Original Article

Assessment of immunohistochemical expression of p16 in head and neck squamous cell carcinoma and their correlation with clinicopathological parameters

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Abstract

Introduction: Oral squamous cell carcinoma is a major cause of death throughout the developed world. It is associated with tobacco chewing, paan chewing and alcohol consumption. Human papillomavirus (HPV) type 16 has also been suggested to play a role in the etiology of head-and-neck squamous cell carcinoma (HNSCC). p16 expression is now being used as a surrogate marker of HPV infection in squamous cell carcinoma.

Materials and Methods: In this cross-sectional observational study, a total of 100 cases of HNSCC were taken. p16 expression was determined by immunohistochemical (IHC) staining and correlated with clinicopathological parameters. The obtained results were analyzed and evaluated using Chi-square test, value of $P < 0.05$ was taken significant.

Results: P16 was positive in 60% of cases. A statistically significant direct association was observed between p16 with age, site of the tumor, abnormal sexual habits and lymph node involvement.

Conclusion: IHC expression of p16 can be used as a surrogate marker of HPV. Study of p16 expression may provide clinicians with more exact information in order to evaluate tumor aggressiveness, treatment modalities and can provide support for vaccination program in a high-risk group.

Keywords: Head-and-neck squamous cell carcinoma, human papillomavirus, immunohistochemistry, p16

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Submitted: 14-Jun-2020, **Revised:** 27-Oct-2020, **Accepted:** 07-Jan-2021, **Published:** 14-May-2021

INTRODUCTION

Head-and-neck cancer (HNC) is the tenth most common malignancy globally.[1] In India, it ranks among the top three types of cancer.[2] More than 90% of HNCs are squamous cell carcinomas (HNSCC). Others are quite uncommon types which include lymphoma and adenoid cystic carcinoma.[3] Squamous cell carcinoma of the upper aerodigestive tract typically occurs in older patients in their fifth to seventh decades of life and older. Commonly, there is a prolonged history of tobacco exposure and alcohol abuse.[4]

Access this article online

Quick Response Code:

Website: www.jomfp.in

DOI: 10.4103/jomfp.JOMFP_252_20

How to cite this article: Pandey P, Ralli M, Dixit A, Agarwal S, Chaturvedi V, Sawhney A, *et al*. Assessment of immunohistochemical expression of p16 in head and neck squamous cell carcinoma and their correlation with clinicopathological parameters. J Oral Maxillofac Pathol 2021;25:74-81.

According to the current literature, the risk factors of HNSCC are surprisingly similar to those of cervical cancer and cervical intraepithelial neoplasia, including the number of sexual partners, younger age at first sexual intercourse, practice of oral sex, history of genital warts and younger age.[5] Human papillomavirus (HPV) status is associated with p16 expression, and HPV-positive tumors are less likely to harbor p53 mutations.[6] The prognosis for patients with HNSCC is determined by the stage at presentation. Early-stage tumors are treated with surgery or radiotherapy and have a favorable prognosis.[7]

The loss of expression of p16 has been observed in oral premalignant lesions and primary tumors of the oral cavity. Mechanisms of inactivation include homozygous gene deletion, gene mutation and hypermethylation of upstream CpG island regions.[8] HPV-associated cancers are caused by the expression of HPV's E6 and E7 proteins that bind to and inactivate tumor suppressor proteins p53 and retinoblastoma protein respectively, leading to malignant transformation of HPV infected cells.[9] As with female genital (or cervical) carcinogenesis, the immunohistochemical detection of p16 protein (p16 IHC) has been proposed as a surrogate marker of HPV infection in HNC.[10]

Aim

The aim of this study was to study p16 expression in head-and-neck squamous cell carcinoma (HNSCC) on immunohistochemistry and correlate the expression of IHC markers with clinicopathological parameters.

