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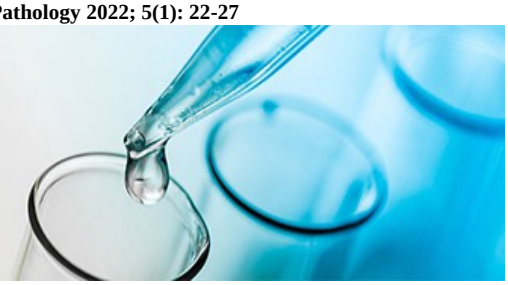

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To study the expression of P16 in squamous cell carcinoma of head and neck

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Abstract
Background: p16 is a cyclin dependent kinase inhibitor (CKI), a tumour suppressor gene, and is the second commonly affected gene next to p53 in HNSCCs. The main function of p16 is to control the phosphorylation of retinoblastoma (Rb) gene and block the progression of cell cycle. The immunohistochemical (IHC) expression of p16 is convenient as it is employed to tissue blocks and is less tedious than the other methods. Hence detection of p16 and its correlation with the head and neck SCCs is mandatory for patient management and outcome. The present study aimed to study the expression of P16 in squamous cell carcinoma of head and neck.
Materials and Method: The present study was a Comparative and longitudnal study of All new diagnosed cases of primary head and neck squamous cell carcinoma of either sex was conducted in the Department of Pathology, Government Medical College, Amritsar. As p16 staining is both nuclear and cytoplasmic, its expression was evaluated in the stained sections for all patients.
Results: HNSCC was more common in males with male to female ratio of approximately 6: 1. Oral cavity accounted for the most common site of occurrence of HNSCC (60%). Cases of nasal cavity and eye were the most common site for p16 positivity in HNSCC cases (100%). Among the oral cavity SCC cases, buccal mucosa was the most common site involved (37.6%). Among the p16 positive cases most cases are HNSCC Grade 1 (50%). Of the HNSCC cases, most cases (44%) showed intermediate staining of p16 over expression. It was observed that p16 over expression was most common in HNSCC moderately differentiated cases (66.70%).
Conclusion: Further, DNA detection based studies are needed to validate the utility of IHC detection of p16 as a surrogate marker for HPV associated HNSCC.

Keywords: HNSCC, IHC, squamous cell

Introduction

The burden of cancer in India is on rise due to increase in longevity of the growing population. Overall 57.5% of global head & neck cancers occur in Asia, Out of which around 30-35% occur in India [1-3]. Its incidence is on the rise in the Indian subcontinent with new patients being diagnosed every day [4-5]. Over 2, 00,000 Head and neck cancers (HNSCC) occur every year in India [5-7]. Nearly 80,000 are diagnosed every year in the country [8]. The etiology of Head and neck squamous cell carcinoma is multi-factorial. Genetic factors, diet, occupational exposure, tobacco and alcohol consumption. Human Papilloma Virus (HPV) also considered as risk factor for Squamous cell carcinomas independent of the traditional risk factors which include tobacco abuse and ethanol consumption [9-11].

Human papillomavirus (HPV) infects keratinocytes in the mucosa or skin, and persistent infection with HPV may lead to premalignant lesions and invasive cancer, especially cervical cancer. Human papilloma virus (HPV) has Subtypes 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and 89 are considered as low oncogenic effect viruses which can be isolated from low grade epithelial cell [12]. High-risk HPV, that is, HPV-16, 18 has been found to be associated with OSCC and HPV-16 with oral leukoplakia (OL) including proliferative verrucous leukoplakia (PVL) [13] p16 is a cyclin dependent kinase inhibitor(CKI), a tumour suppressor gene, and is the second commonly affected gene next to p53 in HNSCCs. The main function of p16 is to control the phosphorylation of retinoblastoma (Rb) gene and block the progression of cell cycle [13, 14]. Similarly over expression of p16 in cervical cancer is due to functional inactivation of Rb by HPV E7 oncoprotein. Thereby, it clearly shows that HPV causes inactivation of p16 pathway which leads to malignant transformation and carcinogenesis.

The immunohistochemical (IHC) expression of p16 is convenient as it is employed to tissue blocks and is less tedious than the other methods. Hence detection of p16 and its correlation with the head and neck SCCs is mandatory for patient management and outcome. Identifying peripheral surrogate markers and screening and prevention methods is an important field of future investigations in HNSCCs. Several studies have evaluated the expression of p16 in HNSCCs. However, the lack of standardization hinders comparison of the results between the various publications and makes a practical application of the results very difficult.

