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Original Research Article

p16^{INK4a} An Independent Prognostic Marker in Head and Neck Squamous Cell Carcinomas

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Abstract

Introduction/Background: The aim of the present study is to determine the expression of p16^{INK4a} and its prognostic utility in the head and neck squamous cell carcinomas (HNSCC). Squamous cell carcinoma of the head and neck is a major public health problem in developing countries. A subset of head and neck cancer is associated with the human papillomavirus (HPV) [1]. Viral infection is closely correlated with expression of p16^{INK4a} in these tumours. p16^{INK4a} is the strongest independent prognostic marker in HNSCC and is associated with favourable prognosis [1]. *Materials and Methods:* The present study is retrospective study done during the period from June 2016 to July 2017. A total of 52 cases were taken and histological grading was done according to WHO criteria. p16^{INK4a} expression was determined by immunohistochemical staining on the paraffin embedded tissue sections. *Results:* Out of 52 cases, 41/52 cases (78.8%) were positive for p16^{INK4a} and remaining 11/52 cases (21.2%) were negative for p16^{INK4a}. Expression of p16^{INK4a} was graded accordingly. *Conclusion:* p16^{INK4a} is an independent prognostic marker for HNSCC. p16 protein overexpression is very sensitive for the presence of transcriptionally active HPV and is widely available and also easy to interpret the test result. Expression of p16^{INK4a} has major impact on treatment response and survival in patients with HNSCC.

Keywords: Head and Neck Squamous Cell Carcinomas (HNSCC); Human Papilloma Virus (HPV); Immunohistochemistry; p16^{INK4a}.

Introduction

Squamous cell carcinoma of the head and neck is a major public health problem in developing countries. A subset of head and neck cancer is associated with the human papillomavirus (HPV). Viral infection is closely correlated with expression of p16^{INK4a} in these tumours [1]. p16, also designated MTS1 (multiple tumour suppressor 1) and p16^{INK4a}, is one of the most extensively studied proteins in the past decades due to its critical roles in cell cycle progression, cellular senescence, and the development of human cancers [2].

HPV-associated cancers are caused by expression of HPV's E6 and E7 proteins that bind to and inactivate tumour suppressor proteins p53 and

retinoblastoma protein (pRb) respectively leading to malignant transformation of HPV infected cells [3]. pRb is functionally inactivated by binding of viral protein and no longer acts as a cell cycle inhibitor. HPV E7 binds to hypophosphorylated form of Rb. The binding occurs in Rb pocket that sequesters E2F transcription factors. Thus, pRb, unable to bind the E2F transcription factor is functionally inactivated and transcription factor are free to cause cell cycle progression [4,5]. Together, these findings demonstrate that both the transcriptional level and translational status of p16 are critical for its overall ability to mediate cellular activities. Nuclear and cytoplasmic positivity of p16 can be explained on this mechanism.

p16 expression is now being used as a surrogate marker of HPV infection in head and neck squamous cell carcinoma and thus is expected to be showing differences depending on endemicity of HPV infection [6]. In the developing countries like India, tobacco chewing and smoking are the etiological risk factors for the causation of head and neck squamous cell carcinomas [1] [3] [7].

P16 expression can help in providing important prognostic information and future therapies aimed at targeting this pathway of HPV tumorigenesis. P16 expression is associated with better response to treatment, favourable prognosis and increased disease free survival rate [1] [8]. The present study has been undertaken to study expression of P16 in HNSCC.

Materials and Methods

The present study is a retrospective study done during the period of one year in Chalmeda Anand Rao institute of medical sciences, Karimnagar from July 2016 to June 2017. A total of 52 archival primary HNSCC specimens received in the pathology department were included in the study. All the specimens were fixed in 10% formalin and embedded in paraffin. Routine hematoxylin and eosin staining was done on the paraffin embedded blocks. Histopathological grading was given according to CAP protocols as well differentiated, moderately differentiated and poorly differentiated.

Expression of p16 protein in 5 μM FFPE sections on poly-L-lysine coated slides were evaluated using G175-405 clone (Biogenix, USA). After epitope retrieval, sections were incubated with prediluted primary mouse antihuman p16INK4a antibody for 30 min followed by chromogenic substrate diaminobenzidine and counterstaining with harris hematoxylin. P16 expression in all immunostained slides were evaluated and scored independently by two of the pathologists. Differences in interpretation were reviewed jointly to obtain a consensus.