MATERIALS AND METHODS

This was a cross-sectional observational study done in the Department of Pathology, in duration of January 2017 to June 2018. Hundred histologically diagnosed cases of HNSCC were studied. Patients with other than HNSCC such as adenocarcinoma, melanoma, sarcoma and metastasis were excluded. Data obtained were analyzed with other clinicopathological parameters including age and sex of the patient, history of tobacco use, paan chewing, alcohol abuse, abnormal sexual habits, site of lesion, grade of tumor and lymph node metastasis.

The tissue was fixed in buffered formalin (pH = 7.0) and embedded in paraffin. The tissue block was sectioned at 4–5 μm and the sections were stained for hematoxylin and eosin as per standard guidelines and examined. Histopathologic grading was done according to the World Health Organization criteria based on three parameters: (1) flattened polyhedral, round, or ovoid epithelial cells; (2) intracellular or extracellular keratinization and (3) intercellular bridges.[7]

- Grade I: Well differentiated
- Grade II: Moderately differentiated
- Grade III: Poorly differentiated or anaplastic.

IHC profile of the tumor was assessed by subjecting one representative section from tumor block to p16. Immunohistochemistry was performed on 4-μm thick sections from 10% formalin-fixed paraffin-embedded specimens, according to the streptavidin-biotin immunoperoxidase technique. Positive and negative controls were run simultaneously.

Interpretation of results

The IHC expression of p16 was classified according to nuclear and cytoplasmic positivity. These were scored as positive when more than 5% of cells (cutoff) stained positive. Biopsies with diffuse pattern (>30%–85% of labeled cells with strong positivity, spreading in several tissue areas) were considered to have high IHC expression of p16 (Grade III). Focal distribution (>10%–30% of labeled nuclei and cytoplasm strongly positive, spreading in one tissue area) was considered as moderate expression (Grade II) and sporadic positivity (5%–10% of nuclei and cytoplasm with weak and scattered positivity as low expression (Grade I).[11]

A descriptive study was carried out for all the variables included in the study. The whole data were entered in Microsoft Excel Master Sheet and analyzed using Statistical Package for the Social Sciences (SPSS) 15.0 IBM SPSS Statistics, India software. As the data were qualitative, Pearson's Chi-square test was used to assess the association between these parameters. $P < 0.05$ was taken as significant and <0.01 as highly significant; whereas $P > 0.05$ was taken as nonsignificant.

Observations

In this study age of the patients ranged from 17 to 92 years, with the mean age of 46.56 ± 14.40 years and most common age group was 31–40 years. The mean age of the males and females was 46.03 ± 14.33 years and 50.08 ± 14.91 years, respectively. Eighty-seven (87%) patients were male and 13 (13%) were female with a sex ratio of approximately 6.7:1.

Eighty-six (86%) patients were tobacco users, intake being in the form of gutka or smoking, 24 (24%) were alcoholics, 60 (60%) were paan chewers and 24 (24%) had history of abnormal sexual habits.

The most common site of involvement was the oral cavity (79%) [Figure 1] followed by the oropharynx (12%), hypopharynx (3%) and the larynx (2%).

Sixty-one (61%) cases were histologic Grade I [Figure 2], 35 (35%) were histologic Grade II [Figure 3] and 4 (4%) were histologic Grade III tumors [Figure 4]. In 26% of cases, lymphadenopathy was present.

P16

It was observed that 60 (60%) cases were positive for p16 (inclusive of all grades), while 40 (40%) cases were negative [Figure 5]. Out of 60% positive cases, 23 (23%) showed Grade 1 (low expression) [Figure 6], 18 (18%) showed Grade 2 (moderate expression) [Figure 7] and 19 (19%) showed Grade 3 (strong expression) [Figure 8] p16 expression.

Table 1 illustrates the correlation of p16 expression with various clinical parameters.

Out of 60 cases with p16 positivity, 43 (70.2%) were <50 years of age, 47 (78.33%) were males and 53 (88.33%) had cancer in the oral cavity.

