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A Comparative Study on the Expression of p16 - a Cell Cycle Regulator Protein in Oropharyngeal and Laryngeal Squamous Cell Carcinoma.

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ABSTRACT

The association of oropharyngeal squamous cell carcinoma (OPSCC) with Human Papilloma Virus (HPV) infection has been established in recent years. HPV infection is associated with overexpression of p16 (a cell cycle regulator), which improves the survivability of these patients irrespective of the stage and grade. However, p16 expression among Laryngeal Squamous cell carcinoma (LSCC) has not been established clearly till date. This study aims to study and compare the pattern of p16 expression in OPSCC and LSCC. Out of a total 503 cases of OPSCC and LSCC, 30 cases each were selected from these two sites and subjected to Immunohistochemical (IHC) analysis for p16 expression. Statistical analysis was done using chi square test in SPSS 21.0 software. Our data showed that p16 overexpression was noted in 70% of OPSCC and 40% of LSCC and the most common sites involved were the base of tongue and hypopharynx. Comparative analysis showed that p16 expression in OPSCC was statistically significant (p-value=0.01). However, no significant association was observed among tumor grades. From our study, we conclude that p16 analysis by IHC alone can be used as a single reliable prognostic marker in OPSCC and LSCC.

Keywords: Cyclin-Dependent Kinase Inhibitor p16, Human Papillomavirus Viruses, Immunohistochemistry, Laryngeal squamous cell carcinoma, Orophayngeal squamous cell carcinoma, Squamous Cell Carcinoma of Head and Neck.

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INTRODUCTION

Cancer is a leading cause of death worldwide. According to World Health Organization (WHO) statistics 2020, nearly 10 million deaths occur due to cancer every year [1]. Moreover, it has been projected that cancer related deaths may increase up to 13.2 million by 2030. The characteristic feature of cancer is the rapid division of cancer cells that evade the activity of tumor suppressor genes and this occurs due to genetic and environmental alterations. The major risk factors come from environmental influences like cancer causing infections, usage of tobacco, alcohol consumption, smoking and increased body mass index [International agency for research on cancer (IARC)]. Cancer causing infections like human papilloma virus and hepatitis constitutes about 30% of cancer [2] cases in middle income countries [3].

Oropharyngeal and laryngeal SCC involve a multistep carcinogenesis process in which compound genetic events occur earlier and modify the normal function of proto-oncogenes and tumor suppressor genes. p16 is the cell regulatory and tumor suppressor protein encoded by CDKN2A gene. This acts by inhibiting cyclin dependent kinases (CDK4 and CDK6) leading to maintaining of retinoblastoma gene (Rb gene) in hypophosphorylated state. Hence, progression of cell cycle from G_1 phase to S phase is inhibited thereby, promoting cell to go for senescence or apoptosis [4,5].

Human papilloma virus (HPV) infection is one among the common infections causing squamous cell carcinoma (SCC) at the Oropharynx and larynx. E6 and E7 are oncoproteins expressed by HPV [6]. E6 oncoprotein degrades p53, a tumor suppressor gene. E7 oncoprotein degrades Retinoblastoma gene (Rb gene) which results in p16 overexpression. p16 expression inhibits cell cycle progression and leading to apoptosis of HPV infected cells, thereby remains as a very good surrogate marker for determining the prognosis of the patient.

MATERIALS AND METHODS

The study was conducted retrospectively to study the expression of p16 in different grades of oropharyngeal and laryngeal SCC and to compare the expression of p16 between oropharyngeal and laryngeal SCC at the Institute of Pathology, Madras Medical College for a period of one year. A total of 503 cases of SCC had been reported during that period constituting 242 SCC cases from oropharynx and 261 cases from larynx.

Inclusion criteria

All SCC reported in oropharynx and larynx specimens.

Exclusion criteria

Non-neoplastic, benign, premalignant cases and malignancies other than SCC are excluded from the study.