Materials and Methods

The present study was a Comparative and longitudinal study of all new diagnosed cases of primary head and neck squamous cell carcinoma of either sex was conducted in the Department of Pathology, Government Medical College, Amritsar, in after approval from the institutional thesis and ethics committee. Informed consent of the patient was taken (if required in vernacular language). Relevant history of the patient would be taken as per the proforma attached along with. Patients with carcinomas other than Squamous cell carcinomas as demonstrated by histology, prior treatment with radiation and chemotherapy, prior history of head and neck squamous cell carcinoma were excluded.

Method

From these patients selected, the data pertaining to age at diagnosis, sex, betel nut use, smoking and alcohol intake are collected. Use of tobacco, pan chewing or consumption of alcohol at least five days a week for a minimum period of 2 years is considered as a risk factor. However, smoking status was reported as never or ever smoked (including current and former smokers), and betel nut chewing was reported as never or ever chewed (including current and former chewers). Alcohol use was recorded as history of alcohol drinking or non-drinking. Tumor location, tumor size, lymph nodal status as assessed by histology/cytology (wherever available) is noted in particular. Informed consent is obtained from those patients selected for the study prospectively and depending upon contact information availability in the retrospective cases (Consent form attached). Follow up data was obtained from case files/hospital records/surgeons notes. Hematoxylin and Eosin stained slides from biopsy / resection specimens of primary squamous cell carcinomas was studied particularly to note definite squamous differentiation to grade them as well differentiated, moderately differentiated and poorly differentiated squamous cell carcinomas. Formalin fixed, paraffin embedded tissue sections was first stained with routine IHC stain.

Interpretation of IHC staining

As p16 staining is both nuclear and cytoplasmic, its expression was evaluated in the stained sections a data was recorded.

- Positive control tissue was having colored end product at site of target antigen.
- Negative control sections will not have the above color since there is no antigen antibody reaction.
- If the test shows positivity in the form of specific color it means tissue is having antibody specific antigen.

IHC scoring

The IHC score is calculated by combining an estimate of the percentage of immunoreactive cells (quantity score) with an estimate of the staining intensity (staining intensity score).

Staining intensity score

- Score 0–Negative
- Score 1–Weak
- Score 2–Moderate
- Score 3–Strong

Quantity score

- Score 0 – No staining
- Score 1 – 1 - 10% positive cells
- Score 2 – 11 - 50% positive cells
- Score 3 – 51 - 80% positive cells
- Score 4 – 81 – 100% positive cell

Results

Table 1: Distribution of cases according to p16 staining

P16	Number	%age
Negative expression (0-2)	20	40.0
Intermediate expression (3-5)	22	44.0
Strong expression(6-8)	8	16.0
Total	50	100.0

40% patients in the present study have negative expression, 44% have intermediate expression while in 16% of cases strong expression was observed.

Table 2: Association of p16 with age group

P16	Negative (score 0-2)		Intermediate (3-5)		Strong (6-8)		Total		P Value
Age group	n	%	n	%	n	%	n	%	
Age≤50	6	35.30%	9	52.90%	2	11.80%	17	100.00%	.638
Age>50	14	42.40%	13	39.40%	6	18.20%	33	100.00%	
	20	40.00%	22	44.00%	8	16.00%	50	100.00%	

In present study all the three cyclin P16 groups were comparable on basis of age and no significant association was seen between them.

Table 3: Association of p16 with sex

P16	Negative (score 0-2)		Intermediate (3-5)		Strong (6-8)		Total		P Value
Gender	n	%	n	%	n	%	n	%	
Female	3	42.90%	2	28.60%	2	28.60%	7	100.00%	.532
Male	17	39.50%	20	46.50%	6	14.00%	43	100.00%	
	20	40.00%	22	44.00%	8	16.00%	50	100.00%	

In present study all the three cyclin P16 groups were comparable on basis of gender and no significant association was seen between them. 42.90% females were negative while 39.50% males were negative for p16. While 28.6% females showed strong expression14% males showed strong expression. More no of males showed intermediate expression then females. The association was not statistically significant.

Table 4: Association of p16 with site of HNSCC

P16	Negative (score 0-2)		Intermediate (3-5)		Strong(6-8)		Total		P Value
HNSCC	n	%	n	%	n	%	n	%	
Oral cavity	13	43.30%	14	46.70%	3	10.00%	30	100.00%	.225
Hypopharynx	3	60.00%	2	40.00%	0	0.00%	5	100.00%	
Oropharynx	1	25.00%	1	25.00%	2	50.00%	4	100.00%	
Larynx	3	42.90%	3	42.90%	1	14.30%	7	100.00%	
Nose	0	0.00%	2	66.70%	1	33.30%	3	100.00%	
Near eye	0	0.00%	0	0.00%	1	100.00%	1	100.00%	
Total	20	40.00%	22	44.00%	8	16.00%	50	100.00%	

43.30% cases of oral cavity were P16 negative, 46.7% showed intermediated expression while in 10% there was a strong expression.