Immunostaining in tumors was quantified based on the intensity score of nuclear (N) and cytoplasmic (C) staining and percentage of the cells stained in nuclear and cytoplasmic staining. Final score is given by multiplying intensity score and positivity score, the minimum score being 0 and a maximum score being 12 as shown in Tables 1&2 [9].

Table 1: Immunostaining of tumors

Score	0	1	2	3	4
Intensity score of nucleus and cytoplasm	None	Weak	Moderate	Strong	
Positivity score of nucleus and cytoplasm	0%	1%-10%	11%-50%	51%-80%	81%-100%

Table 2:

Total score	
0-3	Negative staining of p16
4-12	Positive staining of p16

Table 3: Socio-Demographic Factors

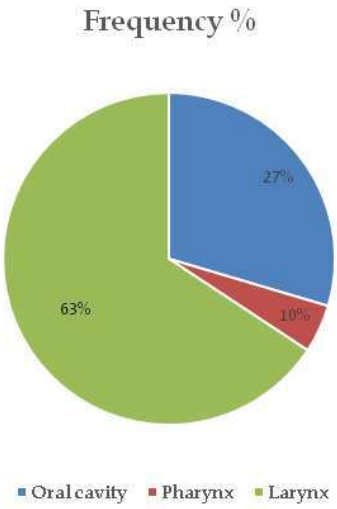
Age (years)	Frequency (%)
<40	05 (9.6%)
41-50	09 (17.3%)
51-60	17 (32.7%)
61-70	11 (21.2%)
>70	10 (19.2%)
	52 (100%)
Sex	
Male	42 (80.8%)
Female	10 (19.2)

Results

A total of 52 cases diagnosed as HNSCC (oral cavity, pharyngeal and laryngeal carcinomas) at the Chalmeda Anand Rao institute of medical sciences (Karimnagar) from June 2016 to July 2017 were recruited. The age of the patients ranged between 31 to 85 years with a mean age of 58 years. The males were significantly more affected than females as shown in Tables 3,4,5,6, 7 and 8.

Table 4: Etiological Risk Factors

	Smoking	Tobacco Chewing	Both	None	Total
Males	09(17.3%)	18(34.6%)	10(19.2%)	05(9.6%)	42
Females	02(3.8%)	06(11.5%)	NIL	02(3.8%)	10
Total	11(21.1%)	24(46.2%)	10(19.2%)	07(13.4%)	52



Graph 1: Site of Tumour

Table 5: Tumour Differentiation

Diagnosis	Cases
Well Differentiated	13 (25%)
Moderately Differentiated	31 (59.6%)
Poorly Differentiated	08 (15.4%)

Table 6: Correlation of Histopathological Grade and Demographic Features

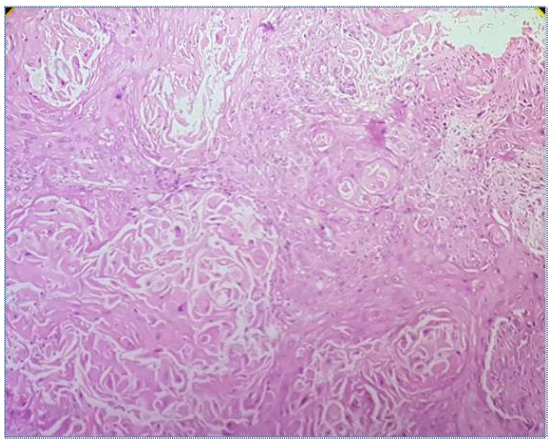
	Well Differentiated	Moderately Differentiated	Poorly Differentiated
Age Group (Years)	13	31	08
<40	03 (5.8%)	01 (1.9%)	01 (1.9%)
41-50	01 (1.9%)	07 (13.4%)	01 (1.9%)
51-60	05 (9.6%)	10 (19.2%)	02 (3.8%)
61-70	02 (3.8%)	09 (17.3%)	Nil
>70	02 (3.8%)	04 (7.7%)	04 (7.7%)
Sex			
Male	09 (17.3%)	25 (48.1%)	08 (15.4%)
Female	04 (7.7%)	06 (11.5%)	Nil