All cases of SCC had been graded as well, moderately and poorly differentiated grades according to the WHO grade of differentiation. A total of 60 cases were randomly selected, 30 each from oropharyngeal and laryngeal SCC comprising of 10 each from the three different grades. The paraffin blocks from these patients were taken and subjected to immunohistochemical analysis for p16 using "Super-sensitive polymer system" based on non-biotin polymeric technology. Heat induced antigen retrieval was done. The antigen was bound with mouse monoclonal antibody against p16 (PathnSitu). The expression was detected by adding secondary antibody which was conjugated with horse-radish peroxidase-polymer and DAB (diaminobenzidine) substrate. Ca Cervix slide was taken as positive control.

Expression of block positivity (intense nuclear and cytoplasmic positivity) was evaluated based on the five-point scale by James S. Lewis et al [7].

0	-	No staining
1+	-	Staining of 1 to 25% of tumor cells
2+	-	Staining of 26-50% of tumor cells
3+	-	Staining of 51-75% of tumor cells

4+ - Staining of 76-100% of tumor cells

These scores are further grouped into negative (score 0) and positive (score 1+ to 4+).

The results were tabulated in MS Excel sheet. The statistical analysis was done using Chi square test in SPSS 21.0 software. The p-value below 0.05 was considered significant.

All procedures performed in the current study were approved by the Institutional Ethical Committee (ECR/270/Inst./TN/2013, Ref No: 21092015 dated 08/09/2015) in accordance with the 1964 Helsinki declaration and its later amendments. Informed consent was obtained from all individual participants included in the study.

OBSERVATION AND RESULTS

Out of all the cases (n=503) included from the SCC of oropharynx and larynx, the major subtype was conventional type of SCC (98%) followed by papillary (1%), basaloid (0.6%) and spindle type (0.4%) (Table-1). The maximum incidence of oropharyngeal SCC was noted in the tongue (47.90%) predominantly in the posterior 1/3rd followed by buccal mucosa (17.4%) and tonsil (16.10%) (Table-2). In laryngeal SCC, the incidence was maximum in the hypopharynx (36.8%) followed by supraglottis (28%) and glottis (26%) (Table-3).

The expression of p16 was studied in 30 cases each of oropharyngeal and laryngeal SCC constituting 10 cases each from well differentiated (Figure-1A,1B), moderately differentiated (Figure-2A,2B) and poorly differentiated grades (Figure-3A,3B). 21 cases (70%) from Oropharyngeal SCC (Table-4) and 12 cases (40%) from laryngeal SCC (Table-5) showed positivity for p16 expression. The expression of p16 in Oropharyngeal SCC was more common compared to laryngeal SCC and was also statistically significant (Table-6).

DISCUSSION

In head and neck malignancies, squamous cell carcinoma remains the most common malignancy which has a great impact not only on patient survival, but also on the speech, swallowing and better well-being of the patient [8]. It remains the 6th most common cause of malignancy worldwide [9]. The highest incidence was noted in developing countries. These tumors were associated with multistep carcinogenesis which causes accumulation of mutations leading to precancerous lesions with progression to cancerous lesions and associated invasion and metastasis [10,11]. Various genetic and environmental factors were involved in these sequences.

It had been established many decades ago about the association of HPV infection and cervical cancer. This paved the way for the administration of HPV vaccine and the same had been included in the routine vaccination schedule among female population in reproductive age group [12]. With this concept, various studies were conducted and established the association of HPV infection with oropharyngeal squamous cell carcinoma (OPSCC) [13]. Much light had not been thrown to establish the association of HPV infection in laryngeal SCC [14,15]. Our study aimed to assess the incidence of p16 expression in OPSCC and laryngeal SCC, and also compare the pattern of p16 expression between OPSCC and laryngeal SCC.

Current study included 503 cases of SCC from oropharynx and larynx. The most common site of involvement in OPSCC was tongue (47.9%), followed by buccal mucosa (17.4%) and tonsil (16%). This was in concurrence with the study by Subha Bhat et al [16]. But in a study by Fischer C A et al [17], the incidence of SCC in tonsil was 46% which was much higher than buccal mucosa (7%) (Table-7).

In laryngeal lesions, it was observed that the site of distribution was maximum in hypopharynx. This distribution was compared with the study conducted by Eugenia Allegra et al [18] (Table-8).