In 60% and 40% cases of hypo-pharynx negative expression and intermediate expression was seen respectively. In 25% cases each of ORO-pharynx negative and intermediate expression was seen while 50% Cases in the ORO-pharynx in the present study showed strong positivity for P16.

In larynx 42.9% cases each showed negative and intermediate expression was seen while 14.30% had strong expression for P16.

66.7% cases in the nasal cavity had intermediated expression while 33.3% had strong expression. 100% case present near eye had strong expression for p-16.

Table 5: Association of p16 with SCC in oral cavity

P16	Negative (score 0-2)		Intermediate (3-5)		Strong(6-8)		Total		P Value
HNSCC	n	%	n	%	n	%	n	%	
Absent	5	27.80%	8	44.40%	5	27.80%	18	100.00%	.191
Buccal mucosa	5	41.70%	5	41.70%	2	16.70%	12	100.00%	
Tongue	2	22.20%	7	77.80%	0	0.00%	9	100.00%	
Floor of mouth	4	80.00%	1	20.00%	0	0.00%	5	100.00%	
Alveolus	2	100.00%	0	0.00%	0	0.00%	2	100.00%	
Hard palate	1	100.00%	0	0.00%	0	0.00%	1	100.00%	
Lower jaw	1	50.00%	0	0.00%	1	50.00%	2	100.00%	
Lower lip	0	0.00%	1	100.00%	0	0.00%	1	100.00%	
Total	20	40	22	44	8	16	50	100	

27.80% cases of ones not present in oral cavity were P16 negative, 44.4% showed intermediate expression while in 27.8% there was a strong expression.

In 41.7% cases each of buccal mucosa negative expression and intermediate expression was seen respectively while in 16.7% there was a strong expression.

In 22.2% and 77.8% cases each of tongue was negative and intermediate expression was seen for P16.

In floor of mouth 80% cases showed negative expression while 20% had intermediate expression for P16.100% cases in the alveolus and lower had negative expression for P16 while 50% cases in lower jaw had strong expression and other 50% had negative expression for P16.

Table 6: Association of p16 with size of tumor

P16	Negative (score 0-2)		Intermediate (3-5)		Strong (6-8)		total		P Value
Size	n	%	n	%	n	%	n	%	
<2cm	13	34.20%	17	44.70%	8	21.10%	38	100.00%	.638
>2cm	7	58.30%	5	41.70%	0	0.00%	12	100.00%	
total	20	40.00%	22	44.00%	8	16.00%	50	100.00%	

In 34% cases of tumor size <2cm p16 expression was negative while in 44.7% it was intermediate. In 21.10% it was strong.

In 58.3% cases of >2cm p16 expression was negative while in 41.7% it was intermediate.

No significant association was seen in both the groups that shows size of tumor has no association with p16

Table 7: Association of p16 with involvement of lymph node

P16	Negative (score 0-2)		Intermediate (3-5)		Strong(6-8)		Total		P Value
Involvement of lymph node	n	%	n	%	n	%	n	%	
Absent	8	27.60%	16	55.20%	5	17.20%	29	100.00%	.221
Single Ipsilateral Lymph Node< 3-6 Cm	9	52.90%	5	29.40%	3	17.60%	17	100.00%	
Ipsilateral multiple lymph-node >6cm	3	75.00%	1	25.00%	0	0.00%	4	100.00%	
Total	20	40.00%	22	44.00%	8	16.00%	50	100.00%	

83.4% cases in which there was no lymph node involvement were positive for P16 while 27.60% were negative.
In 52.90% cases of single Ipsilateral <3-6cm in size p 16 was negative, in 29.40% it had intermediate expression while in 17.6% it had strong expression. In 75% of Ipsilateral multiple lymph-node >6cm had negative expression while 25% had intermediate expression. No significant association was seen among both.

Table 8: Association of p16 with tumor differentiation

P16	Negative (score 0-2)		Intermediate (3-5)		Strong(6-8)		total		P Value
Tumor differentiation	n	%	n	%	n	%	n	%	
Well Differentiated	10	34.50%	11	37.90%	8	27.60%	29	100.00%	.017
Moderately differentiated	5	33.30%	10	66.70%	0	0.00%	15	100.00%	
Poorly differentiated	5	83.30%	1	16.70%	0	0.00%	6	100.00%	
Total	20	40.00%	22	44.00%	8	16.00%	50	100.00%	

27.60% (11), 37.90% (8) and 34.50% (10) were strongly positive, intermediately positive and negative for P16 in well differentiated HNSCC.
In 33.30% cases of moderately differentiated HNSCC was negative while 66.70% were intermediately positive for P16 In 83.3%cases of poorly differentiated cases P16 had a negative expression while in 16.70% of cases it was intermediately positive. Significant association was seen among both.

