DOI: https://doi.org/10.53350/pjmhs221611072

ORIGINAL ARTICLE

Expression of P16 in Head and Neck Squamous Cell Carcinoma

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ABSTRACT

Introduction: Head and neck squamous cell carcinoma (HNSCC) is a malignant neoplasm that arises in the oral cavity, nasal cavity, paranasal sinuses, oropharynx, nasopharnx, and larynx. Among various prognostic markers in HNSCC, demonstration of p16 protein expression is of great importance.

Objective: To determine the frequency of p16 expression in head and neck squamous cell carcinoma by Immuno-histochemistry (IHC) in patients reporting at Rehman Medical Institute.

Methodology: This was descriptive, cross sectional study piloted at the Department of Histopathology, Rehman Medical Institute (RMI) for a period of six months from 10/March/2016 to 10/September/2016. This study was carried out on a total of 103 patients diagnosed with head and neck squamous cell carcinoma. On Formalin Fixed Parafin embedded blocks of already diagnosed OSCC cases, IHC staining was done by using p16 (monoclonal) antibody on FFPE blocks of OSCC. Statistical analysis of the data was done by using the software SPSS version 20.

Results: p16 was positive in 95 cases (92.2%) and negative in 8 cases (7.8%). Chi-square test was performed between p16 and site of biopsy, a significant relation was observed with P-value 0.023 (<0.05). Of all the positive cases, p16 was positive in 51 cases (53.7%) of oral cavity SCC, 17 cases (17.9%) of pharyngeal SCC, 23 cases (24.2 %) of laryngeal SCC and 4 cases (4.2%) of nasal cavity SCC.

Conclusion: High expression of p16 was observed in HNSCC cases with correlation to various sites of Head and Neck.

Keywords: HNSCC, p16; Prognosis, Cancer

INTRODUCTION

Head and neck cancers are malignant neoplasms arising in the oral cavity, nasal cavity, paranasal sinuses, oropharynx, nasopharnx and larynx. The majority of head and neck cancers histologically belongs to squamous-cell type and hence designated as Head and neck squamous cell carcinoma (HNSCC). It is one of the major emerging health problems, ranked sixth commonest cancer worldwide, and accounting for approximately 4% of all the tumors [1, 2].

It is the second commonest malignancy in Pakistan [3, 4]. Rapid advancements in treatment and early detection of HNSCC have significantly impacted the overall survival rates of the affected patients (about 50-65% at 5 years) [5]. In addition, development of novel biomarkers for early detection offers an opportunity to improve overall cancer outcomes.

A number of risk factors and genetic mutations are involved in the development of HNSCC. Cigarette smoking and alcohol abuse are known risk factors for HNSCC, although infection with high-risk Human Papilloma Virus (hrHPV) is another emerging risk factor, accounting for 20-25% of all cases of HNSCC globally [2, 6, 7]. In particular, HPV16 and 18 play a role in the pathogenesis of HNSCC. HPV16-positive patients have been reported to have better survival than HPV16-negative patients [8, 9]. HPV 16 is also etiologically associated with various benign and malignant neoplasms of the skin and mucosa [10].

Prevalence of HPV in HNSCC in different continents varies, as in Europe and North America the prevalence is about 40.5% to 72.2%, [11] in Australia it is 63.5 % [12] and 33 % in Asia [13]. In different countries the prevalence also varies; in United States of America the prevalence is 70%,[14] in Netherland it is about 29.0% [15] and in England the prevalence during the last few decades is estimated to be 58 % [16]. In India it is about 33.6% to 67% while in Pakistan the estimated prevalence of HPV related HNSCC is 89.4% to 90% [17].

During the past few decades molecular and genetic abnormalities have been identified as the underlying cause of HNSCC. A large variety of genes suppression mutations, deletions and translocations have been detected by different methods which are involved in the development of HNSCC. Among them the most common are p16, p53, cyclin D1, p63 PPTEN, Rb and Epidermal growth factor receptor.

p16 a tumor suppressor gene involved in many malignant processes of the body is also identified to have a role in the etiology of HNSCC. The main function of p16 in the body is to suppress the tumor process, but genetic mutation may result in the development of HNSCC [18]. The expression of p16 protein can be detected by different methods like; Immunohistochemistry (IHC), Real Time-Polymerized Chain Reaction (PCR) and fluorescent in situ hybridization (FISH) [19]. Different IHC markers are available for the detection of p16 [20, 21]. Detection of p16 by IHC has been established in 78 to 88%of HNSCC [22].

