Evaluation of the Role Played by Cyclin D1 as a Diagnostic and Prognostic Marker in the Progression of Oral Carcinogenesis

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Abstract Cyclin D1 is linked with the development and progression of oral squamous cell carcinoma (OSCC). This case–control study was directed to characterise the immunoreactivity of the protein cyclin D1 and its correlation with the clinicopathological parameters of patients with OSCC and potentially malignant disorders (PMD). A group of patients with OSCC were followed up after treatment, and the cyclin D1 expression was reviewed for correlation of cyclin D1 expression with prognosis of the patients.

Methodology Sixty individuals were included in this study: OSCC (20), PMD (20) and Control (20). Immunohistochemistry assay was evaluated. The clinicopathological parameters were correlated with the staining intensity of cyclin D1. The results were subjected to Pearson's correlation test.

Results Age, gender and site showed no statistically significant correlation with cyclin D1 expression in OSCC and PMD. The cyclin D1 score did not show a significant difference with histopathological diagnosis of OSCC. Cyclin D1 was not expressed in 60% of the Control and 30% PMD cases while the expression of cyclin D1 was seen in 100% of OSCC cases although cyclin D1 score did not show a statistically significant association in the prognosis of the disease among the OSCC patients.

Keywords Cyclin D1 · Oral cancer · Potentially malignant disorder

Introduction

Oral squamous cell carcinoma is the sixth most common cancer in humans with a 5-year mortality remaining at 50% despite advances in technology with regard to diagnosis and management. Therefore, oral squamous cell carcinoma remains a significant health burden. Oral squamous cell carcinoma forms 4% of all malignancies in the West and 40% of all malignancies in India [1].

Carcinogenesis is a process which takes place when multiple factors play a role to bring about a discoordination in the cell cycle. The various phases of the cell cycle are regulated by a number of proteins called “cyclins”. Cyclin D1 is a 45-kilodalton protein which is found on chromosome 11q13 and regulates the G1-S phase transition.

Since published data are full of evidence that cyclin D1 is an important marker for oral squamous cell carcinoma, this case–control study was directed to find if there was indeed a correlation between cyclin D1 expression and clinicopathological parameters of oral squamous cell carcinoma and potentially malignant disorders. The study also

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attempts to assess the correlation between cyclin D1 expression and the follow-up status of patients with OSCC.

Methodology

This study included a total of sixty individuals of which twenty cases (20) were oral squamous cell carcinoma (OSCC) and twenty cases (20) of potentially malignant disorder (PMD) and twenty normal healthy individuals (20). The OSCC group had 8 females and 12 males between the age group of 35–79 years of age (Mean $\pm$ SD 59 ± 11.48). The PMD group had 8 females and 12 males between the age group of 26 and 62 years of age (Mean $\pm$ SD 46.9 ± 10.53). The healthy individual group had 8 females and 12 males between the age group of 26 and 86 years of age (Mean 52.7 ± 18.1). (Table 1).

97.95% of cases had associated habits of tobacco chewing/beedi smoking of an average period of 15 years. 80% of OSCC cases presented at Stage 4. OSCC is staged clinically into four stages depending on the size of the lesion, the lymph node involvement and distant metastases. Stage 4 is when size of the lesion is more than 4 cms and adjacent structures such as skin, bone, nerves, masticator muscles or infratemporal fossa are involved by the primary lesion. Most common site involved in this series in both oral squamous cell carcinoma and potentially malignant disorder was the buccal mucosa (Table 2).

Informed consent was taken from the individuals participating in the study, and Institutional Ethics Committee approval was obtained from Sri Ramachandra University and Vinayaka Missions University. Incisional biopsy was done and histopathological grading was done on the haematoxylin- and eosin-stained sections.

Immunohistochemistry study was carried out using polymer-labelling technique (Dako Envision).

The immunohistochemistry reports were done by two senior oral and maxillofacial pathologists. Presence of brown-coloured end product at the site of target antigen in the cytoplasm and nucleus was indicative of positive immunoreactivity. Absence of staining or weak staining was considered negative. In every slide, positive tumour cells per hot spot were calculated and mean percentage per slide (labelling index) was determined. Labelling score of 1, 2, 3 and 4 was assigned for labelling indices 1–25, 26–50, 51–75 and $> 75\%$, respectively. All the relevant clinical, histopathological and IHC data were tabulated and subjected to Pearson's correlation test and Chi-square test. Thirteen of the twenty cases from the OSCC group were followed up for 12 months following treatment, and their cyclin D1 report was reviewed and correlated with the follow-up details.

