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# IMMUNOHISTOCHEMICAL LOCALIZATION OF P16 PROTEIN IN TONGUE SQUAMOUS CELL CARCINOMA

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## ABSTRACT
Tongue squamous cell carcinoma is the second most prevalent anatomic site of oral cancer in India which is associated with use of tobacco in various forms. p16INK4A a tumor suppressor gene and found inactivated by many mechanisms in cancer. This study role of p16 protein expression in tongue cancer patients. In this retrospective study, a total of 50 tongue cancer patients were enrolled. P16 protein was evaluated by immunohistochemistry on formalin fixed paraffin embedded tongue tumor tissues. Further, p16 protein expression was correlated with clinicopathological parameters and disease status. In this study, p16 expression was noted in 24% of tumor tissue of tongue cancer patients. In relation to clinical parameters, a significant higher p16 expression was seen in tobacco habituated patients. In relation with pathological parameters, a trend of higher p16 expression was noted in patients with T1 tumor size and poorly differentiated tumors. In univariate survival analysis, patients with p16 expression showed a trend of better diseases free survival and overall survival than patients with p16 negative expression. In summary, p16 overexpression can be considered as good prognosticator in tongue cancer. It may induce cellular senescence and thereby slower the tongue cancer growth.

## INTRODUCTION
Oral cancer is a major health problem in India and accounts for more than 30% of all cancers. Around 77,000 new cases and 52,000 deaths are reported annually, which is approximately one-fourth of global incidences [1]. Oral cancer occurs due to extensive exposure to various forms of tobacco like gutka, zarda, mawa, kharra, khaini, bidi, hookah, and alcohol. It arises from malignant lesions such as oral submucous fibrosis, leukoplakia, erythroplakia, or oral lichen planus. Ninety percent of the oral cancer are histopathologically squamous cell carcinomas (SCC). Tongue is the second most anatomic site after buccal mucosa in oral cavity cancer.

Inactivation of tumour suppressor genes and activation of oncogenes have been shown to play a role in the multi-step process of oral carcinogenesis. A tumor suppressor gene encodes a protein that acts to regulate cell division and when it is inactivated by a mutation cause loss of protein or encodes an abnormal protein. As a result, uncontrolled cell division may contribute to the development of a cancer. p16INK4A (p16) a tumor suppressor gene that negatively regulates the G1 phase of the cell cycle. It is an inhibitor of Cyclin D-CDK4 and CDK6 complex which prevents phosphorylation of retinoblastoma (Rb) protein and the release of E2F, subsequently leading to the inhibition of the transition from G1 to S phase in the cell cycle. Inactivation of p16 induces Rb protein phosphorylation, and cell cycle progression from the G1 to the S phase, resulting in DNA synthesis and abnormal cell proliferation. P16 inactivation occurs via homozygous deletions, methylation of promoter region, or point mutations [2]. P16 overexpression is also associated with HPV infection. HPV oncogenes E6 and E7 cause inactivation of pRB and lead to p16 overexpression. HPV positive tumors have been previously characterized by high expression for p16. p16 protein expression by immunohistochemistry used as a surrogate marker for HPV infection in both oropharyngeal as well as non-oropharyngeal squamous cell carcinomas [3]. HPV is considered as leading cause for oropharyngeal cancer in western countries.

To study the role of p16INK4A in tongue cancer, p16 protein is evaluated by immunohistochemistry on tongue tumor tissues and correlated with clinical and pathological parameters as well as diseases status.

## MATERIALS AND METHODS

### Patients

This retrospective study included 50 tongue cancer patients who had been diagnosed and treated at GCRI during 2015 to 2018 were included in the study. The detailed clinical history such as patient's age, tobacco habit, disease stage, histopathological findings, treatment offered and disease status was recorded from the case file maintained at the Institutional Medical Record Department. Formalin fixed paraffin embedded tumor tissue (FFPE) blocks were collected from Histopathology department of the institute. Disease staging was done according to UICC TNM classification. Disease status was assessed by clinical examination, radiological investigations and biochemical investigations. The study was approved by Institutional Scientific Review Board and Ethics Committee. Patients subjected to neo-adjuvant therapy (either Radiotherapy or Chemotherapy before surgery) were excluded from the study.

