_{cal}$ = 2.38 ) was less than ($F_{crit}$ =5.14) i.e. (p>0.05) for (2, 6) df, hence accepting the null hypothesis that there exist no significant difference between age groups.

**Table 5** ANOVA analysis

| ANOVA | | | | | | |
|---|---|---|---|---|---|---|
| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
| Rows | 289.5 | 2 | 144.75 | 2.387082 | 0.172704 | 5.143253 |
| Columns | 1164.917 | 3 | 388.3056 | 6.403573 | 0.026717 | 4.757063 |
| Error | 363.8333 | 6 | 60.63889 | | | |
| Total | 1818.25 | 11 | | | | |

For p16 levels of expression, the results showed $F_{cal}$ = 6.40 > $F_{crit}$ = 4.75 i.e. p<0.05 thus signifying that there is significant difference in the expression levels of p16 within an age group, rejecting the null hypothesis.

Immunohistochemical evaluation of oral premalignant and malignant lesions for p16 expression using an anti p16 antibody has given variable results with some studies showing decreased expression and others showing over expression.

Regardless of the mechanism involved, our findings suggest that p16 immunohistochemistry is not helpful in differentiating dysplastic from nondysplastic mucosa in oral cavity biopsies, and thus is not a reliable biomarker for use in routine clinical practice**.** Our observations were in accordance with the findings of Klaes R, *et al* 2001.

The regulation of p16 function is multifactorial. It integrates mechanisms that target the DNA, RNA, and protein levels through independent and overlapping pathways, some of which remain to be further explored. While the general role of p16 in tumor suppression is well-established, the specific contributions of p16 deregulation to the development of a particular tumor depend on the nature of the p16 deficiency and the coordination of other mediating molecular events occurring in the same tumor microenvironment. This complex orchestration of direct and indirect mechanisms of growth control derived from alterations of p16 function may best be addressed by a molecular assessment of the intricate roles of p16 in cancer progression.

## CONCLUSION

It can be inferred from the present data that high p16 expression levels is associated with well differentiated OSCC (low grade), but however there existed no significant difference in the expression range for the different grades of tumors. The percentage of expression with respect to the portions of tissue involved suggests that $p16^{INK4A}$ cannot be regarded as a significant prognostic tumor marker (p=0.499) on the stand alone results of IHC. Also there exists no relation between the levels of p16 expression among age groups, sex, gender and site of biopsy**.**

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