Immunohistochemical expression of cell cycle proteins P16 and Cyclin D1 (expressed duding G1 phase of cell cycle in association with clinical and pathological features of squamous cell carcinoma of head and neck

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Introduction

Squamous Cell Carcinomas represent a bulk of the cancers related to the head and neck originating from the stratified squamous epithelial lining of the upper airways, oral and digestive system. The incidence of these Squamous cell carcinomas of the head and neck (HNSCCs) is on the rise in the Indian subcontinent with new patients being diagnosed every day [1].

In India, it is the most common cancer in men and third most common cancer in women, with incidence rates per 1,00,000 people as 12.8 and 7.5 in men and women respectively. The main risk factors are tobacco in the form of chewing and smoking and alcohol consumption. Survival of patients with oral squamous cell carcinoma has not improved in the last 40 years, despite recent advances in surgical procedures and the availability of new chemotherapeutic agents. Surgical resection results in significant functional and cosmetic defects, therefore it is important to develop conservative therapeutics, whereupon identification of markers representing oral squamous cell carcinoma aggressiveness would be worthwhile to decide the most suitable treatment for each patient from therapeutic options [2].

The etiology of HNSCC is multi-factorial. Genetic factors, diet, occupational exposure have been implicated along with traditional risk factors such as smoking, tobacco and alcohol consumption have been implicated in the etiopathogenesis of Head and Neck Squamous Cell Carcinomas (HNSCCs) and especially, the Human Papilloma Virus (HPV), when these classical risk factors are absent. Studies have postulated that patients with HPV associated HNSCCs have better response to treatment than their stage matched non-HPV related HNSCC[]. Thus, HPV related HNSCCs define a unique population of patients with distinct biology that should be treated separately from non-HPV related HNSCCs. Greater than 86% of HPV associated tumors overexpress p16, a cyclin dependent kinase inhibitor, with only 3% of non-HPV associated tumors over expressing it. p16 status is also reported as an independent prognostic factor for disease-free survival (DFS) with reported DFS of 84% and 46% for patients with p16 positive and p16 negative tumors respectively [3,4].

Research studies have implicated high–risk Human Papilloma Virus (HPV) as a risk factor for SCCs independent of the traditional risk factors which include tobacco abuse and ethanol consumption. While these high risk HPV associated SCCs appear to be associated with abnormal sexual behavior such as oral sex and increasing numbers of sexual partners, they lack an association with smoking and drinking. It is interesting to note that high risk HPV type 16 is the most prevalent subtype constituting 86% of HPV positive Oropharyngeal SCC. It is important to know the association, as these patients with HPV positive HNSCC are clinically distinct from their HPV negative counterparts: they present with lymph node positive disease and originate from oropharynx while histologically these tumors are usually high grade and can be described as exhibiting basaloid morphology [4].

The Human Papilloma virus contributes to carcinogenesis by expressing E6 and E7 oncoproteins, which bind to p53 and Retinoblastoma gene (Rb) gene respectively. These latter proteins are critical for apoptosis and cell cycle regulation and when bound by the viral proteins, they get disrupted causing cells to proliferate with marked over expression of the tumour suppressor protein p16. This marked over expression of p16 which is very uncommon in non HPV related SCC makes p16 a good surrogate marker of HPV which indirect indicates the pathogenesis for squamous cell carcinoma [4].

Literature review has highlighted p16 detection systems by various techniques. It has been detected by immunohistochemistry as well as Fluorescence in Situ Hybridization (FISH) on formalin fixed paraffin embedded tissues.

Higher HPV prevalence is observed in Indian population and is probably due to higher incidence of HPV infection in oral SCC as compared to other countries. Hence detection of p16 and its correlation with the head and neck SCCs is mandatory for patient management and outcome. In the majority of studies, HPV-associated HNSCCs have a better prognosis compared to the stage-matched non-HPV-

related HNSCC. Clinical trials are now centering on de-intensification of treatment to reduce treatment associated morbidity and focus is directed on the development of HPV-targeted therapies. Identifying peripheral surrogate markers and screening and prevention methods is an important field of future investigations in HNSCCs.

Cyclin D1 proto-oncogene is also an important regulator of G1 to S-phase transition in numerous cell types from diverse tissues. Binding of cyclin D1 to its kinase partners, the cyclin dependent kinases 4 and 6 (CDK4/6), results in the formation of active complexes that phosphorylates the Retinoblastoma tumor suppressor protein (Rb). Hyper phosphorylation of Rb results in the release of Rb-sequestered E2F transcription factors and the subsequent expression of genes required for entry into S-phase. More recently, cyclin D1 has also been shown to act as a cofactor for several transcription factors. Initial studies indicated that cyclin D1 is localized predominantly in the nuclei of asynchronously growing cells [5].