Table 7: p16INK4a Expression

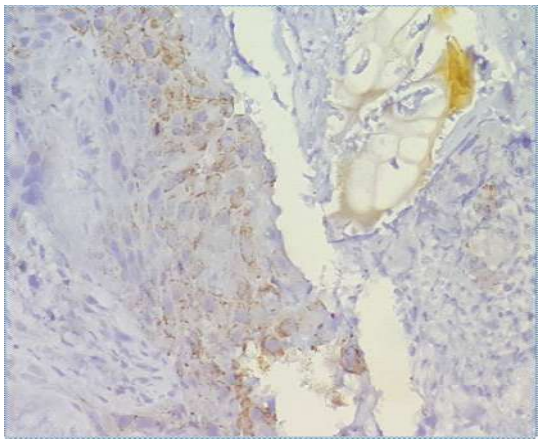
HNSCC	Frequency
P16 positive	41 (78.8%)
P16 negative	11 (21.2%)

Table 8: P16INK4a Expression in Relation to Socio-Demographic Factors

	Males	Females	Well Differentiated	Moderately Differentiated	Poorly Differentiated	Total
p16 positive	35(67.3%)	06(11.5%)	07(13.5%)	26(50%)	08(15.4%)	41(78.8%)
p16 negative	07(13.5%)	04(7.7%)	06(11.5%)	05(9.6%)	NIL	11(21.2%)
Total	42	10	13	31	08	52

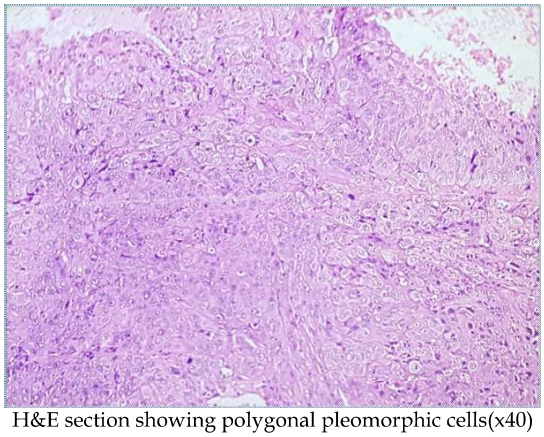


H&E section showing keratin pearls (x40)

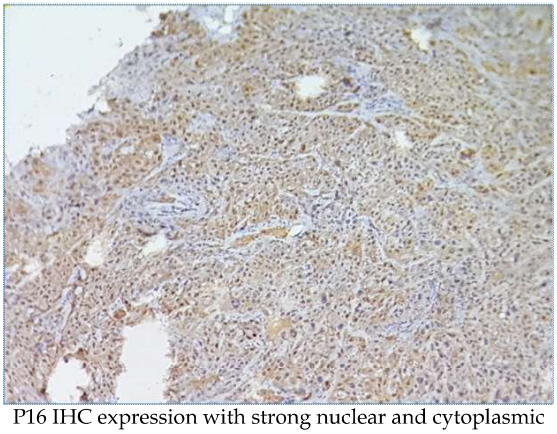


p16 IHC showing weak cytoplasmic positivity

Fig. 1&2: Well Differentiated Squamous Cell Carcinoma

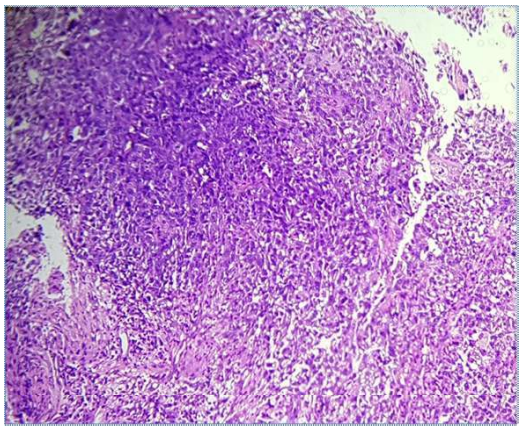


H&E section showing polygonal pleomorphic cells(x40)

