A STUDY ON EXPRESSION OF P16 AS A SURROGATE MARKER OF HIGH RISK HPV INFECTION IN OROPHARYNGEAL, HEAD & NECK SQUAMOUS CELL CARCINOMA & ITS CORRELATION WITH DIFFERENT HISTOMORPHOLOGICAL FEATURES

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ABSTRACT

Aim of this study is to assess the expression of p16 as an immunohistochemical marker (which is a predictor or surrogate marker of high risk HPV infection) in oropharyngeal, head and neck squamous cell carcinoma and its correlation with different histomorphological features.

Our study was an observational, descriptive study with cross-sectional design, done in the Department of Pathology, in collaboration with the Department of Plastic Surgery. It was conducted on a group of patients admitted in Department of plastic Surgery, R G Kar Medical College & Hospital, and Kolkata. The samples included 54 specimens of, oropharyngeal & head neck SCC. Here, in this study, the expression of P16 in oropharyngeal & head neck SCC was shown & correlated with their histomorphological features.

In present study median age of p16 positive patients were 54 years and male were more commonly affected than female. According to Socio economic status p16 positive cases were most commonly found in Lower status. Present study also showed that most of the p16 positive patients with SCC were married and literate. In most of the p16 positive cases history of one or more predisposing factors were present.

Tobacco chewing came out as a most common risk factor. Most of the p16 positive cases were located at Oropharynx that came out as most common site. In all p16 positive cases, most of them had Non Keratinizing SCC which was statistically significant.

KEYWORDS

P16, Surrogate Marker, Oropharyngeal, Head & Neck Squamous Cell Carcinoma

INTRODUCTION

Squamous cell carcinoma of the head and neck (SCCHN) is the sixth most common type of cancer worldwide. Every year 600,000 new cases are diagnosed. In the past decades, it was determined that along with alcohol and tobacco consumption, human papilloma virus (HPV) infection is one important risk factor among others for the development of oral cancer¹.

Infection with high-risk HPV types is particularly linked to cancer. HPV-16 and HPV-18 are detected in the majority of HPV-positive SCCHNs. Approximately 26% of all SCCHN cases and 36% of oropharyngeal SCCs are positive for HPV. Remarkably, the number of HPV-positive SCCHNs has been increasing over the past 20 years.²

The p16/Rb/cyclin-D1 pathway is a key controller of the cell cycle, which controls the passage of cells from G1 to S phase. Consequently, its inactivation is a frequent event in all types of cancer. The cyclin-dependent kinase (CDK) inhibitor p16 targets CDK4 and prevents Rb phosphorylation. On one hand, the p16 gene is often mutated or epigenetically silenced in SCCHN. On the other hand, p16 protein overexpression is caused by viral E7 protein. Consequently, p16 is supposed to be an indirect marker of HPV-induced SCCHN. Some studies describe p16 protein detection as a helpful marker of HPV (especially HPV-16) activity.^{3,5}

In the Indian subcontinent, one of the top three types of cancer is oral cancer. Oral cancer is in approximately 30% of all cancers in India. Oral squamous cell carcinoma (SCC) is more common in men between the sixth and eighth decades of life, and the incidence of oral SCC in young age varies between 0.4% and 6.6%.⁶

Prognostic implication of age on the survival of young age is controversial. Numerous studies had shown that the disease was more aggressive with poor prognosis in young adults as compared to older age group. In contrast, Goldenberg et al. have not established any significant difference in survival between different age group.⁷

Aim of this study is to assess the expression of p16 as an immunohistochemical marker (which is a predictor or surrogate marker of high risk HPV infection) in oropharyngeal, head and neck squamous cell carcinoma and its correlation with different histomorphological features.

MATERIALS AND METHODS

This study was conducted on a group of patients admitted in Department of Surgery, R G Kar Medical College & Hospital, Kolkata. Approval from an institutional review board was obtained at the

initiation of the study. All patients were provided with signed informed written consent.

STATISCAL ANALYSIS:

For statistical analysis data were entered into a Microsoft excel spreadsheet and then analyzed by SPSS (version 25.0; SPSS Inc., Chicago, IL, USA) and Graph Pad Prism version 5. Data had been summarized as mean and standard deviation for numerical variables and count and percentages for categorical variables. Unpaired proportions were compared by Chi-square test or Fischer's exact test, as appropriate. P-value ≤ 0.05 was considered for statistically significant.