Expression of p16

Strong nuclear and cytoplasmic staining was considered as positive for p16 expression. In our study, p16 expression was positively noted in 70% of OPSCC which was similar to other studies with p16

expression ranging between 65-80% (Table-9). Among laryngeal SCC group, p16 expression was noted in around 40% in our study which correlated with other studies which showed p16 expression in only 25-30% (Table-9). Among the various grades of SCC, maximum p16 expression was noted in well differentiated SCC followed by moderately differentiated and poorly differentiated SCC in both OPSCC and laryngeal SCC but there was no statistically significant correlation between grades and p16 expression [19].

In our study, we also compared p16 expression in OPSCC and laryngeal SCC. The expression of p16 in oropharyngeal SCC was more common compared to laryngeal SCC and was also statistically significant. This finding strongly supports the use of HPV vaccination in OPSCC patients as shown in previous studies by Giraldi et al [22]and Kumar et al [23] which revealed that HPV vaccination decreased the risk for HPV infection, head and neck SCC and various HPV associated diseases in both the genders.

A major limitation of our study is the small number of cases subjected to IHC which may explain the lesser incidence of p16 expression among laryngeal SCC. In spite of this, p16 positive cases of laryngeal SCC are likely to have good prognosis as shown by Eugenia Allegra et al [18] with less aggressive nature of malignancy and lowered risk of metastasis. In laryngeal lesions, HPV vaccine is already in use for the cases of recurrent papillomatosis [12]. A definite albeit reduced p16 expression in laryngeal SCC favours more widespread administration of HPV vaccination in at risk patients with a goal of reducing HPV associated laryngeal SCC.

Table 1: Histological subtypes of SCC of Oropharynx and Larynx

Histological subtypes	No. of cases (503)	Percentage (%)
Conventional	493	98%
Papillary	5	1%
Basaloid	3	0.6%
Spindle	2	0.4%

Table 2: Distribution of the site of involvement in SCC in Oropharynx

Site	Frequency	Percentage (%)
Buccal mucosa	42	17.4
Floor of mouth	7	2.90
Gingivobuccal sulcus	4	1.70
Hard palate	14	5.80
Lip	4	1.7
Retromolar trigone	9	3.70
Tongue	116	47.90
Tonsil	39	16.10
Soft palate	7	2.9
Total	242	100

Table 3: Distribution of the site of involvement in Laryngeal SCC

Site	Frequency	Percentage (%)
Glottis	68	26
Hypopharynx	96	36.8
Subglottis	24	9.2
Supraglottis	73	28
Total	261	100

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Table 4: Grade wise comparison of p16 expression in OPSCC

Grade	No. of cases	p16 (n=30)	
		Positive	Negative
Well Differentiated	10	9	1
Moderately Differentiated	10	7	3
Poorly Differentiated	10	5	5
Total	30	21 (70%)	9 (30%)
Chi square value- 3.8095; p-value = 0.148858			

Table 5: Grade wise expression of p16 in Laryngeal SCC

Grade	No. of cases	p16 (n=30)	
		Positive	Negative
Well Differentiated	10	6	4
Moderately Differentiated	10	4	6
Poorly Differentiated	10	2	8
Total	30	12 (40%)	18 (60%)
Chi- square value- 3.3333; p-value = 0.188876			

Table 6: Comparison of p16 expression between OPSCC and Laryngeal SCC

Site	p16- Positive	p16- Negative
Oropharyngeal SCC	21	09
Laryngeal SCC	12	18
Total	33	27
Chi square test = 5.4545; p-value = 0.019517		

Table 7: Comparison of distribution of OPSCC location

Tumor location	Current study	Subha bhat et al [16]	C A Fischer et al [17]
Tongue	47.9%	31%	40%
Buccal mucosa	17.4%	22%	7%
Tonsil	16.1%	9%	46%

Table 8: Comparison of distribution of laryngeal SCC location

Site	Current study	Eugenia Allegra [18]
Hypopharynx	36.8%	37.8%
Supraglottis	28.0%	27%
Glottis	26.0%	35.2%
Subglottis	9.2%	-