The correlation of p16 expression with sex and site of the primary tumor was found to be statistically significant (P = 0.004 and 0.03 respectively), while the correlation of p16 expression with age was statistically insignificant (P = 0.224).

Table 2 illustrates the correlation of p16 expression with various risk factors.

Majority of our patients were tobacco users (86 patients), which 54 (62.79%) cases had positive p16 expression.

History of alcohol abuse was present in 24 patients, of which 16 (66.67%) had positive p16 expression.

Most of our patients were paan chewers (60 patients), of which 40 (66.67%) cases had positive p16 expression, but the correlation of p16 expression with tobacco abuse, alcohol abuse and paan chewing habit was statistically insignificant (P = 0.16, 0.44 and 0.09).

History of abnormal sexual habit was present in 24 patients, of which 19 (79.17%) were having positive p16 expression. The correlation of p16 expression with a history of abnormal sexual habit was found to be statistically significant (P = 0.02).

Table 3 illustrates the correlation of p16 expression with histologic grading.

The correlation of p16 expression with histologic grading was not found to be statistically significant (P = 0.36).

Table 4 illustrates the correlation of p16 expression with lymphadenopathy.

Of the 100 cases, in 74 patients lymphadenopathy was absent at the time of presentation and amongst them 50 (67.56%) cases showed positive grade p16 expression. The correlation of p16 expression with lymphadenopathy was found to be highly statistically significant (P = 0.009).

DISCUSSION

In the present study, grade 3 p16 expression was observed in 19 cases (19%), grade 2 in 18 cases (18%) and grade 1 in 23 cases (23%), while 40 cases (40%) were negative for p16 and scored grade 0. Our results were concordant with the study of Fregonesi *et al*., Yuen *et al*. and Ai *et al*., whereas wide variability and discordance was observed when the criteria for grading used were different.[11-13]

The age and sex distribution of present as well as previous studies indicates that the incidence of head-and-neck malignancies is higher in older age group and in males. This can be attributed to the

Table 1: Correlation of P16 expression with various clinical factors

Clinical parameters	P16 Grade 0 (n=40)	P16 Grade 1 (n=23)	P16 Grade 2 (n=18)	P16 Grade 3 (n=19)	Total cases (n=100)	Pearson's χ^2 (P)
Age (years)						1.48 (0.224)
≤50	24	17	12	14	67	
>50	16	6	6	5	33	
Sex						8.49 (0.004)*
Male	30	22	17	18	87	
Female	10	1	1	1	13	
Site						8.924 (0.03)*
Oral cavity	26	23	16	14	79	
Oropharynx	8	0	2	2	12	
Larynx	2	0	0	0	2	
Hypopharynx	2	0	0	1	3	
Face	2	0	0	2	4	

*Statistically significant

Table 2: Correlation of p16 expression with various risk factors

Risk factors	P16 Grade 0 (*n*=40)	P16 Grade 1 (*n*=23)	P16 Grade 2 (*n*=18)	P16 Grade 3 (*n*=19)	Total cases (*n*=100)	Pearson's χ^2 (*P*)
Tobacco chewing						1.993 (0.16)
Positive	32	20	17	17	86	
Negative	8	3	1	2	14	
Alcohol						0.9 (0.44)
Positive	8	5	5	6	24	
Negative	32	18	13	13	76	
Paan chewing						2.8 (0.09)
Present	20	13	12	15	60	
Negative	20	10	6	4	40	
Abnormal sexual habits						4.8 (0.02)*
Present	5	6	8	5	24	
Absent	35	17	10	14	76	

*Statistically significant

Table 3: Correlation of P16 expression with histological grading

Histological grading	P16 Grade 0 (*n*=40)	P16 Grade 1 (*n*=23)	P16 Grade 2 (*n*=18)	P16 Grade 3 (*n*=19)	Total cases (*n*=100)	Pearson's χ^2 (*P*)
I (WDSCC)	28	15	8	10	61	2.25 (0.36)
II (MDSCC)	11	7	10	7	35	
III (PDSCC)	1	1	0	2	4	

