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A STUDY OF THE ROLE OF p16 IN THE CLASSIFICATION AND PREDICTIVITY OF SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

Pathology

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ABSTRACT

Background: Head and neck squamous cell carcinomas (HNSCC) associated with smoking, tobacco products and alcohol are in declining trend whereas those associated with Human papilloma viruses are rising, especially in young sexually active individuals. p16 expression is regarded as a surrogate marker for HPV related carcinomas which have peculiar clinical and morphological attributes.Therefore,immunohistochemical study of p16 expression in comparision with p53 and Ki67 expressions were undertaken to establish epidemiological and clinical profile, classify and analyse with grade and stage of tumours and lastly their predictivity to treatment amongst serving and dependent family members of Armed Forces in a tertiary level hospital.

Methods: 300 cases of HNSCCs including 122 resection specimens were studied both retrospectively and prospectively for a period of five years .

Result: Of all immunohistochemical tests carried out in 122 resection specimens, 54 cases (44.2%) were found to be p16 positive. Statistically significant association ($p < 0.05$) were found between tumor location, tumor grade, keratinistaion, LVI and TILs and p16. No significant correlation($p>0.05$) of p16 with age, sex, etiology, depth of invasion, perineural invasion and stage found. Non keratinizing HNSCCs were found to have more p16 expression while keratinizing type showed more of p53 expression.

Conclusion: Patients with head and neck squamous cells carcinomas with p16 expression have peculiar clinical and morphological features and have good prognosis to treatment.

KEYWORDS

Head and Neck Squamous cell carcinomas, Human papilloma viruses, Immunohistochemistry, Keratinisation.

INTRODUCTION

Most of the Head and neck squamous cell carcinomas (HNSCCs) arise from the mucosal lining of upper aerodigestive tract comprising of nasal cavity, paranasal sinuses, oral cavity and oropharynx, nasopharynx, hypopharynx and larynx. 57.5% of worldwide head and neck cancers occur in Asia, especially in India which accounted for 30% of all cancers. In India, 60 to 80% of patients present with advanced disease as compared to 40% in developed countries[1]. With increasing awareness amongst general population there is a decrease in prevalence of HNSCC due to smoking and alcohol but increase in those associated with Human Papilloma viruses (HPV) infection , especially HPV 16 , through sexual route [2]. Smokers of pipes and cigars and heavy drinkers showed a more elevated risk of cancer of the oral cavity [3,4]. Both HPV related and smoking related HNSCC have distinct epidemiological, demographic, clinical and pathological features[5, 6]. HPV related HNSCC has an improved prognosis and favourable response to treatment as compared to those related to smoking [7, 8]. HPV related HNSCCs reveal a diffuse p16 positivity as compared to those related to smoking, which decrease/ eliminate p16 expression[9]. The relationship of HPV positive HNSCC with conventional prognostic parameters such as stage, grade, lymphovascular invasion (LVI), perineural invasion (PNI) is less well understood. There is also an overlap of such positivity in tobacco related cancers which conventionally show positivity for the p53 and a high proliferative index. In view of the above , we felt a need for study on the role of p16 immunohistochemistry in the classification and predictivity of HNSCC in Indian context especially amongst member of armed forces and their dependants.

MATERIALS AND METHODS:

Both prospective(02years) and restrospective (03 years) observational study was done at a tertiary care hospital for a period of five years in 300 patients Cases of HNSCC in oral cavity, oropharynx , pyriform fossa and metastatic deposits in cervical lymph nodes were studied to predict the clinico-epidemiological and histopathological attributes.

Clinical details of patients were accessed from Stats section of the hospital including personal habits like smoking, tobacco and alcohol. The morbid anatomy of the resected specimens were studied with respect to the site, external and cut appearances and extent of spread were accessed from their pathology reports.

Immunohistochemical study involving p16, p53 and ki67 expressions on 122 resected specimens was carried out to study the pattern of p16 immunohistochemistry in the classification and predictivity factors of HNSCC.