To the best of our knowledge, frequency of p16 in HNSCC patients has not been reported in the local population of Khyber Pukhtunkhwa. Therefore, the objective of this study was to determine the frequency of p16 expression in head and neck squamous cell carcinoma

by Immunohistochemistry (IHC) in patients reporting to Rehman Medical Institute. On the basis of our study findings, it will provide us with local statistics of the magnitude of p16 expression in HNSCC and therefore, open a window for further research.

MATERIALS AND METHODS

This was descriptive, cross sectional study piloted at the Department of Histopathology, Rehman Medical Institute (RMI) for a period of six months from 10/March/2016 to 10/September/2016. The inclusion criteria in our study were consecutive, already diagnosed and H&E stained cases of head and neck SCC, FFPE tissue blocks of OSCC cases and patients of both the gender from 18-60 years. The exclusion criteria were cases where site of the biopsy was other than Head and Neck, cases where site of the biopsy was not mentioned, poorly fixed biopsies and fragmented biopsies where stain interpretation is not possible. Sample size calculation was done by using WHO sample size calculator, with confidence level (1 alpha) = 95%, absolute precision (d) = 0.8% and anticipated proportion of p16 expression in SCC (p) = 78% [22]. Total sample size calculated was 103. The study was conducted after written permission was obtained from hospital ethical and research committee. The newly diagnosed cases of head and neck squamous cell carcinoma i.e. nasal, nasopharyngeal, oral cavity, oropharyngeal and laryngeal SCC from both indoor and outdoor patients, from 10/ March/ 2016 to 10/ September/ 2016, meeting the inclusion criteria and presented to the Rehman Medical Institute(RMI) laboratory were included in the study. The purpose and benefits of the study was explained to all researchers of this project and a written informed consent was obtained.

The biopsy specimens were received in 10 % buffered formalin labeled with patient's name and hospital number. These samples after assuring site of biopsy and patient profile were given specific histopathological number.

The small biopsies as a whole tissue and from large biopsies representative sections were taken and placed in plastic cassettes, properly labeled with graphite pencil. These cassettes were then transferred to tissue processor containing different chemicals in different concentration, to be processed for 16 hrs.

In tissue processor the tissue were first dehydrated by the increasing strengths of alcohol e.g. 70% for two hrs, 90% for four hrs and absolute alcohol for four hrs. During dehydration the water in the tissue has been replaced by alcohol. In the next step alcohol was cleared from the tissues by placing in Xylene for two hrs. Finally the tissues were impregnated with paraffin wax by placing them in wax for four hrs.

After processing the tissues, paraffin blocks were made by placing them in a metal labeled mould and then fresh molten wax was poured in it and allowed to settle and solidify. From this paraffin embedded blocks thin sections of 3 µm were cut and placed on a glass slide.

The glass slides were stained with Haematoxylin and Eosin stain by using the international staining protocols. After staining, the slides were air dried and mounted by using cover slips and DPX (Distyrene, a plasticizer, and xylene) and labeled accordingly.

The stained and labeled slides were examined under light microscope (Olympus CX22LED) and diagnosis of squamous cell carcinoma was made. Those cases in which the diagnosis was not straight forward, an Immunohistochemical marker p63 was applied, which is positive in squamous cell carcinoma.

All the cases diagnosed as HNSCC were then stained with the Immunohistochemical marker p16, by using the following protocols. From the paraffin embedded blocks sections were taken on glass slide and were pretreated (antigen retrieval) by microwaving of tissue in citrate buffer to unmask the antigens.

The primary antibody; p16 (Dako;AntiCDKN2A/p16INK4a antibody[EPR1473]) was applied, which will bind to the antigen of interest and the excess primary antibody was washed with distilled water. Then biotinylated anti-IgG antibody (secondary antibody), was added which binds to the primary antibody present. After avidin-biotin-peroxidase complex, was applied which binds the secondary antibody.

Finally 3, 3' diaminobenzidine (DAB) was added as a chromogen (color changing reagent), and counterstaining with hematoxylin stain was done. Antigen antibody complex formation imparts color to the nuclei and cytoplasm of neoplastic squamous cells and was taken as positive. The p16 stained slides were air dried and mounted by using DPX and cover slips.