### Immunohistochemical localization

The 4µm thin sections were cut on microtome (Leica, Germany) and taken on 3-aminopropyl triethoxysilane (APES) coated slides. Immunohistochemical localization of p16 protein was performed on FFPE tissue blocks containing primary tumor and evaluated by Haematoxylene and Eosin (H&E) staining, on Ventana Benchmark XT autoimmunostainer using Ventana reagents (Ventana, USA). Briefly, the protocol includes following steps of deparaffinization using EZ solution, antigen retrieval using cell conditioning (CC1), incubation with ultra view DAB inhibitor for 4 minutes, 100µl of ready to use CINTEC p16 antibody (Company: Ventana), ultra view HRP multimer for 8 minutes, ultra view DAB detection kit for 8 minutes, counterstain with haematoxylin for 8 minutes, bluing reagent for 4 minutes and mounted with DPX.

### Scoring

Two individual observers scored the sections under microscope. Nuclear/Cytoplasmic staining pattern was noted for p16. For p16, scoring was done using the ASCO and CAP guidelines 2007 where immunoreactivity scored as 0 for negative (no cytoplasmic/nuclear staining), 1+ (faint or incomplete cytoplasmic/nuclear staining), 2+ (10-30% with cytoplasmic/nuclear staining) and 3+ (<30% tumour cells with complete cytoplasmic/nuclear staining). For statistical analysis p16 positive 2+ and 3+ were clubbed as positive.

## STATISTICAL ANALYSIS

Statistical analysis was carried out using SPSS statistical software version 20 (SPSS Inc, USA). Pearson's Chi-square test with Pearson's correlation coefficient (r) was used to assess correlation and significance between the two parameters. In case of patient number less than 5 in the cells of 2 x 2 tables, Yates' Continuity Correction value along with its significance was taken into consideration. Univariate survival analysis was carried out by Kaplan and Meier method and Log Rank statistics was used to assess the prognostic significance of disease free survival (DFS) and overall survival (OS). P values ≤ 0.05 were considered significant.

## RESULTS

### p16 expression

p16 protein encoded by tumour suppressor p16 gene. Cytoplasmic and nuclear p16 expression was noted in 24% (12/50) of tongue cancer patients (Figure 1), while p16 expression was noted negative in 76% (38/50) in tongue cancer patients (Figure 2).