Processing of Specimen:

Grossing of the specimens fixed in 10% neutral buffered formal saline were done as per CAP protocol [10]. Type of specimens in the study include punch biopsies, partial glossectomy specimen, wide local excision specimen, hemimandibulectomy specimen, laryngectomy specimen and neck dissection

All tumour slides for both old and new cases, stained by Haematoxylin and Eosin method were examined for keratinisation, grade, LVI, PNI, TILs and depth of invasion. A pathological stage was assigned to each resection specimens as per TNM classification. One representative slide for each patient was selected for IHC with p16, p53 and KI67 antibodies.

p16 and p53 expressions are considered **p**ositive when more than 50% of the tumour cells show with nuclear and/or cytoplasmic staining.

Data Analysis

An Excel data sheet was generated to analyze the complete data collected. SPSS software version 11.5 was used to analyze the data. Pearson's correlation co-efficient two tailed was used to compare grade, stage and tumour type with p16, p53 and Ki67 immunostaining. Fisher exact test was applied wherever applicable. In all the tests, a p value of <0.05 was taken as significant.

RESULT

A total of 300 cases of HNSCCs were studied in a tertiary level hospital of Indian Armed Forces for a period of five years both retrospectively and prospectively including 122 resection cases (Table 1 and 2)

Table 1.Distribution of cases (n=300)

Parameters	**Small Specimens** (n=178)	**Resection specimens (n=122)**	**Total (n=300)**
Sex			
Male	156	89	245
Female	22	33	55

Age			
<50 yr	14	19	33
>50 yr	164	103	267
Etiology			
Smoke+alcohol	130	83	213
Smokers	26	28	54
Alcohol	3	3	6
None	19	8	27
Location			
Oral cavity	88	84	172
Oropharynx	56	29	85
Hypopharynx	34	9	43
GRADE			
WD	40	46	86
MD	120	56	176
PD	1	20	38
Keratinisation			
Keratinizing	59	81	140
Non keratinizing	119	41	160

Table 2.Distribution of 122 cases as per histological findings

	No of cases	Percentage(%)
Size		
<2.0cms	37	30.4
<2.0cms	85	69.6
Depth		
≤ 1.0cm	60	49.2
>1.0 cm	62	50.8
LVI		
Positive	32	26
Negative	90	74
PNI		
Positive	30	24.6
Negative	92	75.4
TILs		
Positive	88	72.1
Negative	34	27.9

Figure 1.Exophytic growth(A) and Ulcerative growth(B) in the retromolar trigone region and buccal mucosa respectively.

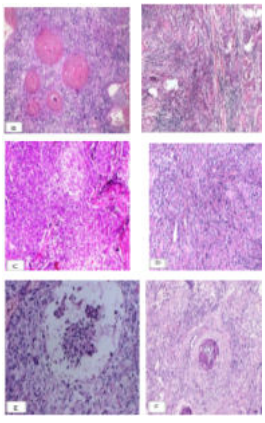

Figure 2. WDSCC-K with TILS(A) , MDSCC-K with TILs (B) , MDSCC- NK with TILS(C)PDSCC – NK with TILs (D), Vascular invasion(E), Neural invasion(F) (H& E, 100X, 200X)

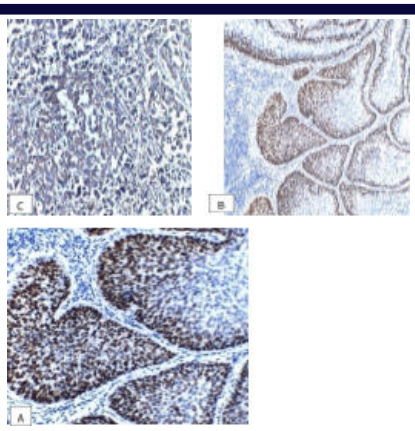

Fig.3 Positive p16(A), p53(B) and Ki67(C) (H& E 100x, 200X)

Figures- 1, 2 and 3 depicts various gross, microscopic and immunostaining patterns of HNSCCs.

Immuno-histochemistry tests and correlation studies were performed on 122 resection specimens as pathological stage of the tumor could be established in larger specimens(Table 3) .