Table 9: Association of p16 with tumor stage

P16	Negative (score 0-2)		Intermediate (3-5)		Strong (6-8)		total		P Value
Tumor Stage	n	%	n	%	n	%	n	%	
Stage 1	6	24.00%	14	56.00%	5	20.00%	25	100.00%	.191
Stage 2	2	50.00%	2	50.00%	0	0.00%	4	100.00%	
Stage 3	10	52.60%	6	31.60%	3	15.80%	19	100.00%	
Stage 4	2	100.00%	0	0.00%	0	0.00%	2	100.00%	
Total	20	40.00%	22	44.00%	8	16.00%	50	100.00%	

No significant association was seen among tumor stage and P16

Discussion

In the present study, we evaluated p16 expression in HNSCC. p16 is a tumor suppression gene, inactivation of which is considered as the major oncogenic event in the carcinogenesis of OSCC.

Clinico-pathological correlation with p16

Age

Out of total cases <50 years 35.5% were negative and rest were positive while >50 years 42.40% were negative which shows that as age increases the p16 expression decreases however all the three cyclin P16 groups were comparable on basis of age and no significant association was seen between them. Similar results were obtained by pinky *et al.* [16] who observed 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. Rally *et al.* [17] showed that Out of 26 cases with Grade III expression of p16, 16 (61.54%) cases belonged to age more than 50 years. However the result in their study was also statistically non-significant but it was similar to the present study as we also observed that cases with more than 50 years showing more strong expression of p16(18.2%) then <50 years (11.8%).

Gender

In present study all the three cyclin P16 groups were comparable on basis of gender and no significant association was seen between them. 42.90% females were negative while 39.50% males were negative for p16. While 28.6% females showed strong expression14% males showed strong expression. More no of males showed intermediate expression then females. The association was not statistically significant. In a study by rally *et al.* 17no significant association was seen between p16 expression and sex ($P = 0.331$) with mostly males showing intermediate and strong positivity for p16. Males 79.6% were positive for p16 while female 45.5% were positive with no significant difference which was similar to present study.
Hasmi *et al.* [18] in their study observed no significant association of gender with p 16 which was in accordance to the present study. However in a study by pinky pandey *et al.* [16] significant association was observed between sex and p 16 with expression being more in males. This was in contrast to the present study.

Site

In the western world, HNSCC of the oral cavity and oropharynx are becoming more prevalent which may be related to an increase in oral cavity and oropharynx HPV infections.[19]All the cases of nose and eyes were positive for P16 which was followed by oropharynx where 75% cases(25%intemediate and 50%strong) showed positive expression. In larynx 42.9% cases each showed negative and intermediate expression was seen while 14.30% had strong expression for P16. In oral cavity 43.30% cases were P16 negative, 46.7% showed intermediated expression while in 10% there was a strong expression. In hypo-pharynx 60% cases were negative while and 40% showed intermediate expression was seen respectively.

Tumor size and lymph node metastasis

In 34% cases of tumor size <2cm p16 expression was negative while in 44.7% it was intermediate. In 21.10% it was strong. In 58.3% cases of >2cm p16 expression was negative while in 41.7% it was intermediate. No significant association was seen in both the groups that shows size of tumor has no association with p16. Hashmi *et al.* also observed no significant association between tumor size and p 16. In a study by C.A. Fischer also no significant association was seen between tumor size and p16 positivity

both above mentioned studies were in accordance to the present study [20].

In case of lymph node as well there were no significant difference was observed between the two. This was in discordance with the study by pander *et al.* where 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 ($P = 0.009$). This was also in discordance 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 under expression/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 [21-22].

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.[23-24] but in present study both tumor size and lymph node have negative association with p 16.

No significant association was seen among tumor stage and P16.hashmi *et al.* also showed no significant association between tumor stage and p16 positivity. Chrystiano de C. Ferreira *et al.* [25] observed significant association between p16 and tumor stage with early stage tumors showing more positivity. In present study also early stage tumors were more positive but it wasn't statistically significant. De Cicco *et al.* [26] and Du *et al.* [27] reported similar results.

Conclusion

Histological grade is a means of quantitating the degree of differentiation by applying a set of histological criteria. Usually well differentiated tumors are low grade lesions, whereas poorly differentiated tumors are high grade neoplasms. Grade is a strong and independent factor associated with distant metastasis in head and neck carcinomas. Thus, it adds important information to clinical and pathologic staging. It helps to identify patients at high risk for distant metastasis for whom an efficient systemic treatment is mandatory. Further, DNA detection based studies are needed to validate the utility of IHC detection of p16 as a surrogate marker for HPV associated HNSCC.

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