Hence, the present study intends to analyze the immunohistolochemical expression of chosen cell cycle proteins P16 (cycline dependent kinase inhibitor)and Cyclin D1) expressed duding G1 phase of cell cycle in association with clinical and pathological features of squamous cell carcinoma of head and neck especially in our Indian population where we have a significantly higher incidence of squamous cell cancers than the West .

1. Materials and Methods

This is a hospital based cross sectional study, it was conducted in the department of Pathology at Sri Lalithambigai Medical College and Hospital, Chennai, Tamil Nadu . Duration of this study was one year. Totally 60 patients were chosen consecutively. All diagnosed cases of Squamaous cell carcinoma of head and neck diagnosed at histopathology by single histo pathologist to avoid inter- observer variations. All the patients were selected based on the inclusion and exclusion criteria. In inclusion criteria includes Patients diagnosed on histopathology as squamous cell carcinoma of head and neck and both gender was taken in this study. All specimens will be fixed in 10% formalin solution and paraffin blocks was prepared and subsequently stained with haematoxylin and eosin. The 3 micron thickness tissues were used for Immunohistochemistry analysis to determine the Cyclin D1 and P16 expression in them. Squmaous cell carcinoma of head and neck was categorized as per WHO classification wherever possible. Immunohistochemistry was performed on all the selected slides using Cycline D1 and P16 as primary antibody.

Statistical Analysis

All the data`s were collected and analyzed by using SPSS -16 version software.

2. Results

Age distribution

In the present study 60 patients were included and the age of the patients ranged from 14 -80 years, with the mean age of 54.68years. The majority of the patients 29 out of 60 patients studied were from 41-60 years age group making the major group constituting 48.33%. (Table.1)

Table .1 Age distribution

Age groups	**No.**	**Percent**
<20 years	1	1.67%
21-40 years	4	6.67%
41-60years	29	48.33%
61-80 years	26	43.33%
TOTAL	**60**	**100.00%**

Gender distribution in total number of cases

Out of 60 patients, 45 were males and 15 out of 60 patients were females, constituting 75% and 25% respectively, with a male preponderance. With male: female ratio 3:1. **(Table. 2)**

Table 2 Gender distribution

Gender	**Number**	**Percent**
Male	45	75.00%
Female	15	25.00%
TOTAL	**60**	**100.00%**

Distribution of cases according to site

In 60 cases of squamous cell carcinoma of head and neck 10 different sites of location of squamous cell carcinoma was observed. Tongue was the most common site seen in 36.67% (n=22) followed by larynx 25.00% (n=15), cheek 15.00% (n=9), mandible 10.00% (n= 6), posterior pharyngeal wall and tonsil were seen in equal numbers and percentage 3.33% (n=2),nasopharynx , ethmoid recess, soft palate, scalp were also seen in equal number and percentage1.67% (n=1). **(Table No. 3)**

Table 3. Distribution of cases according to site

code	**Site of lesion**	**n**	**Percent**
1	Tongue	22	36.67%
2	Larynx	15	25.00%
3	Cheek	9	15.00%
4	Mandible	6	10.00%
5	Tonsil	2	3.33%
6	Pharyngeal wall	2	3.33%
7	Nasophrynx	1	1.67%
8	Soft palate	1	1.67%
9	Ethmoid recess	1	1.67%
10	Scalp	1	1.67%
TOTAL		**60**	**100.00%**

Distribution of cases according to IHC p16 expression:

In the present study 15% showed p16 positive (n=,9) and 85% showed p16 negative (n=51). Table no 8 Distribution of cases according to IHC p16 expression. **(Table.4)**

Table.4. Distribution of Cases According to IHC p16 Expression

Expression	**P16**	**Percent**
NEGATIVE	51	85.00%
POSITIVE	9	15.00%
TOTAL	**60**	**100.00%**

Distribution of cases according to IHC CyclinD1 expression

Cyclin d1 showed moderate expression in , 46.67%(n =28) , 18.33% showed strong expression(n= 11) and 35.00% showed weak expression (n= 21). **(Table 5)**

Table 5. Distribution of cases according to IHC CyclinD1 expression

	Cyclin D1	Percent
MODERATE	28	46.67%
STRONG	11	18.33%
WEAK	21	35.00%
TOTAL	**60**	**100.00%**

Distribution of cases according to histopathological grading

Out of 60 patients of squamous cell carcinoma of the head and neck , on the basis of histopathological grading they were divided into 3 grades by a single pathologist and was graded as well differentiated squamous cell carcinoma 45.00% (n=27), moderately differentiated squamous cell carcinoma 30.00% (n=18) and poorly differentiated squamous cell carcinoma 25% (n-=15). **(Table.6)**