Table 3 (a) Result of IHC of p16

Parameters	p16 positive (54)	p16 negative (68)	**p value**
1.Age			
≤ 50	19 (15.6)	12(22.2)	0.07
>50	103 (84.4)	42(77.80)	
2.Sex			
Female	33 (27)	12(22.2)	0.28
Male	89 (73)	42(77.80)	
3.Etiology			
A+S	83(68)	38 (70.4)	0.001
S	28(23)	9(16.7)	
A	3(2.5)	3(5.6	
None	8(6.6)	4(7.4)	
4.Location			
Oral cavity	84(68.9)	31(57.4)	0.02
Oropharynx	29(23.8)	16(29.6)	
Hypopharynx	9 (7.4)	7(13	
5.Depth of invasion			
≤1 cm	60 (49.2)	27(50)	0.87
>1cm	62 (50.8)	27(50)	
6.Grade			
WD K	44(36.1)	11(20.4)	0.001
WD NK	2 (1.6)	2(3.7)	
MD K	37(30.3)	13(24.1)	
MD NK	19(15.6)	16(29.6)	
PD	20(16.4)	12(22.2)	
7.Keratinisation			
Positve(K)	81(66.4)	24(44.4)	0.004
Negative(NK)	41(33.6)	30(55.6)	
8.PNI			
Positive	30(24.6)	15(27.8)	0.48
Negative	92(75.4)	39(72.2)	
9.LVI			
Positive	32(26.2)	19(35.2)	0.04
Negative	90(73.8)	35(64.8)	
10.TILs			
Positive	88(72.1)	47(87)	0.001
Negative	34(27.9)	7(13)	
11.STAGE			
I	30(24.6)	13(24.1)	0.133
II	44(36.1)	19(35.2)	
III	12(9.8)	2(3.7)	
IV	36(29.5)	20(37.0)	

Table 3(b) Result of IHC of p53

Parameters	p53 positive (84)	p53 negative (38)	p value
1.Age			
≤ 50	15(17.9)	4(10.5)	**0.30**
>50	69(82.1)	34(88.5)	
2.Sex			
Female	26(31)	7(18.4)	**0.14**
Male	58(69)	31(81.6)	
3.Etiology			
A+S	53(63.1)	30(78.9)	**0.10**
S	23(27.4)	5(13.2)	
A	3(3.6)	-	
None	5(6)	3(7.9)	
4.Location			
Oral cavity	61(72.6)	23(60.5)	**0.20**
Oropharynx	19(22.6)	10(26.3)	
Hypopharynx	4(4.8)	5(13.2)	
5.Depth of invasion			
≤1 cm	41(48.8)	19(50)	**0.20**
>1cm	43(51.2)	19(50)	
6.Grade	`		
WD K	37(44)	7(18.4)	**0.02**
WD NK	0	2(5.3)	
MD K	26(31)	11(28.9)	
MD NK	10(11.9)	9(23.7)	
PD	11(13.1)	9(23.7)	
7.Keratinisation			
Positve(K)	63(75)	18(47.4)	**0.05**
Negative(NK)	21(25)	20(52.6)	
8.PNI			
Positive	18(21.4)	12(31.6)	**0.22**
Negative	66(78.6)	26(68.4)	
9.LVI			
Positive	17(20.2)	15(39.5)	**0.02**
Negative	67(79.8)	23(60.5)	
10.TILs			
Positive	55(65.5)	33(86.8)	**0.01**
Negative	29(34.5)	5(13.2)	
11.STAGE			
I	21(25)	9(23.7)	**0.04**
II	33(39.3)	11(28.9)	
III	11(13.1)	1(2.60)	
IV	19(22.6)	17(44.70)	