RESULTS AND ANALYSIS

We found that, 40(74.1%) patients were 46-65 years of age, 8(14.8%) patients had ≤ 45 years of age and 6(11.1%) patients were >65 years of age. 43(79.6%) patients were male & 11(20.4%) patients were female. 22(40.7%) patients were of lower, 11(20.4%) patients were lower middle, 14(25.9%) patients were upper lower and 7(13.0%) patients were upper middle socio economic status.

It was found that, 3(5.6%) patients had SCC in neck region, 15(27.8%) patients had SCC in oral cavity, 34(63.0%) patients had oropharyngeal SCC and 2(3.7%) patients had SCC over scalp. 36(66.7%) patients had P16 positive, whereas 18(33.3%) had P16 negative SCC. 34(63.0%) had non-keratinizing SCC, whereas 20(37.0%) patients had Keratinizing. 34(63.0%) patients had Non keratinizing SCC, among keratinizing SCC, 3(5.6%) patients had WDSCC 10(18.5%) patients had MDSCC and 7(13.0%) patients had PDSCC.

It was found that, in positive P16, 4(11.1%) patients had no predisposing factor and 32(88.9%) patients had predisposing factor. Association of predisposing factor vs. P16 was not statistically significant ($p=0.4982984175$). In positive P16, higher number of patients 9(25.0%) had tobacco chewing than other. Association of predisposing factor vs. P16 was not statistically significant ($p=0.6920$). In positive P16, higher number of patients 25(69.4%) patients had oropharyngeal SCC as compared with other sites. Association of site vs. P16 was not statistically significant ($p=0.1703$). Our study showed that, in positive P16, 29(80.6%) patients had Non Keratinizing and 7(19.4%) patients had Keratinizing histological features. Association of Keratinizing vs. P16 was statistically significant ($p=0.0004$). In positive P16, higher number of patients 5(13.9%) had PDSCC. Association of grade vs. P16 was statistically significant ($p=0.0001$). In positive P16, the mean age (mean $\pm$ s.d.) of patients was 54.9722 ± 7.9192 years. Distribution of mean age vs. P16 was not statistically significant ($p=0.6538$).

DISCUSSION

Among the patients with positive P16, most of them, 27 (75.0%) patients belonged to 46-65 years age group. However, association of age with P16 positivity was not found to be statistically significant ($p=0.1144$).

This finding is also in concordance with the World Health Organization WHO (2017)⁵ data which states that the median age in p16 positive OPSCC & HNSCC is 50-56 years.

Maura L. Gillison, et al⁹ (2008) also found similar results. When they compared the age of patient with HPV-16-negative and HPV-16-positive head neck squamous cell carcinoma, they noticed most HPV-positive patient were younger (median age: 54 vs 58 years, $P = .002$).

In present study it was found that association of sex vs. P16 was not statistically significant ($p=0.5503$).

As per World Health Organization WHO (2017)⁵ data which states that patient of OPSCC HPV are most commonly male patients. Male to female ratio is 4:1.

Similarly in a study by Chernock RD et al¹⁰ (2009) it was observed that most p16 positive cases were younger male. So present study corroborate with Chernock RD et al¹⁰ (2009).

As per WHO (2017)⁵ OPSCC HPV are more prevalent in patients from higher socioeconomic status. Similarly Maura L. Gillison, et al⁹ (2008) also found that maximum HNSCC-HPV have an annual income of \$50000 or higher.

But in the present study it was observed that 22(40.7%) patients were of lower, 11(20.4%) patients were of lower middle, 14(25.9%) patients were upper lower and 7(13.0%) patients were upper middle socioeconomic status. In positive P16, the higher number of patients 15(41.7%) were from lower socioeconomic status.

So present study does not corroborate with Maura L. Gillison, et al⁹ (2008) regarding prevalence HNSCC associated with socioeconomic status. Our study shows OPSCC is more prevalent in lower or lower middle socioeconomic status.

In a study by Maura L. Gillison, et al⁹ (2008) found that patients with HPV-16-positive head and neck tumour (90 out of 217) were more likely to be married ($P < .001$) & college educated ($P = .003$).

Similarly in our study out of 36 p16 positive cases 32(88.9%) patients were married. So present study corroborates with Maura L. Gillison, et al⁹ (2008). Whereas 25(69.4%) patients were literate among 36 p16 positive cases others were illiterate that didn't corroborated with study of Maura L. Gillison, et al⁹ (2008) regarding literacy.