WDSCC: Well-differentiated squamous cell carcinoma, MDSCC: Moderately differentiated squamous-cell carcinoma, PDSCC: Poorly differentiated squamous cell carcinoma

Table 4: Correlation of p16 expression with lymphadenopathy

Lymphadenopathy	P16 Grade 0 (*n*=40)	P16 Grade 1 (*n*=23)	P16 Grade 2 (*n*=18)	P16 Grade 3 (*n*=19)	Total cases (*n*=100)	Pearson's χ^2 (*P*)
Present	16	4	2	4	26	6.79 (0.009)*
Absent	24	19	16	15	74	

*Statistically significant

Figure 1: Patient with fungating growth on the right lateral border of the tongue

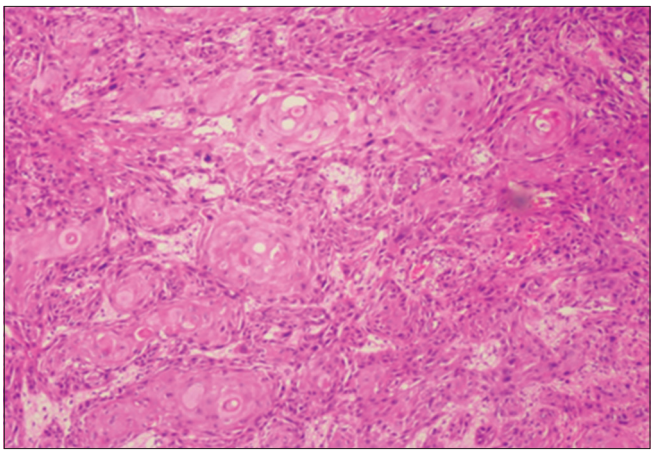

Figure 2: Well-differentiated squamous cell carcinoma showing keratin pearls and intracytoplasmic keratinization (H&E, ×100)

habit of tobacco chewing or smoking or alcohol consumption being more common among males in our part of the world which play an important role in the etiopathogenesis of HNSCC. The age and sex distribution in our study was in concordance with the study conducted by Yuen *et al.* and Ralli *et al.*[12,14]

In our study, significant association was seen between p16 expression and sex of the patient ($P = 0.004$), while in study by Shinohara *et al.*, there was no significant correlation between p16 expression and sex ($P = 0.18$).[15]

No significant association was seen between p16 expression and age ($P = 0.224$) in our study, which was in concordance with other studies like Yuen *et al.*, Rischin *et al.* and Dragomir *et al.*[12,16,17] while in study by Shinohara *et al.*, there was a significant correlation between p16 expression and age ($P = 0.03$).[15]

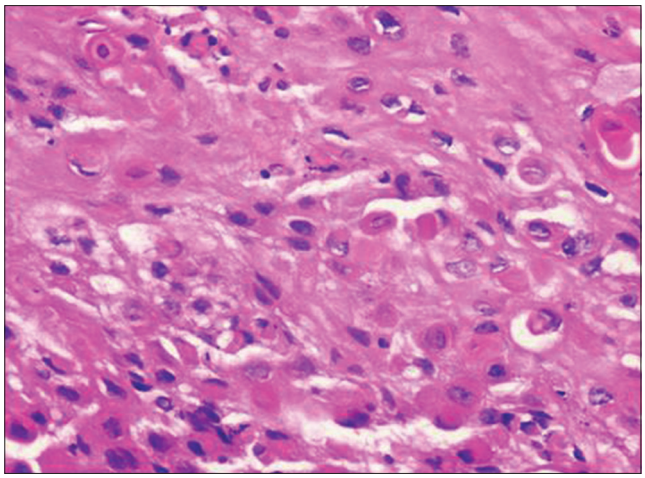

Figure 3: Moderately differentiated squamous cell carcinoma showing intracytoplasmic keratinization and moderate nuclear pleomorphism (H&E, ×400)