Table 3 (C) Result of IHC of Ki67

Parameters	Low (35)%	Moderate (75)%	High (12)%	p value
1.Age				
≤ 50	4(11.4)	14(18.7)	1(8.3	0.58
>50	31(88.6)	61(81.3)	11(91.7)	
2.Sex				
Female	12(34.2)	21(28)	0	0.04
Male	23(65.7)	5472)	12(100)	
3.Etiology				
A+S	24(68.6)	49(65.)	11(91.7)	0.26
S	10(28.6)	18(24)	-	
A	-	2(2.7)	-	
None	1(2.8)	6(8.0)	-1(8.3)	
4.Location				
Oral cavity	30(85.7)	48(64)	6(50)	0.001
Oropharynx	5(14.2)	22(29.3)	4(33.3)	
Hypopharynx	-	5(6.7)	2(16.7)	
5.Depth of invasion				
≤1 cm	20(57.1)	33(44)	7(58.3)	0.26
>1cm	15(42.9)	42(56)	5(41.7)	
6.Grade				
WD K	21(60)	22(29.3)	1(8.3)	0.04
WD NK	0	2(2.7)	0	
MD K	11(31.4)	24(32)	1(8.3)	
MD NK	3(8.6)	17(22.7)	0	
PD	0	10(13.3)	10(83.3)	
7.Keratinisation				
Positve(K)	33(94.2)	46(61.3)	2(16.7)	0.03
Negative(NK)	4(11.4)	29(38.7)	10(83.3)	
8.PNI				
Positive	9(25.7)	18(24)	3(25)	0.41
Negative	26(74.2)	57(76)	9(75)	
9.LVI				
Positive	6(17.1)	24(32)	2(16.7)	0.32
Negative	29(82.9)	51(68)	10(83.3)	
10.TILs				
Positive	22(62.9)	57(76)	9(75)	0.48
Negative	13(37.1)	18(24)	3(25)	
11.STAGE				
I	11(31.4)	16(21.3)	3(25)	0.06
II	8(22.9)	30(40)	6(50)	
III	5(14.2)	7(9.3)	0	
IV	11(31.4)	22(29.3)	3(25)	

There were no significant correlation of p16 expression with age, sex, etiology, depth of invasion, perineural invasion and stage of disease however it does have significant association with tumour location, grade, keratinisation, TILs and LVI.

Majority of patients in our study are males, understandably due to male predominant nature of Armed forces with cut off value of 50 years to label as adult. 42 patients out of 122 resection cases were between 51 to 60 years and showed high p 16 positive (50%). Percentage of p16 positivity may have been much higher (approximately 61.1% out of 54 p16 positives) if we use the cut off value of 60 years.

58 % of the tumors were located in oral cavity and 28% in oropharynx. 55 % of tumours in oropharynx shown positive p16 whereas those of oral cavity showed 40 % p 16 positivity. Hypopharyngeal tumors showed high positivity for p16 as 7 out 9 pyriform fossa tumours (77.8%). This finding was statistically significant (p value<0.05). Whereas p53 expression in tumors of oral cavity is higher (77.4%) followed by oropharynxv(29.6%). Tumors of pyriform fossa are less positive for positive p 53(2.8%)

Of 122 resected cases, 54 cases (44.2%) were p16 positive cases. p16 expression is commoner in patients who both smoked and consumed alcohol (68%) whereas p53 expression was more amongst smokers (63%). Those who did not use either of them showed mixed expression of p16 and p53. There are 12 cases out of 122 cases which showed both positivity for both p16 and p53 with varying expression of Ki67.

p16 expression is found more in moderately differentiated SCC (29.6%) and poorly differentiated SCC (22.2%). p53 expression is more in well differentiated carcinomas.

Non keratinizing SCC showed maximum positivity of p16 in our study (55.6%). P53 expression was found more with Keratinizing SCC. There was a significant correlations of p16 and p53 expression with keratinization in our study (p value< 0.05)

In our study out 122 cases 88% of the cases showed presence of TILs and it was statistically significant with p16 and p53 immunostaining. Staging was done only in resected specimens. Of 122 cases, 30(24.6%) were in stage I, 44(36.1%) in stage II, 12(9.8%) in Stage III & 36(29.5%) in stage IV.