Results

Table 1 shows the age- and sex-wise distribution of the study population. The study population was divided into three groups, namely the OSCC group, PMD group and Control group. Twenty individuals were included in each of the three groups. Male-to-female ratio was 0.6: 0.4. The minimum age of the study group was 26 years, and the maximum age was 79 years. Table 1 shows that in OSCC group, between the age 51–80 years the cyclin D1 expression was 80%. In PMD group, the age group of 21–50 years had cyclin D1 expression of 65% (Table 1). Gender-wise distribution among the OSCC and the PMD patients had shown that the cyclin D1 score did not have a

Table 1 Age- and sex-wise distribution of the study population

Age group	Gender	Control	PMD	OSCC	Total
21–30	Male	2 (10%)	1 (5%)	0	3 (5%)
	Female	3 (15%)	1 (5%)	0	4 (6.6%)
31–40	Male	2 (10%)	1 (5%)	2 (10%)	5 (8.3%)
	Female	0	3 (15%)	0	3 (5%)
41–50	Male	0	6 (30%)	1 (5%)	7 (11.6%)
	Female	0	1 (5%)	1 (5%)	2 (3.3%)
51–60	Male	1 (5%)	3 (15%)	4 (20%)	8 (13.3%)
	Female	5 (25%)	2 (10%)	4 (20%)	11 (18.3%)
61–70	Male	4 (20%)	1 (5%)	2 (10%)	7 (11.6%)
	Female	0	1 (5%)	3 (15%)	4 (6.6%)
> 70	Male	3 (15%)	0	3 (15%)	6 (10%)
	Female	0	0	0	0
Total		20 (100%)	20 (100%)	20 (100%)	60 (100%)
Mean $\pm$ SD		52.7 $\pm$ 18.1	46.9 $\pm$ 10.53	59 $\pm$ 11.48	52.8 $\pm$ 13.3

Table 2 Cyclin D1 score based on site of occurrence among the OSCC patients

Site of occurrence	Cyclin D1 score				Total	<i>p</i> value
	1	2	3	4		
Buccal mucosa	2 (66.7%)	4 (66.7%)	2 (33.3%)	2 (40%)	10 (50%)	0.621
Mandibular alveolus	0	1 (16.7%)	3 (50%)	2 (40%)	6 (30%)	
Maxilla	1 (33.3%)	0	1 (16.7%)	1 (20%)	3 (15%)	
Tongue	0	1 (16.7%)	0	0	1 (5%)	
Total	3 (100%)	6 (100%)	6 (100%)	5 (100%)	20 (100%)	

statistical significant difference among males and females ($p > 0.05$) (Table 1). The most common site of occurrence of cancer among the OSCC patients in this series was the buccal mucosa, followed by mandibular alveolus, maxilla and tongue, and the cyclin D1 score did not show statistical significant difference on the site of occurrence of OSCC ($p > 0.05$).

TNM staging of cancer was done for all patients with OSCC. The minimum stage was T2N1, and the maximum stage of the disease was T4N2. TNM staging did not show statistically significant association with cyclin D1 scoring ($p > 0.05$) (Table 2).

Among the diagnosis of OSCC patients, the most common was well-differentiated squamous cell carcinoma (WDSCC) ($N = 11$) followed by moderately differentiated squamous cell carcinoma (MDSCC) ($N = 8$) and poorly differentiated squamous cell carcinoma (PDSCC) ($N = 1$). The cyclin D1 score did not show a significant difference ($p 0.337$) among the different histopathological diagnoses of OSCC.

Among the 20 patients with OSCC, 13 patients were given different modalities of treatment like surgery, radiotherapy and chemotherapy either alone or in combination. Cyclin D1 scoring did not have significant association ($p 0.296$) with the treatment modality among the patients with OSCC. Thirteen patients for whom treatment was given were followed for 12 months to assess the prognosis. Among them nine were alive at the end of 1 year, three died and one was lost to follow-up. Cyclin D1 score did not show a statistically significant association ($p 0.239$) with the prognosis of the disease among the OSCC patients. The association of cyclin D1 scoring between all the three groups is shown in Table 3. Among the Control group, the cyclin D1 score was between 0 and 2, whereas among the PMD group it was between 0 and 3 and in OSCC group it was between 1 and 4. A statistically significant difference among the cyclin D1 scoring was observed between the Control and PMD group, PMD and OSCC group and between the Control and OSCC group ($p < 0.001$). (Figure 1).

Discussion

Malignant changes in a cell can be a result of disruption in cell signalling, cell cycle and/or the mechanism that repairs cell cycle damage or eliminates dysfunctional cells. Cellular and tissue markers to measure cellular alterations have gained popularity as the traditional methods of clinical and histopathological examinations do not always give satisfactory information for the diagnosis and prognosis of malignant lesions [1, 2].