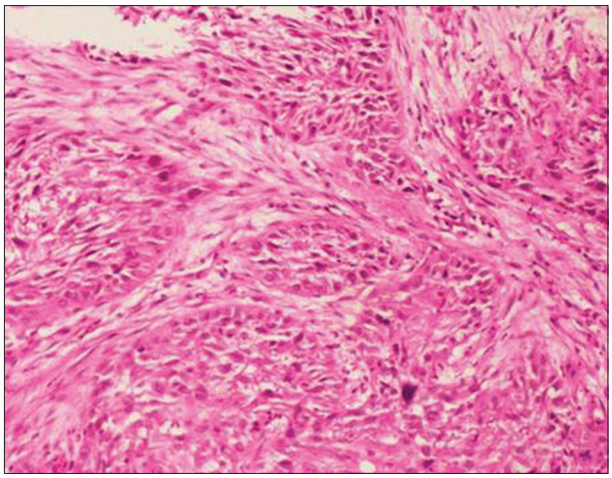

Figure 4: Poorly differentiated squamous cell carcinoma showing clusters of highly pleomorphic tumor cells with hyperchromatic nucleus and fair number of mitotic figures (H&E, ×200)

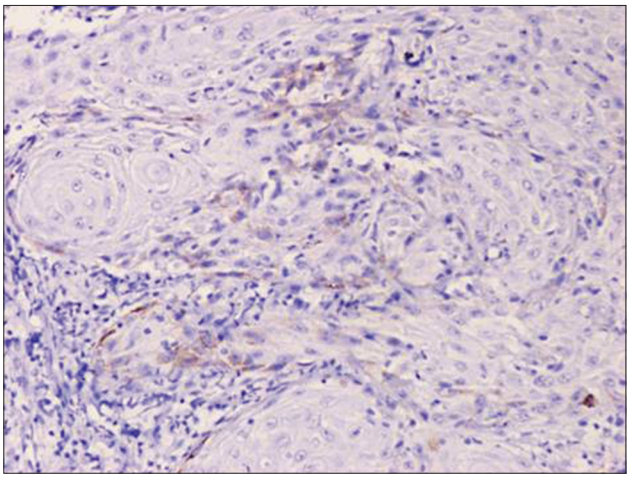

Figure 5: IHC p16 – Score 0, 0%–5% of nuclei and cytoplasm positive (IHC, ×200)

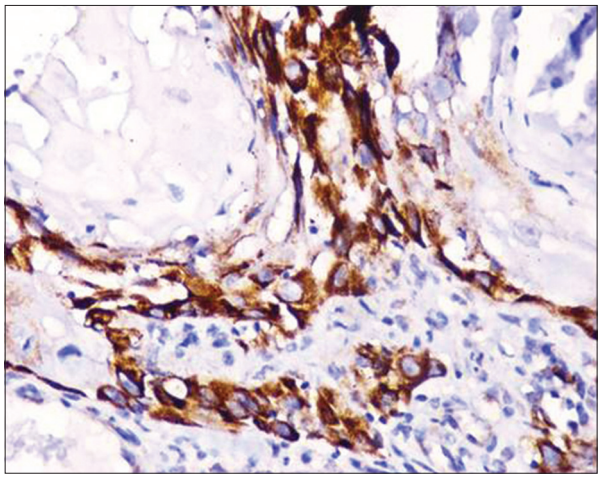

Figure 6: Immunohistochemical p16 – Score 1, Sporadic (5%–10% of nuclei and cytoplasm with weak and scattered positivity) (IHC, ×400)

The commonest site of involvement in our study was the oral cavity (79% cases). The second most common site was the oropharynx (12% cases), followed by hypopharynx (3% cases) and larynx (2% cases). A similar distribution of tumors was seen in the study by Boslooper *et al*.,[18] while the larynx was the most common site of involvement in most of the studies.[19,20] The difference in site involved could be because of different geographical distribution and occurance of risk factors.

