Cyclin D1 expression of different histological grades in oral squamous cell carcinoma patients from northern India

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SUMMARY

Cyclin D1 expression was positive in 35 cases of oral cancer with a Cyclin D1 positivity of 22.16±22.18. The percentage of positive cases as well as Cyclin D1 positivity showed an increase as the grade of differentiation advanced. No significant association was found between Cyclin D1 positivity and degree of differentiation of tumors (p=0.138). A significant difference in Cyclin D1 positivity was observed (p=0.043) comparing well differentiated (16.61±17.89) and poorly differentiated (37.0±32.51) tumors, as well as between well differentiated (16.61±17.89) and moderately differentiate tumors (24.38±21.93; p=0.002). Similarly, significant difference in Cyclin D1 positivity was observed comparing moderately differentiated (24.38±21.93) and poorly differentiated tumors (37.0±32.51; p=0.043). Cyclin D1 expression was more frequently seen in hard palate (75%), buccal mucosa (67%) and lip (60%) while expression of Cyclin D1 was less frequent in sites like gingiva (0%), tongue (40%) and floor of mouth (43%). There was no association between Cyclin D1 expression and primary site of oral cancer (p=0.528) in tobacco and betel quid chewers of northern India.

Keywords: *Cyclin D1, oral carcinoma, Northern India, betel quid chewers*

INTRODUCTION

Oral cancer is one of the ten most common cancers worldwide (1). There is wide geographical variation in the incidence of oral cancer, with approximately two-thirds of patients in the developing countries of Southeast Asia, Eastern Europe and Latin America (2). In India, oral cancer ranks in the top three of all cancers and accounts for over thirty percent of all cancers reported in the country (3). Incidence of oral cancer is increasing day by day due to more intakes of various forms of tobacco and alcohol drinking, which are considered to be the two most important etiological factors in the development of oral cancer (1). It is estimated that 75-90% of all head and neck cancers are caused due to the tobacco use. Tobacco users are from 20-40 times more likely to develop head and neck cancer than non-consumers, depending upon the amount as well as age, sex and race of the user. Human Papilloma Virus (HPV) has also been shown to be associated with incidence of oral cancer. The IARC classifies human papillomavirus 16 (HPV16) as a cause for cancers of the oral cavity and pharyngeal tonsils, and HPV18 as possible causes of oral cancer (http://monographs.iarc.fr/ENG/Classification/index.php). Evidence shows that HPV contributes to carcinogenesis by two virus-encoded proteins, one E6 protein which promotes the degradation of p53 tumor suppressor gene product and second E7 that promotes the degradation of the tumor suppressor gene product pRb (retinoblastoma protein) leading to deregulation of the cell cycle control (1).

Tobacco may be taken in various ways - like smoking, chewing, etc. The most common form of tobacco chewing in India is betel quid. The 'quid' for chewing consists of areca nut and pieces of unripe betel fruit or areca nut wrapped in a piece of betel leaf together with white or red lime. Betel quid chewing has a strong association with oral cancer which arises predominantly from surface epithelium with evolution from early premalignant lesions. Oral Squamous Cell Carcinoma (OSCC) arise as a consequence of multiple molecular events induced by the effects of various carcinogens from habits such as areca nut and betel quid chewing, influenced by environmental factors, possibly viruses in some instances, against a background of inheritable resistance or susceptibility (4). An individual difference in the susceptibility to chemical carcinogens is one of the most important factors in the estimate of risk of human cancer as some patients appear susceptible because of inherited trait(s) in their ability or inability to metabolize carcinogens or pro-carcinogens, possibly along with an impaired ability to repair DNA damage (5).

Oral carcinogenesis is a multi-step process in which 6-10 genetic events lead to the disruption of the normal regulatory pathways that control basic cellular functions. In recent years, several alterations in the expression of tumor suppressor genes and oncogenes in the development of OSCC have been described (6-8). Based on these facts, the present study was done to investigate the expression of Cyclin D1 and to further examine the relationship between Cyclin D1 expression with different histological grades in oral squamous cell carcinoma (OSSC) patients from north India possessing tobacco and betel quid chewing habits.

EXPERIMENTAL

Tissue specimens

Biopsy tissue specimens from 60 untreated primary Oral Squamous cell Carcinoma (45 men and 15 women) were obtained from Aligarh Muslim University, Jawaharlal Nehru Medical College, Department of Otorhinolaryngology (Aligarh, India) in November 2004 to May 2007. The patients were grouped into four age groups: 0-25, 25-50, 50-75 and above 75 years. Tumors were classified into grades I, II, III according to cellular differentiation which is equivalent to well, moderately and poorly differentiated tumors. Clinicopathological data as well as age, gender, areca nut and betel quid intake history and location were obtained in each case.