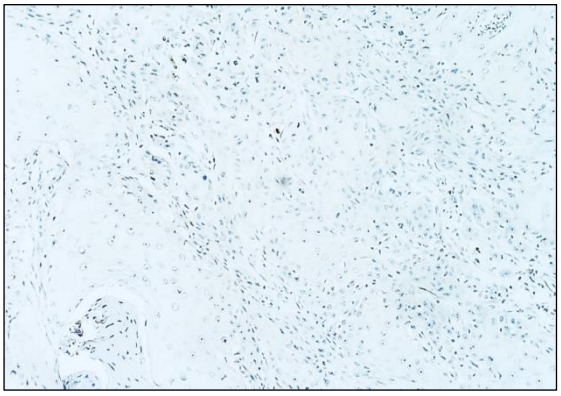

Figure 1: Negative expression of p16 in tongue cancer

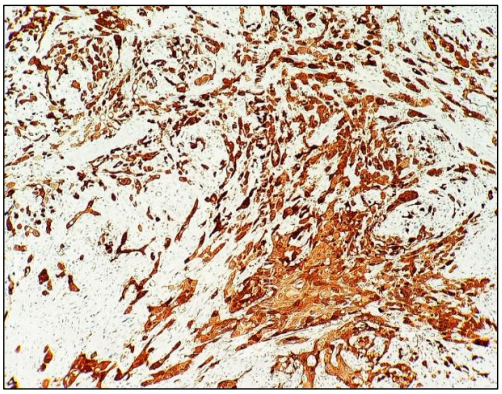

Figure 2: Cytoplasmic and nuclear expression of p16 in tongue cancer

**Correlation of p16 expression with clinical parameters**

Out of 50 patients, 54% (27/50) of the patients were ≤47years, whereas 46% (23/50) had >47years age. With respect to age, similar incidence of p16 expression was noted in patients with age ≤47 years (26%, 06/27) and with age >47 years (22%, 06/23; p=0.75). When patients categorized according to gender, 62% (31/50) patients were males, and 38% (19/50) were females and no difference in incidence of p16 expression was noted between males (26%, 05/31) and females (23%, 07/19; p=0.76) patients. With respect to habit, 48% (24/50) were non-habituated and 52% (26/50) were tobacco habituated. A trend of higher incidence of p16 expression was noted in patients with habituated (38%, 10/26) as compared to patients with non-habituated (08%, 02/24; p=0.01, Table 1)

Table 1: Correlation of p16 expression with clinical parameters

| Parameters | | p16 Expression | | $\chi^2$ | r | P value |
|---|---|---|---|---|---|---|
| | N (%) | Negative N (%) | Positive N (%) | | | |
| Age (years) | 50 (100) | 38 (76) | 12 (24) | 0.10 | -0.04 | 0.75 |
| ≤ 47 years | 27 (54) | 17 (74) | 06 (26) | | | |
| > 47 years | 23 (46) | 21 (78) | 06 (22) | | | |
| Gender | 50 (100) | | | 0.09 | -0.04 | 0.76 |
| Male | 31 (62) | 14 (74) | 05 (26) | | | |
| Female | 19 (38) | 24 (77) | 07 (23) | | | |
| Habit | 50 (100) | | | 0.697 | 0.165 | 0.01 |
| Non-Habituated | 24 (48) | 22 (92) | 02 (08) | | | |
| Habituated | 26 (52) | 16 (62) | 10 (38) | | | |

**Correlation of p16 expression with pathological parameters**

In relation with the size of tumor, 64% (32/50) patients had T2 tumor size followed by 32% (16/50) with T1, and 04% (02/50) with T3 tumor size. A trend of higher incidence of p16 expression was noted in patients with T1 tumor size (31%, 05/16) than T2 tumor size (23%, 07/32), however none of the patients with T3 tumor size exhibited p16 expression (00%, 00/02; p=0.42). In relation to lymph node status, 44% (22/50) patients had negative lymph nodes and 56% (28/50) patients had positive lymph nodes. A similar incidence of p16 expression was noted in patients with positive lymph node (25%, 07/25) and negative lymph node status (23%, 05/22; p=0.85). With respect to disease stage, 44% (22/50) had early stage (I+II) and 56% (28/50) patients had advanced stage disease (III+IV). A similar incidence of p16 expression was noted between early stage disease (23%, 05/22) and advanced stage disease (25%, 07/28; p=0.85). With respect to histological grade of the tumor, 28% (14/50), 48% (24/50) and 24% (12/50) were well differentiated, moderately differentiated and poorly differentiated tumors, respectively. A trend of higher incidence of p16 expression was noted in poorly differentiated (33%, 04/12) as compared to well differentiated (21%, 03/14) and moderately differentiated tumors (21%, 05/24: p=0.69). Perineural invasion (PNI) is the process of neoplastic invasion of nerves and is an under-recognized route of metastatic

spread. In 64% (32/50) of patients with perineural invasion and 36% (18/50) patients without perineural invasion, a similar incidence of p16 expression was noted (22%, 07/32 vs 28%, 05/18, p=0.63, Table 2).