Table 6. Distribution of cases according to histopathological grading

SCC	Diagnosis	Percent
Well Differentiated	27	45.00%
Moderately Differentiated	18	30.00%
Poorly Differentiated	15	25.00%
TOTAL	**60**	**100.00%**

Cyclin D1 Expression in different grades of squamous cell carcinoma

Out of 27 patients of well differentiated squamous cell carcinoma 66% showed weak staining (n=18) and 34%showed moderate expression(n=9) O ut of 18 patients of moderately differentiated squamous cell carcinoma 66% showed moderate staining(n=12) 17% showed weak and moderate staining (n=3)..Out t of 15 patients of poorly differentiated squamous cell carcinoma 46% showed moderate staining,(n=7) and 54 % showed strong staining ,(n=8) weak staining was not seen in at all. **(Table 7)**

Table 7. Cyclin D1 Expression in different grades of squamous cell carcinoma

Cyclin D1				
Diagnosis	**MODERATE**	**STRONG**	**WEAK**	**TOTAL**
MODSCC	12(66%)	3 (17 %)	3 (17 %)	**18**
POOR SCC	7(46%)	8 (54%)	0 (0%)	**15**
WDSCC	9 (34%)	0 (0%)	18 (66%)	**27**
TOTAL	**28**	**11**	**21**	**60**

Chi square value =32.28 p value <0.001

p16 expression in different grades of squamous cell carcinoma.

Out of 27 patients of well differentiated squamous cell carcinoma 34% showed positive expression (n=9) and 66% showed negative expression (n=18).in moderately differentiated scc and poorly sscc p16 expression was negative.

Table 8. Distribution of cases according to IHC p16 expression

P16			
Diagnosis	**NEGATIVE**	**POSITIVE**	**TOTAL**
MODSCC	18(,100%)	0,(O%)	**18**
POOR	15(,100%)	0(,9O%)	**15**

WDSCC	18 (66%)	9,(34%)	**27**
TOTAL	**51**	**9**	**60**

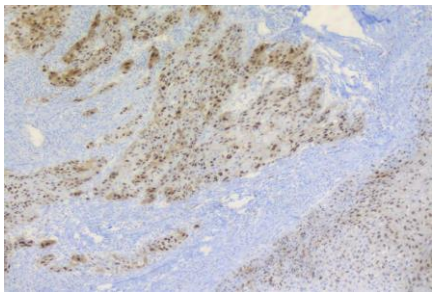
Chi square value = 12.94

P value =0.0015

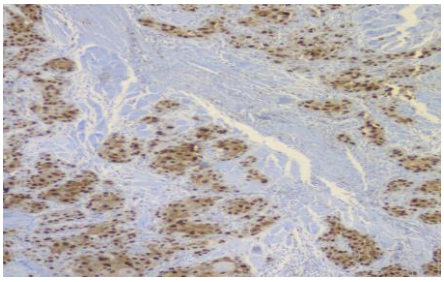
Statically analysis between Cyclin D1 and histopathological grading shows significant association (p value=0.0015) between expression of Cyclin D1 and histopathological grading of squamous cell carcinoma. Over expression of Cyclin D1 was seen in moderately and poorly differentiated squamous cell carcinoma (Figure.1). Statically analysis between p16 and histopathological grading shows significant association (p value =0.0015) exist between p16 and grading of squamous cell carcinoma.

P16 shows positive expression in well differentiated squamous cell carcinoma and loss of p16 expression in moderately and poorly differentiated squamous cell carcinoma (Figure.2).

Figure. 1 IHC image of squamous cell carcinoma showing Cyclin D1 strong expression

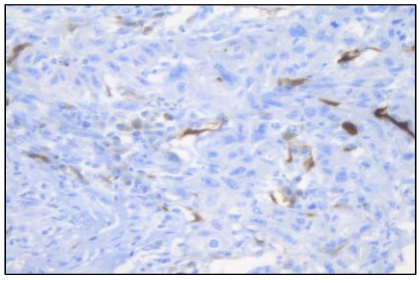


a.IHC image of poorly differentiated squamous cell carcinoma showing Cyclin D1 strong expression

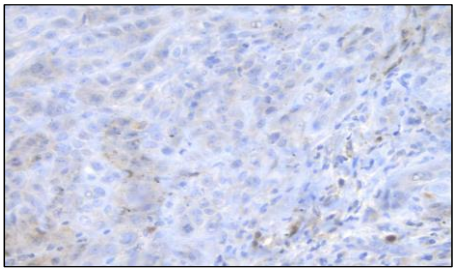


b. IHC image of well differentiated squamous cell carcinoma showing CyclinD1 expression.