Immunostaining

Primary antibody for Cyclin D1 (H-295, Santa Cruz Biotechnology, USA) was added to sections and incubated overnight at 38 °C at room temperature in a moist chamber. The sections were then washed with TBS (x3) for 10 min each, incubated with biotinylated secondary (Link) antibody for 30 minutes at room temperature in a moist chamber and washed in TBS (x3) for 10 min. Sections were incubated with streptavidin for 45 min at room temperature in moist chamber, washed in TBS and incubated in freshly prepared 3, 3' diaminobenzidine tetrahydrochloride (DAB) solution. DAB was prepared by diluting chromogen (1 drop) in 1 ml of substrate and used as the substrate for localizing antibody binding. Sections were than washed in distilled water, counterstained in hemotoxylin (1-2 dips), dehydrated through graded alcohols, cleaned in xylol and mounted in DPX. The positive control slides were incubated with primary antibody, whereas in negative controls primary antibodies were replaced with normal mouse serum. For protein expression, only nuclear positivity (strong brown staining) was assessed quantitatively. Percentage of positively stained cells in the whole layer of epithelium was determined and recorded by assigning them to one of the following categories: 0 = No epithelial cells stained, + = up to 25% of cells positive, ++ = 26 to 50% of cells positive, +++ = >50% of cells positive (9, 10).

Statistical Analysis

An SPSS for Windows computer programme (SPSS Inc. Chicago 11, USA, version 13) was used for statistical analysis. The association between protein expression and tumor location was analyzed by the Chi-square test. The relationship between protein expression and histolopathological grade was analyzed by Kruskal-Wallis analysis of variance (ANOVA). Wilcoxon paired sample test was used to analyze the differences within the three categories of histopathological grade and protein expression. A probability (p value) of less than 0.05 was accepted as statistically significant.

RESULTS AND DISCUSSION

Cyclin D1 Expression

Tissues of OSCC patients with tobacco and betel quid chewing habit (60 specimens) and 10 normal oral tissues were subjected to immunohistochemical staining for expression of Cyclin D1 using H-295 antibody (Santa Cruz Biotechnology, USA). The strong brown nuclear staining of epithelial cells was considered positive. Histological sections with good intensity were assessed for Cyclin D1 scoring.

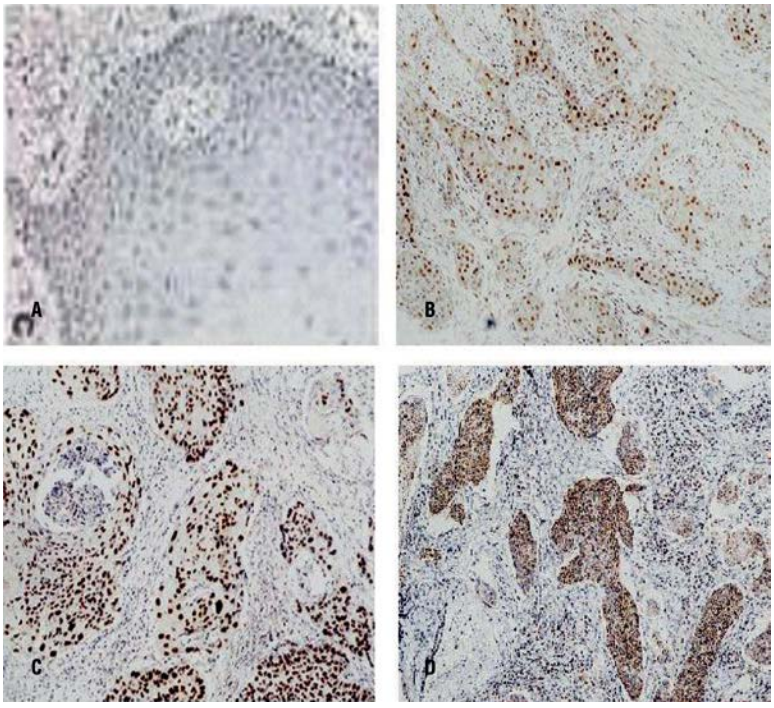

Figure 1: Immunohistochemical detection of Cyclin D1 using Cyclin D1 antibody in tissues obtained from normal and oral cancer patients. Expression of Cyclin D1 in normal tissue (A), in well differentiated OSCC (B), in moderately differentiated O SCC (C) and in poorly differentiated OSCC (D).