The slides were examined by under light microscope and p16 positive and p16 negative cases were separated. The positive cases were then compared to the control positive case of uterine serous adenocarcinoma. Statistical analysis of the data was done using the Statistical Package for Social Sciences (SPSS version 20). Frequency and percentages was calculated for categorical variable. Mean ± SD was calculated for continuous variable. p16 protein expression was stratified among age, gender and site of biopsy. Post stratification chi square test was applied, keeping p value <0.05 as significant.

RESULTS

Total of 103 patients were studied for frequency of p16 protein in HNSCC, there were 62 males and 41 females who were diagnosed as HNSCC. Mean age of the patients under study was 48.55 years (± 11.01 SD).

The frequency of oral cavity SCC cases, pharynx SCC cases, larynx SCC cases and nasal cavity SCC cases are given in Table 1.

IHC p16 positivity was seen in 95 cases (92.2%) of the total 103 cases of HNSCC and negativity was seen in 8 cases (7.8%). (Figure 1) The positive cases of p16 in oral cavity SCC cases, pharynx SCC cases, larynx SCC cases and nasal cavity SCC cases are given in table 2.

Stratification of IHC p16 according to gender showed that of total 62 males, p16 was positive in 58 cases and negative in 4 cases. Of total 41 females, p16 was positive in 37 cases and negative in 4 cases. There was no association found between IHC p16 and gender, which is statistically insignificant, having (P-value = 0.54).

Stratification of IHC p16 according to age was done (P-value= 0.285), showing maximum positive cases

between age 41-51 years, the maximum negative p16 results were of age 29-40 years. (Table 3)

Table 1: Frequency of oral cavity SCC cases according to different anatomic sites

Site of biopsy		Frequency	Percentage
Oral cavity (n=52)	Right Tongue	10	19.2
	Left Tongue	10	19.2
	Base of Tongue	4	7.7
	Gingiva	4	7.7
	Buccal mucosa	10	19.2
	Lower Lip	9	17.4
	Floor of mouth	5	9.6
Pharynx (n=21)	Oropharynx	6	28.6
	Nasopharynx	13	61.9
	Hypopharynx	2	9.5
Larynx (n=26)	Right Vocal cord	6	23.1
	Left Vocal cord	4	15.5
	Pyriform fossa	7	26.9
	Post Cricoid	3	11.5
	Epiglottis	3	11.5
	Supraglottis	3	11.5
Nasal cavity (n=04)	Lateral wall	3	75
	Medial wall	1	25

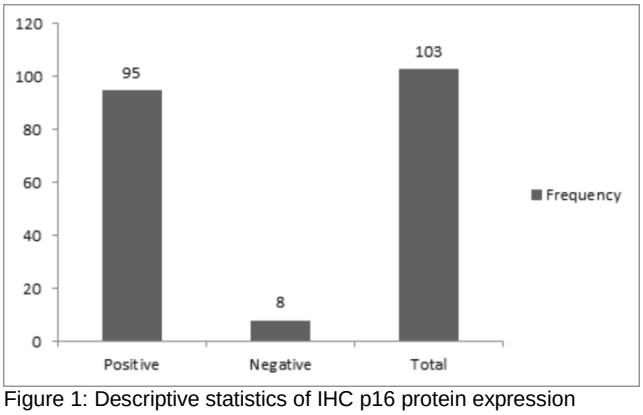

Figure 1: Descriptive statistics of IHC p16 protein expression

Table 2: Stratification of IHC p16 in oral cavity SCC cases, pharynx SCC cases, larynx SCC cases and nasal cavity SCC cases

Site of biopsy	IHC p16		
	Positive	Negative	P-value
Right tongue	10	0	0.023
Left tongue	9	1	
Base of tongue	4	0	
Gingiva	4	0	
Buccal mucosa	10	0	
Lower lip	9	0	
Floor of mouth	5	0	
Oropharynx	6	0	0.023
Nasopharynx	9	4	
Hypopharynx	2	0	
Right vocal cord	6	0	0.023
Left vocal cord	4	0	
Pyriform fossa	6	1	
Post cricoid	1	2	
Epiglottis	3	0	
Supraglottis	3	0	
Lateral wall	3	0	0.023
Medial wall	1	0	

Table 3: Cross tabulation of gender and aee with the IHC p16 result

Parameter		IHC p16 result		P-value
		Positive	Negative	
Gender	Male	58	4	0.540
	Female	37	4	
Age	18-29 years	5	1	0.285
	>29-40 years	34	5	
	>40-51 years	45	2	
	>51 years	11	0	

DISCUSSION

Head and neck squamous cell carcinoma is one of the rapidly emerging malignancies in the developing countries. Besides the site, grade and stage of the tumor, p16 has been shown to have a role in the prognosis of the tumor. For this purpose a study was conducted in RMI to determine the frequency of p16 in the patients with already diagnosed head and neck squamous cell carcinoma.