For risk factor evaluation, it was found in present study that 46(85.2%) patients had predisposing factor. Among them 6(11.1%) patients had h/o alcohol intake, 2(3.7%) patients had h/o alcohol intake along with tobacco chewing, 4(7.4%) patients had family history,

7(13.0%) patients had not any predisposing factor, 5(9.3%) patients had h/o oral sex, 11(20.4%) patients had h/o smoking, 3(5.6%) patients had h/o smoking & tobacco chewing, 16(29.6%) patients had h/o tobacco chewing only. In positive P16 cases, higher number of patients 9(25.0%) had h/o tobacco chewing followed by smoking.

As per World Health Organization WHO (2017)⁵ tobacco smoking is associated with significantly higher oral HPV prevalence thus may play some role in progression from oral HPV infection to OPSCC HPV. So, present study corroborates with World Health Organization data.

Lai S et al¹¹ (2016) found that p16 immunohistochemical expression serves as a surrogate for human papillomavirus (HPV)-positive oropharyngeal squamous cell carcinoma (OPSCC). While HPV-positive OPSCC commonly associate with tobacco-related head and neck cancers. All patients included in this study were male and the majority had significant tobacco (91%) and alcohol (88%) exposure. Overall, 57% of tumors were p16-positive. In their study higher number of patients with heavy tobacco exposure had p16 positive OPSCC.

In the present study it was also observed that in all P16 positive cases, higher number of patients 9(25.0%) had history of tobacco chewing and second higher number of patient had history of smoking. So, present study has also revealed that tobacco exposure is a high risk factor for p16 positive OPSCC and corroborate with Lai S et al¹¹ (2016).

Whereas in a study by Ralli M et al¹² (2016) Expression of p16 was higher in non smokers and non alcoholics and significantly associated with pan chewing habit. No significant correlation was seen with history of abnormal sexual habits, but p16 expression was significantly correlated in cases with multiple sexual partners ($P = 0.003$), with increasing histological grade ($P = 0.045$) and in cases with lymph node metastasis ($P = 0.03$).

But in our study it was observed the increased incidence of P16 positive cases associated with history of tobacco chewing 9(25%) and smoking 7(19.4%). So we couldn't corroborate with this study in terms of risk factors.

In this present study it was also found that, all the cases with history of oral sex were p16 positive. So we couldn't corroborate with above study regarding abnormal sexual behavior.

We couldn't elicit exact history regarding multiple sexual partners as patients were unwilling to reveal these facts due to social stigma.

Taberna M et al¹³ (2017) found that the primary risk factor for developing HPV-related OPSCC is oral HPV-infection and the majority of oral HPV-infections are acquired by oral sex.

Present study corroborate with this study, as all the cases with history of oral sex were p16 positive in present study.

In present study out of all 54 patients 34(63.0%) patients had oropharyngeal SCC, 15(27.8%) patients had SCC in oral cavity, 3(5.6%) patients had SCC in neck region, and only 2(3.7%) patients had SCC over scalp. Where as in 36 P16 positive cases, higher number of patients 25(69.4%) patients had oropharyngeal SCC as compared with other sites. Association of site vs. P16 was not statistically significant ($p=0.1703$).

Similarly, in our study it was also revealed that higher number of p16 positive cases had oropharyngeal SCC. Out of 36 p16 positive cases, 25 cases were from oropharyngeal site.

Thus study of Riener MO et al¹⁴ (2017) corroborates with present study regarding site of tumor site.

A study by Singh S et al¹⁵ (2017) also corroborate with our present study found that head and Neck Squamous Cell Carcinomas (HNSCCs) patients with HPV-infected tumors have more favourable prognosis. All tissue samples for biopsy were subjected to Immunohistochemistry to study p16 expression, a surrogate marker for HPV. Majority of patients had Stage III disease (33 patients – 66%). 16 (32%) patients were HPV positive and 34(64%) were negative. Out of the 16 HPV positive cases, majority, 15 (93.75%) cases were associated with oropharyngeal carcinoma.