The scores obtained were expressed as:

- Positive cases - the percentage of cases showing positive staining with IHC Cyclin D1 staining
- Positivity - the percentage of cells showing a positive staining reaction with IHC Cyclin D1 staining

Cyclin D1 was expressed in 58.33% of the cases (n=35) but was not expressed in controls. The expression of Cyclin D1 in tobacco and betel quid chewers as well as control has been shown in **Fig. 1 (A-D)**. There were 34 cases (56.6%) of differentiated SCC, 18 cases (30%) of moderately differentiated SCC and 8 cases (13.3%) of poorly differentiated SCC.

Statistical Analysis

The **Table 1** depicts positive cases (%) and mean Cyclin D1 positivity (%) in OSCC patients and controls along with their sub categories. As revealed by immunohistochemistry there was no cyclin D1 expression in controls (**Fig. 1A**), while in oral SCC patients with tobacco and betel quid chewing habit the percentage of positive cases as well as cyclin D1 positivity showed an increase with high grade of SCC (**Fig. 2**).

Histological Diagnosis	Total Cases	Cyclin D1 Expression		Positivity	
		Positive cases (%)	Negative cases (%)	Mean±SD	Range
Oral SCC	60	35 (58.33%)	25 (41.6%)	22.16±22.18	0-75
Well differentiated	34	18 (52.94%)	16 (47.05%)	16.61±17.89	0-51
Moderately differentiated	18	11 (61.1%)	7 (38.88%)	24.38±21.93	0-70
Poorly differentiated	8	6 (75.0%)	2 (25%)	37.0±32.51	0-74
Control	10	0	10	0	0

Table 1. Cyclin D1 Expression in OSCC's in tobacco and betel quid chewers

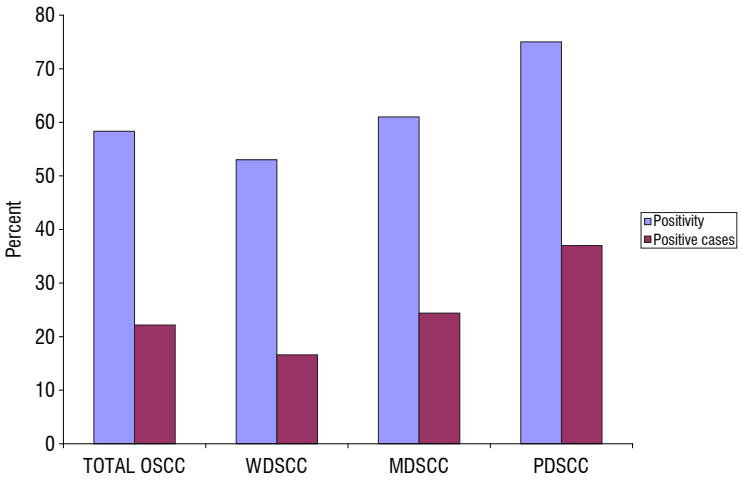

Figure 2. Expression of Cyclin D1 in OSCC's in tobacco and betel quid chewers

WDSCC – well differentiated SCC
MDSCC – moderately differentiated SCC
PDSCC – poorly differentiated SCC

It was found that there was no statistically significant association between Cyclin D1 expression and histological grade in oral cancer in tobacco and betel quid chewers (χ^2=3.954, df=2, p=0.138). However, statistically significant difference (p=0.002) was observed in Cyclin D1 positivity between well differentiated SCC (16.61±17.89) and moderately differentiated SCC (24.38±21.93) as well as (p=0.043) between well differentiated SCC (16.61±17.89) and poorly differentiated SCC (37.0±32.51). Similarly, statistically significant difference (p=0.043) was observed between moderately differentiated SCC (24.38±21.93) and poorly differentiated SCC (37.0±32.51).

Expression of Cyclin D1 in oral cavity was investigated and it was found that Cyclin D1 was more frequently expressed in hard palate (3/4, 75%), buccal mucosa (21/31, 67%) and lip (3/5, 60%) and less frequently in gingiva (0/1, 0%), tongue (4/10, 40%) and floor of mouth (4/7, 43%).

The association between expression of Cyclin D1 and sites of incidence of oral cancer was also evaluated in our study. It was found that there was no significant association between Cyclin D1 expression and primary site of incidence of oral cancer (χ^2=5.122, df=6, p=0.528).

DISCUSSION

Cyclin D1 gene encodes a protein that is a cell cycle regulator (11). The Cyclin D1 gene (CCND1, bcl-1 or PRAD1) located on chromosome 11q 13 (12) encodes a protein that forms a complex with Cyclin dependent Kinases, CDK4 and CDK6. Cyclin D-CDK4 and CDK6 complexes phosphorylate Rb (Retinoblastoma) protein during the G1-S transition which leads to their dissociation from the EF2 transcriptional factor and the initiation of DNA replication (13). Cyclin D1 over expression, either by amplification or transcriptional up regulation, triggers accelerated G1 progression or entering the S phase, with lower cell dependence on growth factors for proliferation (14).