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Table 9: Comparison of p16 expression in OPSCC & Laryngeal SCC with other studies

SCC Location	p16 expression	Reference studies		
	Current study	James Lewis et al [7]	Pallavi Saxena et al [19]	Rally et al [20]
OPSCC	70%	78%	66%	78%
	Current study	Eugenia Allegra et al [18]	S Dai et al [21]	
Laryngeal SCC	40%	27.02%	30.6%	

Figure 1a: Tonsil-Well Differentiated SCC (400X)

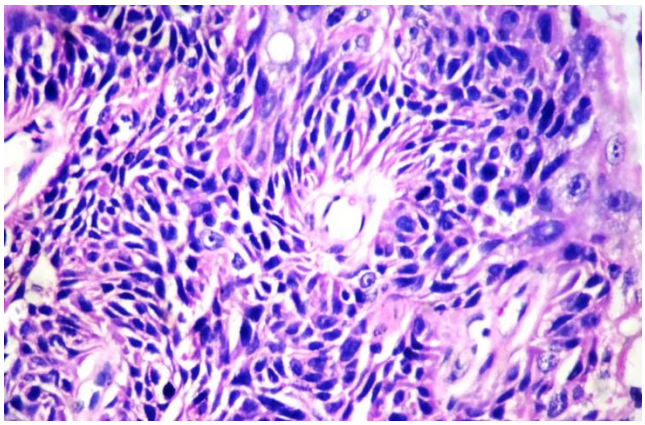

Figure 1b: Tonsil- Well Differentiated SCC IHC p16 –Positive 2+ (400X)

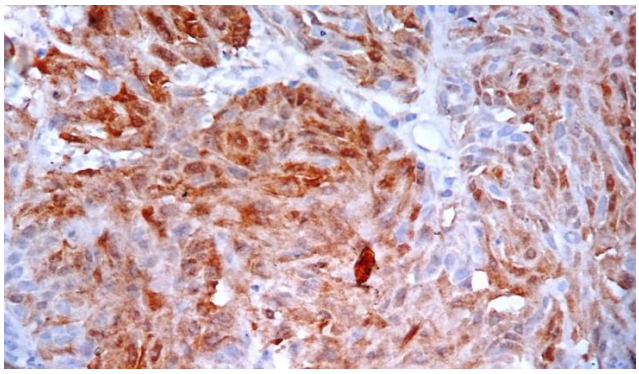

Figure 2a: Tongue- Moderately Differentiated SCC (100X)

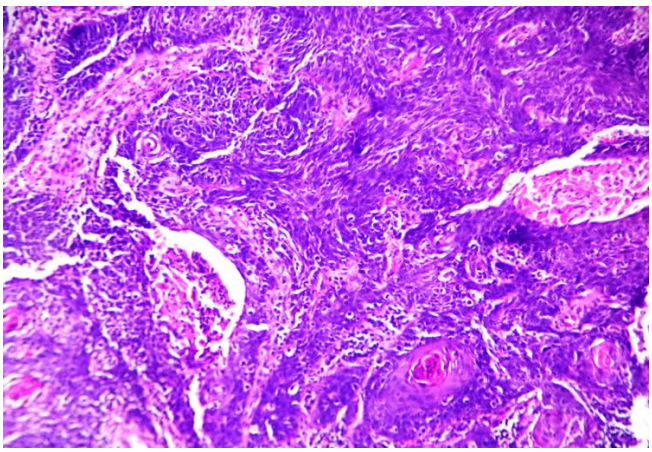

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Figure 2b: Tongue- Moderately Differentiated SCC IHC p16 – Positive 3+ (400x)