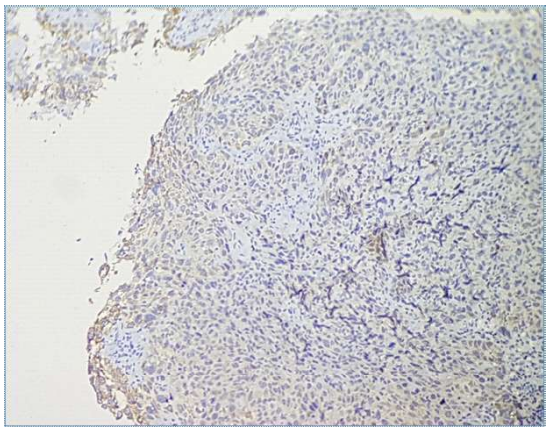


P16 IHC expression with strong nuclear and cytoplasmic positivity

Fig. 3 & 4: Moderately Differentiated Squamous Cell Carcinoma



H&E section showing undifferentiated cells



P16 IHC showing nuclear and cytoplasmic positivity

Fig. 5&6: Poorly Differentited Squamous Cell Carcinoma

Table 9: Comparision of the Studies

Study	Number of Cases	P16 Positive	P16 Negative
Pernille Lassen et al (Denmark 2009)	156	35 (22.4%)	121 (77.6%)
C.A.Fischer et al (Switzerland 2009)	102	41 (40.2%)	61 (59.8%)
James S Lewis Jr et al(St.Louis,MO 2010)	239	187 (78%)	52 (22%)
N Sgaramella et al (Sweden 2015)	109	36 (33%)	73 (67%)
Megha Ralli et al (Haryana 2016)	75	59 (78.7%)	11 (21.3%)
Murthy et al (Maharastra 2017)	170	34 (20%)	36 (80%)
Present study	52	41 (78.8%)	11 (21.2%)

Discussion

A total of 52 cases, diagnosed of primary head and neck squamous cell carcinoma were included in the study. The HNSCC were more common in males than in females at a ratio of 4.2:1. The age range from 31 to 85 years with a mean age of 58 years. The age group most affected was 51-60, followed by 61-70 age groups. These results were similar to findings from a previous study carried out by Murthy et al. [7], which reported that HNSCC was more common in males than females, it was noted that the most affected age group was above 54 years. In our study most of the patients had habit of tobacco chewing about 46% and next followed the smoking habit. Few of them had both the habits. These results were similar to the study of Ralli et al. [10] as shown in Table 9.

Majority of the tumours were from the oral cavity followed by larynx and pharynx in the present study.These findings are similar to those reported by Gathere et al. [11] who found that the most frequent tumour were the oral cancers followed by the nasopharynx, larynx and oropharynx respectively. With regard to histological grade the majority of the tumours were moderately differentiated followed by the well differentiated tumours, and the poorly differentiated.

These findings varied in Murthy et al. [7] where majority of the cases were well differentiated followed by moderately differentiated, and poorly differentiated tumours as shown in figure 1 & 2. These differences could be due to different geographic sites, HPV infection rates, smoking, alcohol and other environmental factors. Multiple studies on the association between histological grade and prognosis have shown that there is a strong association between histological grade and survival rates in patients with HNSCC.

Of the 52 tested HNSCC for p16, (78.8%) were found to be positive for p16. These findings were in concordance with the studies conducted by Lewis et al and Ralli et al. [10]. Majority of p16 positive HNSCC were found amongst males (67%). About half of the p16 positive HNSSC were moderately differentiated followed by the poorly differentiated carcinomas at about 15%. These findings were similar to that of Ralli et al. [10] where majority of the moderately differentiated tumours showed strong p16 expression as shown in figures 3 & 4.

Mendelson et al. [12] found that HPV positive and P16 positive are highly predictive for poorly differentiated tumours. The differences between these studies could be due to the low numbers of poorly differentiated squamous carcinoma in the present study. In the present study the poorly differentiated HNSCC were all p16 positive with strong expression as shown in figure 5 & 6. About 22% of the cases in this study were negative for p16, similar to the Lewis et al. [13] study.

Amidst the epidemic of HPV-related HNSCC, tests are needed for several purposes: for discerning favourable from unfavourable tumour types, for patient counselling about disease and prognosis, for treatment regimens appropriate to their tumour's biology, to identify the actual presence of HPV for virus-specific treatments, and to identify epidemiological trends. The most pressing of these needs is risk stratification. A consensus method for risk stratifying patients with HNSCC is becoming critical.

p16 IHC is emerging as a suitable single test for this purpose. It has been thoroughly studied, is widely available, inexpensive, and is easy to interpret, with clear guidelines on what staining cutoff to use. In fact, the 2011 National Comprehensive Cancer Network guidelines for head and neck cancer recommend p16 IHC testing alone for the purpose of counselling patients with oropharyngeal SCC about their prognosis and utilizing p16 IHC alone as the basis for patient stratification.

Conclusion

p16 protein overexpression is very sensitive for the presence of transcriptionally active HPV

and is widely available and also easy to interpret the test result. p16 expression can be used as a surrogate marker for HPV infection in HNSCC. $p16^{INK4a}$ is an independent prognostic marker for HNSCC. Expression of $p16^{INK4a}$ has major impact on treatment response and survival in patients with HNSCC. $p16^{INK4a}$ expression is associated with a favourable prognosis.

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