Immunohistochemical studies of cyclin D1 expression in SCC of oral cavity has shown over expression of cyclin D1 protein (15-22). In present study, there was no expression of Cyclin D1 protein in control specimen while in oral SCC patients with tobacco and betel quid chewing habit, an increased percentage of positive cases as well as increase in mean cyclin D1 positivity was observed. Thirty five (58.33%) oral SCC cases showed positive Cyclin D1 expression and mean positivity was 22.16 ± 22.18.

Many previous studies have reported similar positivity in oral SCC patients. Arora *et al.* reported that 61% of cases of betel related oral SCC showed Cyclin D1 positivity (17) while Lam *et al.* reported 63% positivity (16). Similarly Staibano *et al.* reported 60% positivity (18) and Gimenez-Conti *et al.* reported 61% positivity (19) for Cyclin D1 in oral SCC patients. Angadi *et al.* (20) and Kuo *et al.* (14) have observed higher cyclin D1 positivity in oral SCC patients and have reported 70.7% and 83% positivity, respectively. However lower values were observed by Takes *et al.* , Xu *et al.* and Akervall *et al.,* which reported 29%, 38% and 43% positivity respectively for Cyclin D1 in oral SCC patients (21, 15, 22).

In our study, we further investigated the Cyclin D1 expression in various sites of oral cavity. Cyclin D1 expression was more frequently expressed in hard palate, buccal mucosa and lip and less frequently in tongue and floor of mouth. There are only few studies that have described the expression of Cyclin D1 in various sites of oral cavity in oral SCC patients. Studies reported that expression of Cyclin D1 in oral SCC patients was more frequently seen in sites like tongue and retromolar region (15, 22). In our study, Cyclin D1 expression was more frequently expressed in hard palate (3/4, 75%), buccal mucosa (21/31, 67%) and lip (3/5, 60%). The correlation between Cyclin D1 expression and primary site of oral cancer was also evaluated in our study. It was found that there was no significant association between Cyclin D1 expression and primary site of oral cancer (p=0.528). Similar results were reported by various studies (14, 23) which found no association between Cyclin D1 expression and primary site of oral cancer.

The relationship between Cyclin D1 expression and tumor grade was also evaluated in our study. An increased positivity with increasing grade was observed in the present study. The difference was found to be significant between well differentiated SCC (16.61±17.89) and moderately differentiated SCC (24.38±21.93, p=0.002) as well as between well differentiated SCC (16.61±17.89) and poorly differentiated SCC (37.0±32.51, p=0.043). Similarly, statistically significant difference was observed between moderately differentiated SCC (24.38±21.93) and poorly differentiated SCC (37.0±32.51, p=0.043). Although most of published data have shown no positive relationship between Cyclin D1 expression and histological grade of oral SCC (24-26), just Angadi and co-workers have observed positive correlation between Cyclin D1 expression and histological grade of oral SCC (20). In our study, we found no significant association between Cyclin D1 positivity and degree of differentiation of tumor (p=0.138) in oral cancer patients with tobacco and betel quid chewing habit. Further in our study, we found a tendency towards higher incidence of Cyclin D1 positivity with high grade of differentiation of tumors. Similar results were reported by Lam *et al*. (26) which found that Cyclin D1 expression was more positive in high grade lesions.

CONCLUSION

Cyclin D1 expression was positive in 35 cases of oral cancer with a Cyclin D1 positivity of 22.16±22.18 (mean ± SD). The percentage of positive cases as well as Cyclin D1 positivity showed an increase as the grade of differentiation advanced. No significant association was found between Cyclin D1 positivity and degree of differentiation of tumors (p=0.138). A significant difference in Cyclin D1 positivity was observed (p=0.043) comparing well differentiated (16.61±17.89) and poorly differentiated (37.0±32.51) OSCC, as well as (p=0.002) between well differentiated (16.61±17.89) and moderately differentiate OSCC (24.38±21.93). Similarly, significant difference (p=0.043) in Cyclin D1 positivity was observed comparing moderately differentiated (24.38±21.93) and poorly differentiated (37.0±32.51) OSCC. Cyclin D1 expression was more frequently seen in hard palate (75%), buccal mucosa (67%) and lip (60%) while expression of Cyclin D1 was less in sites like gingiva (0%), tongue (40%) and floor of mouth (43%). There was no association between Cyclin D1 expression and primary site of oral cancer (p=0.528) in tobacco and betel quid chewers of northern India.

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Declaration of Interests

Authors declare no conflicts of interest.

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