Table 2: Correlation of p16 expression with pathological parameters

| Parameters | p16 Expression | | | | | |
|---|---|---|---|---|---|---|
| | N (%) | Negative N (%) | Positive N (%) | $\chi^2$ | r | P |
| Tumor size | 50 (100) | 38 (76) | 12 (24) | | | |
| T1(≤2cm) | 16 (32) | 11 (69) | 05 (31) | | | |
| T2(≥2cm-≤4cm) | 32 (64) | 23 (77) | 07 (23) | 1.73 | -0.17 | 0.42 |
| T3(≥4cm) | 02 (04) | 04 (100) | 00 (00) | | | |
| Lymph Node status | 50 (100) | | | | | |
| Negative | 22 (44) | 17 (77) | 05 (23) | 0.03 | -0.02 | 0.85 |
| Positive | 28 (56) | 21 (75) | 07 (25) | | | |
| Stage | 50 (100) | | | | | |
| Early stage (I+ II) | 22 (44) | 17 (77) | 05 (23) | | 0.02 | 0.85 |
| Advanced stage (III+IV) | 28 (56) | 21 (75) | 07 (25) | 0.03 | | |
| Histopathology | 50 (100) | | | | | |
| Squamous cell carcinoma | 50 (100) | 38 (76) | 12 (24) | | | |
| Histological Grade | 50(100) | | | | | |
| Well differentiated | 14 (28) | 11 (79) | 03 (21) | 0.75 | 0.09 | 0.69 |
| Moderately differentiated | 24 (48) | 19 (79) | 05 (21) | | | |
| Poorly differentiated | 12 (24) | 08 (67) | 04 (33) | | | |
| Perineural Invasion | 50 (100) | | | | | |
| Negative | 22 (44) | 17 (77) | 05 (23) | 0.22 | 0.06 | 0.63 |
| Positive | 18 (36) | 13 (72) | 05 (28) | | | |

**Univariate Survival Analysis**

**Disease Free Survival (DFS)**

According to Kaplan Meier univariate survival analysis, with respect to DFS, a trend of higher incidence of disease relapse was noted in p16 negative patients (16%, 16/38; 62.05 ± 4.1 months) as compared to p16 positive patients (02%, 01/12; 76.23 ± 4.6 months; Log rank= 1.163, df=1, p=0.28, Table 3, Figure 3).

Table 3: Univariate survival analysis for disease free survival

| p16 Expression | N (%) 50(100) | Remission N (%) | Relapsed N (%) |
|---|---|---|---|
| Negative | 38 | 30 (60) | 08 (16) |
| Positive | 12 | 11 (22) | 01 (02) |
| | | Log rank= 1.163,df=1, p=0.28 | |

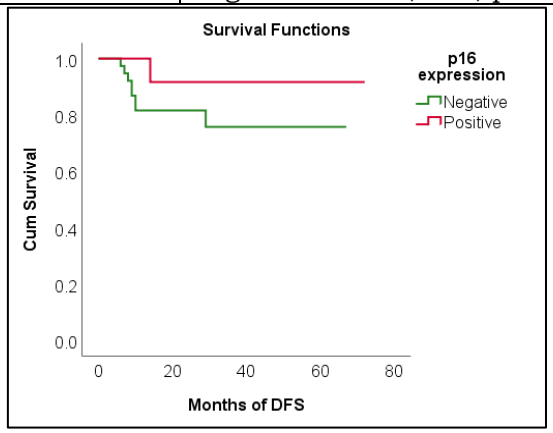


Figure 3: Kaplan-Meier survival analysis for Disease Free Survival (DFS)

**Overall Survival (OS)**

According to Kaplan Meier univariate survival analysis, with respect to OS, a trend of higher incidence of death was noted in p16 negative patients (06%, 03/38) whereas none of the p16 positive patients died during study period (00%, 00/12; Log rank= 1.163, df=1, p=0.28, Table 4, Figure 4).