A significant association was seen between p16 and tumor site ($P = 0.03$) which was in concordance with the study of Yuen *et al*. and Singhi and Westra who observed that p16 expression significantly varies with tumor site,[12,21] while discordant with a study by Shinohara *et al*. and Smith *et al*.[15,22]

Most of the cases included in the present study were tobacco chewers (86%), paan chewers (60%), nonalcoholic (76%) and without multiple sexual partners (76%). Majority of the tobacco users, paan users and alcohol consumers were males. According to Ralli *et al*., HNSCC was associated more with paan chewing and was more in nonalcoholics.[14] One hundred cases of head-and-neck squamous cell cancers were analyzed for expression of p16 which showed no statistically significant association between p16 expression and tobacco chewing ($P = 0.16$), alcohol consumption ($P = 0.44$) and paan chewing ($P = 0.09$).

Similar to our study, Ralli *et al*. and Lazarus *et al*. found no statistically significant association between p16 expression and tobacco use.[14,23] Similarly, a study conducted Ralli *et al*. and Dragomir *et al*. found no significant association between p16 expression and alcohol consumption.[14,17] In contrast, Smith *et al*. found a statistically significant association of p16 expression with alcohol consumption

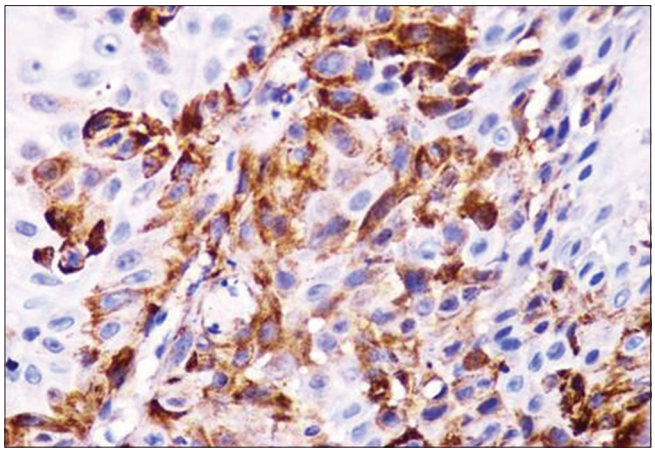

Figure 7: Immunohistochemical p16 – Score 2, focal (>10%–30% of nuclei and cytoplasm strongly positive, spreading in one tissue area) (IHC, ×400)

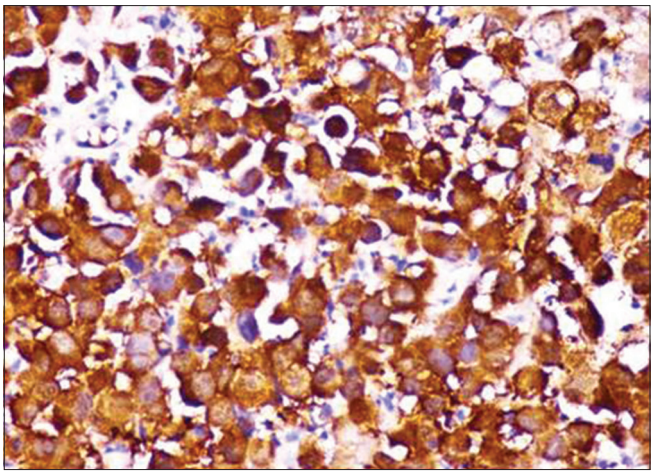

Figure 8: Immunohistochemical p16 – Score 3, diffuse (>30%–85% of cells with strong positivity, spreading in several tissue areas) (IHC, ×400)

and tobacco use ($P < 0.05$).[22] Ralli *et al.* found a statistically significant association of p16 expression with paan chewing ($P = 0.03$).[14]

Association of HPV with p16 expression was correlated with a patient's history of abnormal sexual habits like practice of oral sex and with history of multiple sexual partners. A significant association was seen between p16 expression and history of abnormal sexual habits ($P = 0.02$) in our study.