Regarding most common site of origin present study also consistent with the study of Duxtader EE et al¹⁶ (2012) where they noticed that p16 expression was detected in 54 cases overall, including 25 (74%) of 34 oropharyngeal squamous cell carcinomas, 8 (19%) of 43 non oropharyngeal head and neck squamous cell carcinomas including 3 of 4 from the sinonasal cavity, 6 (30%) of 20 esophageal squamous cell carcinomas, 7 (35%) of 20 lung squamous cell carcinomas, and 8 (40%) of 20 skin squamous cell carcinomas. 54 p16-positive cases, 30 were positive for high-risk human papillomavirus, including 24 (96%) of 25 from the oropharynx. p16 positivity correlates well with high-risk human papillomavirus infection. So this study corroborate with our study as we got most of the OPSCC p16 positive cases among different sites of origin.

Study of both Ursu RG et al¹⁷ (2018) & Maura L. Gillison, et al⁹ (2008) have also shown the similar result regarding the site of origin in p16 positive HNSCC.

In a study by Ursu RG et al¹⁷ (2018) total, 189 formalin-fixed paraffin-embedded tissue samples (99 oral cavity tumors, 28 oropharynx, 48

pharynx, and 14 larynx/hypopharynx) were analyzed for HPV DNA and RNA using Luminex-based assays, and for overexpression of p16INK4a (p16) by immunohistochemistry. Of the 189 cases, 23 (12.2%) were HPV DNA- positive, comprising half of the oropharyngeal cases (14/28, 50.0%) and 9/161 (5.6%) of the non-oropharyngeal cases. In present study we have also shown 25(69.4%) p16 positive OPSCC out of total 36 p16 positive cases.

Maura L. Gillison, et al⁹ (2008) in this study the sample size was 240 among them HPV-16 was detected by in situ hybridization in 82 (72%) of 114 oropharynx cancers, four (67%) of six primary cancers of unknown location, one (14%) of seven paranasal sinus cancers, three (6%) of 49 larynx cancers, two (4%) of 54 oral cavity, and none of the seven hypopharynx or the three nasopharynx cancers. Whereas Sritippho T et al¹⁸ (2016) found that p16 was overexpressed in 8/37 cases (21.6%) of OSCC and 2/4 cases (50%) of VC. HPV-16 was detected in 4/34 OSCC cases (11.8%) and HPV-18 was detected in 1/34 OSCC cases (2.9%). Co-infection of HPV-16/18 was detected in 1/4 VC cases (25%).

In present study it was observed in all 54 cases 36(66.7%) patients were P16 positive, whereas 18(33.3%) were P16 negative SCC. 20(37.0%) patients had Keratinizing, whereas 34(63.0%) had non-keratinizing SCC. In all p16 positive cases, 29(80.6%) patients had Non Keratinizing and 7(19.4%) patients had Keratinizing histological features. Association of Keratinizing vs. P16 was statistically significant (p=0.0004)

Present study also revealed similar trend that in total 36 P16 positive cases, 29(80.6%) patients had Non Keratinizing and 7(19.4%) patients had Keratinizing SCC. Association of Keratinizing vs. P16 was statistically significant (p=0.0004).

Histological grading is not currently advocated in non keratinizing OPSCC according to WHO. So in present study gradation has been done only in keratinizing cases.

In positive P16 Keratinizing SCC, higher number of patients 5(13.9%) had PDSCC. Association of grade vs. P16 was statistically significant (p=0.0001).

In a study Liu SZ et al¹⁹ (2015) concluded that p16 IHC is a reliable surrogate marker for HR HPV testing in OPSCC. Prognostically favorable HR HPV genotypes other than HPV16 are reflected in p16 positivity. Similarly Singh S et al¹⁵ (2017) also found that Patients who were HPV positive had better disease free survival and overall survival. Due to limited timeframe present study couldn't assess the prognosis, disease free survival & overall survival.

Fischer CA et al²⁰ (2010) found that these HPV-positive OPSCC show distinct molecular differences, specific p16 overexpression and a significantly better prognosis. p16 expression in OPSCC and compare its relevance with the established prognostic markers cT and cN classification and the clinical stages I-IV.

Satgunaseelan L et al²¹ (2016) found that p16 expression significantly decreased with increasing pT category (p=0.002). p16 expression was seen in early stage OSCCs.

In situ hybridization (ISH) could not be done in present study due to some limitation. Although out of all 54 OPSCC & HNCC cases 36 cases were p16 positive. So, present study also suggested that p16 IHC is the best test to use for high risk stratification in oropharyngeal SCC.

CONCLUSION

- PDSCC revealed significantly higher P16 positivity among keratinizing squamous cell carcinoma.
- P16 is relatively cheap and easily available surrogate marker in HPV associated HNCC & OPSCC.