According to our study results, mean age was 48.5 yrs at the time of diagnosis with 62 (60.19%) males and 41 (39.81%) females. According to site, 52 cases from oral cavity, 21 cases from pharynx, 26 cases from larynx and 4 cases from the nasal cavity were studied for SCC and p16 protein expression. In our study, no statistical association was found between gender and site of biopsy (P-value = 0.201).

p16 IHC marker positivity was demonstrated in 95 of the total 103 cases (92.2%) which was comparable to the studies conducted by Walline HM et al. [23]Pannone G et al. [7] Geißler C et al. [24] in which the results were 83 % and 100% each respectively. The highest positivity of p16 was seen in the oral cavity SCC cases that is 53.7%, which is far better than the positivity seen by Stephen JK et al. [25] (20%) and Walline HM et al. [23] (26%). Statistical significance between p16 and oral cavity SCC was seen having (P- value = 0.023), which significance is also demonstrated by Stephen JK et al. [25]

Tongue of both sides collectively showed the highest positivity for p16, that was 37.2 % which is near the results of the study conducted by Gonzales-Moles M et al. [26] (68%).

Other sites of the oral cavity, in which p16 positivity was seen in the study were as follow; buccal mucosa (19.6%), lower lip (17.6%), floor of the mouth (9.8%) and base of tongue and gingiva (7.8%).

In pharyngeal SCC the positivity of p16 was seen in 17.9 % of the cases. Statistical significance between p16 and pharyngeal SCC was seen having (P- value = 0.023), which significance is also demonstrated by Stephen JK et al.[25]

In the study the oropharynx cases were 31.6 % positive for p16 which is consistent with the results of the studies conducted by Pannone G et al.[7] (25.6%) and Chandarana SP et al.[27] (57%).

In the nasopharynx the positivity of p16 was seen in 47.4% of the cases, which is closely related to the results of Bishop JA et al.[28] (34%) and Walline HM et al. [23] (50%).

In the hypopharynx the positivity of p16 was seen in 10.5 % of the cases which was consistent with that of Stephen JKet al. [25] (5%).

24.2% of the laryngeal SCC cases were positive for p16, which is closely related to the results of the studies

conducted by Stephen JK et al. [25](15%) and Chernock RD et al. [7] (27.6%). Statistical significance between p16 and laryngeal SCC was seen having (P- value = 0.023), which significance is also demonstrated by Stephen JK et al.[25]. Cases of vocal cord SCC were 38.4 % positive for p16. In pyriform fossa SCC the positivity was seen in 23%, which is consistent with the result of Yang J-Q et al. [29] (22.2%).

In epiglottis and supraglottis SCC p16 was positive in 11.5% of the cases. 3.9% of the post cricoid area SCC showed positivity which do not meet with the results of the study conducted by Yang J-Q et al. [29] (30.8%).

All the cases of nasal cavity SCC were positive for p16, which is in strong association with the study of Bishop JA et al. [28] in which all the HPV16 positive cases by FISH were also positive for p16 IHC.

The negativity of p16 IHC marker was seen in 8 cases (7.8%), which show variability with other studies conducted. In two of the studies none of the case was negative for p16 [8, 23, 24] while two of other studies show 22% and 24 % negativity [9, 21]. Of the total 8, p16 negative cases 4 were of the nasopharynx, 3 of the larynx and 1 case of the oral cavity.

No statistical association was seen between IHC p16 and age and gender (P-value = 0.285 and P-value = 0.540 respectively), similarly no statistical association was also not seen between p16 and age and gender in studies conducted by Chernock RD et al [7] and Stephen et al. [25] There are certain limitations of the study. No data regarding the occupation, marital status, smoking and eating habits of the patient was recorded. No follow up of the patient was done who received the medical care.

CONCLUSION

Head and neck squamous cell carcinoma is one of the most common and prevalent malignancy of this region of the country. Most of the cases of HNSCC are positive for p16 IHC marker, which is one of the prognostic markers. The study will help in the future follow up of the patient regarding their prognosis and survival.

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