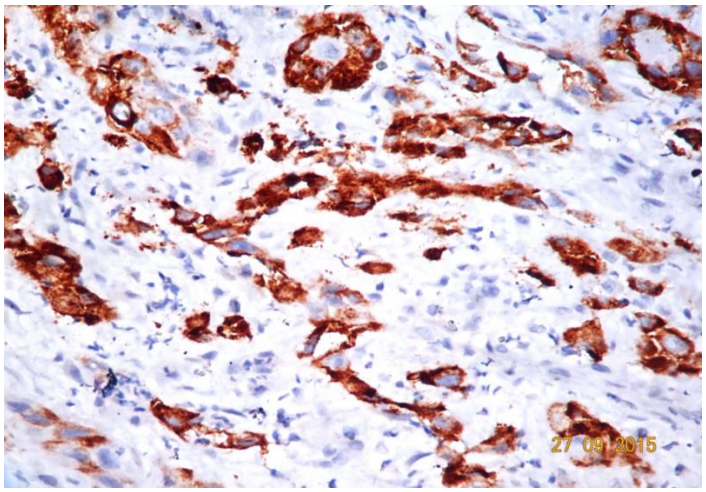


Figure 2b: Tongue- Moderately Differentiated SCC IHC p16 – Positive 3+ (400x)

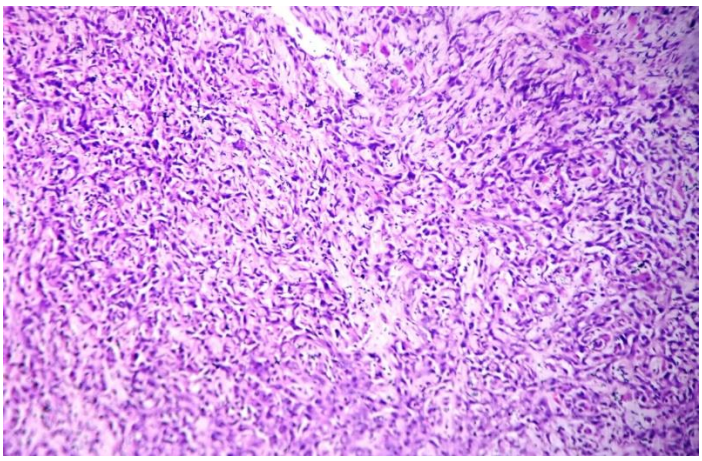
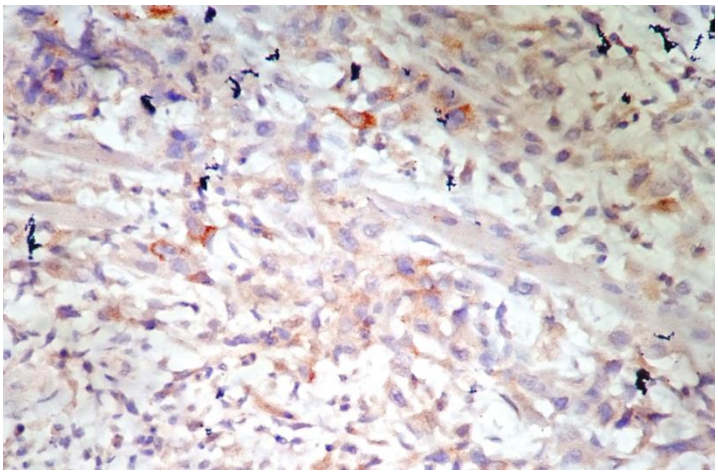


Figure-3b: Pyriform Fossa- Poorly Differentiated SCC IHC p16 – Negative (400X)



CONCLUSION

To summarize, in our study we noticed an overexpression of p16 in both OPSCC and laryngeal SCC. This may be associated with HPV infection or due to non-HPV causes. HPV infection can be detected by HPV-ISH (In Situ Hybridization) technique which is an expensive procedure. Moreover, various studies

done by HPV-ISH technique showed no difference existed between HPV-ISH positive/negative cases and the prognosis varied mainly with the expression of p16. This was due to the regulatory function of p16 protein potentiating the apoptosis of HPV infected cells and also the mutated cells (non-HPV cases).

Based on the data from our study, we conclude that p16 overexpression can be used as a single surrogate biomarker in risk stratification and as a prognostic indicator. To add on, we also suggest considering HPV vaccination which could be beneficial in preventing head and neck squamous cell carcinoma, thereby reducing overall disease burden, morbidity and mortality.

CONFLICT OF INTEREST

The authors report no potential conflicts of interest. The authors alone are responsible for the content and writing of this article.

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