According to a study by Ralli *et al.*, a significant association was seen between p16 expression and history of multiple sexual partners ($P = 0.003$).[14] According to studies conducted by Pannone *et al.*, Fregonesi *et al.*, Singhi and Westra and Smith *et al.*, p16 expression was strongly associated with HPV infected HNSCC.[10,11,21,22]

Histological grading is a method of quantitating the degree of differentiation by applying a set of histopathological criteria. In general, poorly differentiated tumors are high-grade neoplasms, while well-differentiated tumors are low-grade lesions. Grade is a good prognostic factor associated with distant metastasis of tumors in head-and-neck carcinomas. It provides important additional information to clinical and pathologic staging. Thus, it helps to identify patients at high risk for distant metastasis so that the patients at high risk should get efficient systemic treatment.[24]

In our study, a maximum number of cases belonged to histologic grade I (61%), which was in concordance to study by Chen *et al.*[25] Grade III tumors were less frequently observed in our study when compared to study by Solomon *et al.*[26] Studies by Pena *et al.*, Ralli *et al.*, Peltoneon *et al.*, Wilson *et al.* and Smilek *et al.* observed moderately differentiated carcinoma/Grade II forming the most common group.[3,14,19,20,27]

In the present study, p16 expression had no significant correlation with histological grade of the tumor ($P = 0.36$) which was in concordance with Yuen *et al.* and Dragomir *et al.*[12,17] They hypothesized that tumors exhibiting p16 expression effect cell differentiation. Cell division is possibly arrested at a stage in the differentiation process leading to the tumor comprised mainly of poorly differentiated nonkeratinizing areas, while our findings were discordant with the study of Ralli *et al.* ($P = 0.045$), Smith *et al.* ($P = 0.02$) and Muirhead *et al.* ($P = 0.001$) and who observed that there is more probability of p16 overexpression in later stage and high-grade tumor.[14,22,28]

These differences could also be attributed to the difference in geographic distribution of tumor, sample size, scoring criteria and different types of antibodies used by different authors.

Lymph node involvement was present in 26 (26%) cases which was in concordance with various studies in literature,[3,9,16] while higher involvement was reported by Wilson *et al.* and Smilek *et al.*[20,27] Fifty of the 74 (67.56%) lymph node-negative cases showed positive staining of p16 which was statistically significant ($P = 0.009$). This was in accordance with the study conducted by Ai *et al.* ($P < 0.0001$) and Smith *et al.*, who hypothesized that as p16 protein is an important cell cycle regulatory protein, the overexpression of p16 protein inhibits cell proliferation at the G1-S phase and underexpression/weak expression of it allows cancer

cells to proliferate without control and hence effects the tumor stage including the size of tumor and nodal metastasis.[13,22]

However, in study by Yuen *et al.* and Muirhead *et al.*, no statistically significant association was found between p16 expression and lymph node involvement status and they concluded that p16 expression contributes to tumor size and cell proliferation, but it has no prognostic significance for metastasis to lymph node and long-term survival.[12,28]

CONCLUSION

As HPV integration with the transcription of viral oncoprotein induces overexpression of p16, we can use p16 immunohistochemistry as a surrogate marker of HPV. Significant expression of p16 in node-negative patients may guide the type and intensity of the therapy in patients with HNSCC.

Overexpression of p16 has been significantly seen in male patients of HNSCC who had tumors in the oral cavity and had a history of abnormal sexual habits. Vaccination programs may provide prevention from HPV infection in high-risk population.

Future implications

HPV status assessment may be helpful in early identification of cancer, determination of prognosis and posttreatment follow-up. HPV status should be included as an important risk and prognostic factor in future trials. Along with it, trials should be performed to get a proper treatment regime for HPV-positive and HPV-negative HNSCC in order to provide better treatment and lower relapse rates.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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