Table 4: Univariate survival analysis for Overall Survival

| p16 Expression | N (%) 50(100) | Alive N (%) | Died N (%) |
|---|---|---|---|
| Negative | 38 | 35 (70) | 03 (06) |
| Positive | 12 | 12 (24) | 00 (00) |
| | | Log rank=1.163,df=1, p= 0.28 | |

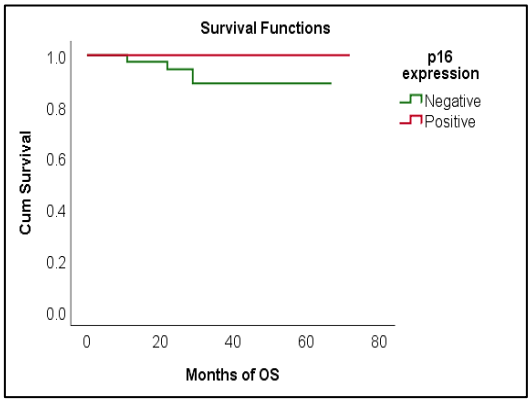


Figure 4: Kaplan- Meier survival analysis for Overall Survival

## DISCUSSION

p16 a cell cycle checkpoint regulator functions as a tumor suppressor, and alterations of the p16/pRB pathway may be implicated in betel nut- and tobacco-related oral tumorigenesis [4]. P16 is overexpressed in HPV-positive oral cancer too due to the degradation of pRb by the viral oncoproteins E6 and E7 and even several HPV-negative oral lesions gave moderate to high p16 expressions. In such cases p16INK4A act as a tumor suppressor cell cycle protein and its dysfunction may not be only HPV-related, other mechanisms and co-factors should be considered. p16 promoter methylation is a common genetic alteration in oral squamous cell carcinoma including tongue cancer. p16 hyper methylation is frequent in pre-cancerous oral lesions and lesions with hyper methylated p16 promoter tend to transform into oral cancers have been shown [5]. p16 is frequently mutated or homozygously deleted in several types of cancers Homozygous p16 deletions have been found in over 50% of gliomas [6], and mutations in p16 was found in several tumors, including esophageal, pancreatic, and non-small-cell lung cancer; lymphomas; and familial melanomas [7,8].

The present study evaluated p16 protein in tongue cancer and observed cytoplasmic and nuclear staining of p16 protein in 24% of tongue cancer patients. Many studies have noted p16 expression between 10% to 57% in oral cancer and oropharyngeal cancer [9-13]. P16 expression was also noted Oral Leukoplakias [14].

In relation to clinical parameters, similar p16 expression was noted in patients with ≤47 years of age and >47 years of age and between Gender. In accordance to our findings many studies showed no significant correlation between p16 and age [13-17]. Contrary to that two studies have shown significant higher incidence of p16 expression with age [18-19]. Many studies showed similar p16 expression in male and female groups [13,14,16-18, 20,21]. Further, significant higher expression of p16 was noted in tobacco habituated patients than non-habituated patients. In accordance, these studies have shown significant correlation of p16 expression with tobacco habit [14,15,19,20].

In relation to pathological parameters, a trend of higher p16 expression was noted in T1 tumor size and poorly differentiated tumors as compared to their respective counterparts. However, similar incidence of p16 expression was noted with lymph node status and disease stage. Contrary to our finding two studies noted similar incidence of p16 expression with tumour size [16,17] and one study showed significant correlation with lymph node status

[17]. Further, in accordance with our study significant association of p16 expression was not observed with lymph node [22], disease stage [13,19,23,24] and histological grade [13,15,16,18,22,25] With perineural invasion, present study and study of Aslan et al. [26] showed no significant correlation of p16 expression, whereas Hong-xue Meng et al. [19] showed significant association.

The disease free survival and overall survival analysis was carried out by Kaplan-Meier survival analysis. With respect to DFS and OS, trend of a higher incidence of disease relapse and death was noted in p16 negative patients as compared to p16 positive patients. Loss of p16INK4A expression predict early relapse and reduced survival in squamous cell carcinoma of the anterior tongue [24], oral cancer [9], and oropharyngeal cancer [19-21].

In summary, p16 overexpression can be considered as good prognosticator in tongue cancer. However, the underlying mechanisms for better prognosis in p16 positive tongue SCC is unclear. It may induce cellular senescence and thereby slower the tongue cancer growth.

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