Table 1: Distribution of all parameters with P16 expression

		P16			Chi-square value	p-value
		Negative	Positive	TOTAL		
Age	≤45	3	5	8	0.0750	0.9632
	Row %	37.5	62.5	100.0		
	Col %	16.7	13.9	14.8		
46-65	13	27	40			
	Row %	32.5	67.5	100.0		
	Col %	72.2	75.0	74.1		

	>65	2	4	6		
	Row %	33.3	66.7	100.0		
	Col %	11.1	11.1	11.1		
	TOTAL	18	36	54		
	Row %	33.3	66.7	100.0		
	Col %	100.0	100.0	100.0		
SEX	Female	5	6	11	0.3568	0.5503
	Row %	45.5	54.5	100.0		
	Col %	27.8	16.7	20.4		
	Male	13	30	43		
	Row %	30.2	69.8	100.0		
	Col %	72.2	83.3	79.6		
Predisposing factor	TOTAL	18	36	54	0.4586	0.4982
	Row %	33.3	66.7	100.0		
	Col %	100.0	100.0	100.0		
	No	4	4	8		
	Row %	50.0	50.0	100.0		
	Col %	22.2	11.1	14.8		
Predisposing factor	Yes	14	32	46	4.7374	0.6920
	Row %	30.4	69.6	100.0		
	Col %	77.8	88.9	85.2		
	TOTAL	18	36	54		
	Row %	33.3	66.7	100.0		
	Col %	100.0	100.0	100.0		
Site	Alcohol intake	2	4	6	5.0206	0.1703
	Row %	33.3	66.7	100.0		
	Col %	11.1	11.1	11.1		
	Alcohol intake, Tobacco chewing	0	2	2		
	Row %	0.0	100.0	100.0		
	Col %	0.0	5.6	3.7		
	Family history	1	3	4		
	Row %	25.0	75.0	100.0		
	Col %	5.6	8.3	7.4		
	NA	3	4	7		
	Row %	42.9	57.1	100.0		
	Col %	16.7	11.1	13.0		
	Oral sex	0	5	5		
	Row %	0.0	100.0	100.0		
	Col %	0.0	13.9	9.3		
	Smoking	4	7	11		
	Row %	36.4	63.6	100.0		
	Col %	22.2	19.4	20.4		
	Smoking, Tobacco chewing	1	2	3		
	Row %	33.3	66.7	100.0		
	Col %	5.6	5.6	5.6		
Site	Tobacco chewing	7	9	16		
	Row %	43.8	56.3	100.0		
	Col %	38.9	25.0	29.6		
	TOTAL	18	36	54		
	Row %	33.3	66.7	100.0		
	Col %	100.0	100.0	100.0		
	Neck	1	2	3		
	Row %	33.3	66.7	100.0		
	Col %	5.6	5.6	5.6		
	Oral cavity	6	9	15		
Site	Row %	40.0	60.0	100.0		
	Col %	33.3	25.0	27.8		
	Oropharynx	9	25	34		
	Row %	26.5	73.5	100.0		
	Col %	50.0	69.4	63.0		
	Scalp	2	0	2		
	Row %	100.0	0.0	100.0		
	Col %	11.1	0.0	3.7		
	TOTAL	18	36	54		
	Row %	33.3	66.7	100.0		
	Col %	100.0	100.0	100.0		

Histomorphology	Non Keratinizing	5	29	34	12.159	0.0004
	Row %	14.7	85.3	100.0	9	
	Col %	27.8	80.6	63.0		
	Keratinizing	13	7	20		
	Row %	65.0	35.0	100.0		
	Col %	72.2	19.4	37.0		
	TOTAL	18	36	54		
	Row %	33.3	66.7	100.0		
	Col %	100.0	100.0	100.0		
Grade	NON KERATINIZING	5	29	34	21.1803	<0.0001
	Row %	14.7	85.3	100.0		
	Col %	27.8	80.6	63.0		
	WDS	3	0	3		
	Row %	100.0	0.0	100.0		
	Col %	16.7	0.0	5.6		
	MD	8	2	10		
	Row %	80.0	20.0	100.0		
	Col %	44.4	5.6	18.5		
	PD	2	5	7		
	Row %	28.6	71.4	100.0		
	Col %	11.1	13.9	13.0		
	TOTAL	18	36	54		
	Row %	33.3	66.7	100.0		
	Col %	100.0	100.0	100.0		

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