Figure 2. IHC image of squamous cell carcinoma showing low expression of p16



a. IHC image of poorly differentiated squamous cell carcinoma showing low expression of p16



b. IHC image of moderately differentiated squmaous cell carcinoma p16 expression.

Discussion

Age & gender distribution of patients

Assessment of the age among all the patients (N=60) revealed, maximum number of the patients belonged to the age 41-60years of age i.e. 29 (48.33%) followed by 61-80 years of age 26 (43.33%) and 21-40 years of age i.e 4 (6.67%). Only 1 (1.67%) of the patients was having age less than 20 years. Among all the patients(N=60), 45 (75%) were males and 15 (25%) were females. Dr Krecicki et al in 2004 studied the cellcycle regulatory proteins in laryngeal carcinoma among 58 patients The average age of the patients was 59 years (range 41–75 years) [5].

Galera et al in 2017 did a study to assess the prognostic values of P16 in laryngeal squamous carcinoma in 236 patients and concluded there were 228 men and 8 women, with a mean age of 58.5 +/- 10.06 years [6]. Hanken et al in 2012 did a study on Cyclin D1 expression in HNSCC and out of 222 OSCCs, 157 male and 67 female patients [7]. Bova et al in 1999 did a study on cyclin D1 & P16 expression in tongue carcinoma and concluded 70 % of patients were male, 63% were <65 years [8].

Site of lesion among all the patients

The assessment of the site of lesion in all patients with head & neck squamous cell carcinoma revealed highest number of patients having carcinoma, tongue carcinoma was seen in highest number of patients i.e 22 (36.6%) followed by laryngeal carcinoma 15(25%). The nasopharyngeal, ethmoid recess, soft palate and scalp carcinoma were the least prevalent site of carcinoma, 1(1.6%) each.

Complaints among the patients with HNSCC

The most common complaint encountered by the patients was ulcer i.e 29(48.33%) followed up by hoarseness of voice 12(20%), erythroplakia & leukoplakia 4(6.67%) each, blood in saliva & odynophagia 3(5%). The least common symptoms were trismus, sore throat & ear ache, 1(1.67%) each.

Expression of P16 among the carcinoma

Among all the patients diagnosed as squamous cell carcinoma of head and neck (n= 60), the expression of P16 was observed among 9 (15%) of the patients and rest of the patients (85%) did not express P16 on imunohistochemistry

Bova et al in 1999 did a study on cyclin D1 & P16 expression in tongue carcinoma. Loss of p16INK4Aexpression was demonstrated in 55% of tumors (78 of 143) and was associated with reduced disease-free (P< 0.007) and overall (P< 0.014) survival. (78) Krecicki et al in his study has found decresed p16 expression in 27 out of 58 cases of laryngeal carcinoma [5].

In our study we found that p16 expression was lost in moderately and poorly differentiated tumors . Statically coreration was found between immunostaining of p16 and histological grading of tumour indicating that tumors with low differentiation show dysfunction of the p16 gene. Krecicki et al 75 and Dey B et al [9] found p16 negative in poorly differentiated tumours and there was a significant correlation between p16 expression and histological grading of the tumour

Expression of Cyclin D1 among all the carcinoma patients

Bova et al in 1999 did a study on cyclin D1 & P16 expression in tongue carcinoma. Overexpression of cyclin D1 occurred in 68% of tumors (100 of 147) and was associated with increased lymph node stage (P< 0.014), increased tumor grade (P < 0.003), and reduced disease-free (P< 0.006) and overall (P < 0.01) survival [8].

Krecicki et al has found overexpression of cyclin D1 in 48 % of the patients [5].

Over expression of cyclin D1 promotes cell proliferation. Abnormal expression of cyclins have been found in various malignancy and in head and neck tumors it is strongly expressed. In our study there was no coreeration between p16 and cyclinD1 immunostaing which corrrealate with Krecicki et al who also found no correlation between p16 and cyclin D1 immunostaining . So Cyclin D1 was found to be an independent prognostic factor which Michalides et al [10] has also quoated in his study. Krecicki et al in his study has also found over expression of Cyclin D1 in advanced stages of the disease .

In our study strong expression of Cyclin D1 was significantly high among moderate & poorly differentiated carcinoma (27.2% & 72.73% respectively) and show a statistically

significant correlation between the cyclin D1 expression and histological grading of the tumour. Other authors also had similar findings. Dey B et al [9], Swati Saawarn et al and Krecicki et al [5].

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3. Conclusion

In the present study we conclude that the Cyclin D1 may be target molecule in chemotherapy as tumor over expressing Cyclin D1 has been found to be more sensitive to chemotherapeutic agent for head and neck squamous cell carcinoma.

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