Of 54 p16 positives cases, 20 (37.0%) belonged to stage IV and 19(35.2%) belong to stage II. Of 84 p53 positives cases, 33 (39.3%) belonged to stage IV and 19(22.2%) belong to stage II . Ki67 expressions were mostly moderate in all the stages.

DISCUSSION

HPV related malignancies represent 5-20% of all HNSCCs and 40-90% of those arising from the oropharynx [11,12,13]. HPV-related oropharyngeal squamous cell carcinomas (OPSCCs) account for 40-80% of OPSCCs diagnosed in the United States [14] HPV related HNSCCs are more common in the age group of 40-55years without any environmental risks factors [5,15]. Traditional HNSCCs were more common in males and elderly persons [16].

HPV related HNSCCs are more common in non smokers and non alcoholics but in our study it is not so. p16 expression is found in 68% patients who both smoked and consumed alcohol Possible reasons for these could be the effect of synergistic action of both tobacco smoking and HPV virus in inducing carcinogenesis. The discussion about association of HPV and smoking with the carcinogenesis of OSCC is controversial in literature [17, 18]. It is known that patients with HPV-associated HNSCC have less heavy nicotine abuse, a significant amount of them are ex or light smokers and smoking might act as an additional risk factor. Nevertheless, positive serum antibodies against HPV E6/E7 revealed a 56.2-fold increased risk for OSCC for patients who had smoked [19]. So, there is a need for further studies to established the interaction between tobacco/nicotine use and HPV.

HPV positive SCCs are more non keratinizing type and this is well correlated with the findings by Chernock RD et al. [20] and LewisJS Jr et al.[21]. NK SCC occurred in slightly younger patients that were more often male. It more frequently presented with lymph node metastases and was surgically resected compared to K SCC [20].

Presence of perineural invasion is associated with poor prognosis in terms of decreased overall survival rate[(22)]. Lymphovascular invasion is associated with poor prognosis and its presence is associated with nodal involvement and ultimately rising the stage of the tumor [23]. Tumor infiltrating lymphocytes are associated with favorable outcome [24, 25]. In our study out 122 cases 88% of the cases showed presence of TILs and it was statistically significant with p16 and p53 immunostaining.

Majority of patients in our study (44%) are in stage II and nearly half of patients are p 16 positive. In advanced stage, disease is associated with poor prognosis however p16 positive advanced disease has a better prognosis as these tumor are sensitive to chemoradiation and thus improved the survival of the patient[26,27,28]. Though better prognosis for early stage disease but still little has altered for advanced disease in the past few decades [29].

Treatment is generally multimodal, consisting of surgery followed by chemoradiotherapy (CRT) for oral cavity cancers and primary CRT for pharynx and larynx cancers. The EGFR monoclonal antibody cetuximab is generally used in combination with radiation in HPV-negative HNSCC where comorbidities prevent the use of cytotoxic chemotherapy. The immune checkpoint inhibitors pembrolizumab and nivolumab are used for treatment of recurrent or metastatic HNSCC and pembrolizumab as primary treatment for unresectable disease [30].

CONCLUSION:

HPV related HNSCCs are on the rise and are commonly seen in sexually active individuals. They have a sexual route of transmission. Non HPV related HNSCCs are on the decreasing trend worldwide while incidence of HPV related HNSCCs are increasing and is expected to increase more in the coming years. Tobacco smoking may still play a role in the HPV related HNSCCs. For this, further studies are required to establish the association of tobacco smoking and HPV related HNSCCs, especially oropharyngeal SCCs. Role of p16 immunohistochemistry as surrogate marker for in HPV infection well documented especially in SCCs of oropharyngeal region but p16 positivity is also found in other site of head and neck, though in lesser extent. Non keratinizing HNSCCs were found to have more p16 expression while keratinizing type showed more of p53 expression. In our study overlap of both p16 and p53 immunohistochemistry was found in 18 cases which require further study to prove its significant association.

Most importantly, follow up and survival data could not be assessed due to loss of follow up of majority of these patients and due to other technical and logistic reasons. Further large-scale studies are required to assess the survival data and outcome of various modes of treatment.

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