Cyclin D1 is a protein encoded by CCND1 gene and is located on the long arm of chromosome 11 on band 11q13. According to the literature, the overexpression of cyclin D1 has been linked to the development and progression of cancer. Deregulated cyclin D1 degradation appears to be responsible for the increased levels of cyclin D1 in several cancers. The ability of these cyclins to activate the cyclin-dependent kinases CDK4 and CDK6 is the most documented mechanism for their oncogenic actions and provides an attractive therapeutic target. The role of cyclin D1 as a prognostic marker still remains controversial [3]. Cyclin D1 is a critical regulator of G1 phase of the cell cycle progression and plays a key role in G1-S phase transition [2, 4, 5, 26]. Cyclin D1 when overexpressed could stimulate two phases:

1. Normal cell cycle that could be hindered.
2. Increased susceptibility to cancer [6].

At the molecular level, the orderly progression of cells through the cell cycle is understood to be regulated by the proteins cyclins and cyclin-dependent kinases. The genes which encode these proteins when altered and these proteins when overexpressed are reported to be involved in the process of carcinogenesis [2]. There are G1 cyclins, which regulate the passage of cells from G1 phase to S phase (Cyclins C, D1–D3, E) and mitotic cyclins that facilitate the passage of the cells during mitosis (cyclins A and B). The role of the cyclins is to activate CDKs. Cyclin D is the first cyclin to increase in the cell cycle. Cell replication is promoted by the activation of CDK4 by cyclin D1 in G1 phase which leads to the phosphorylation of retinoblastoma protein (Rb) which results in the release of E2F. In the S

Table 3 Cyclin D1 score comparison between Control, PMD and OSCC patients

Cyclin D1 score	Control group	PMD patients	OSCC patients	<i>p</i> value
0	12 (60%)	6 (30%)	0 (0%)	< .0001 ¹
1	6 (30%)	3 (15%)	3 (15%)	< .0001 ²
2	2 (10%)	9 (45%)	6 (30%)	< .0001 ³
3	0 (0%)	2 (10%)	6 (30%)	
4	0 (0%)	0 (0%)	5 (25%)	
Total	20 (100%)	20 (100%)	20 (100%)	

¹Association between Control and PMD group²Association between PMD and OSCC group³Association between OSCC and Control group **Fig. 1** Graph depicting follow-up status

phase, E2F stimulates the transcription of cyclin E, which binds to CDK2, stimulating the synthesis of DNA. The transition from G2 to M is initiated by the transcription of cyclin A, which forms a complex with CDK2 and regulates the events of the mitotic prophase. The carcinogenic process involves the acquisition of mutations that culminate with the abnormal expression of proteins, many of them involved in cell cycle control [2, 24].

Overexpression of cyclin D1 has been correlated with a shorter G1 phase, reduced dependence on growth factors and abnormal cell proliferation. This abnormal cell growth may favour the occurrence of other genetic alterations required for tumourigenesis. This overexpression has been correlated with other proliferation markers (Ki67, PCNA) and associated with various tumours such as breast, liver, colon, oesophagus and lung [7–10]. This aberrant expression has also been associated with a poor survival and poor prognosis [2].

This study was undertaken to compare the expression of cyclin D1 in oral squamous cell carcinoma and PMD and to

Fig. 2 Photomicrograph of cyclin D1 uptake in PMD

correlate its expression with clinical parameters such as age, sex, site, TNM staging and histopathological differentiation and follow-up status. The results were statistically analysed using Pearson's correlation test and Chi-square test.

The immunohistochemistry reports were done by two senior oral and maxillofacial pathologists. The IHC reactivity was reported as positive when there was brown staining of cytoplasm and nucleus, which was similar to many related studies [11–13, 15] whereas Vora et al. [14] reported exclusive cytoplasmic staining in their cases as positive (Figs. 2, 3) unlike Gillette et al. who considered cytoplasmic staining as negative. Intensity of staining has been reported on a three-point criteria as weak, moderate and strong [12, 17, 23] or on a two-point criteria as weak and strong [18, 20]. Some investigators have used labelling index score [11, 16, 18, 19, 21] as in our study, while Maahs et al. [16] used stereological method to determine labelling index.

In our series, cyclin D1 expression was seen in basal and parabasal layers in leukoplakia without dysplasia. Erythroplakia and leukoplakia with dysplasia showed cyclin D1 overexpression extending beyond the spinous cell layer. This is evidence that a PMD with cyclin D1 expression

Fig. 3 Photomicrograph of cyclin D1 uptake in OSCC

extending into the spinous cell layer can be indicative of carcinogenesis.

Shintani et al. [22] reported cyclin D1 overexpression frequencies ranging from 17.1 to 83% in OSCC. Angadi and Krishnapillai and Mishra and Das noted an uniformly increasing intensity in relation to histopathological

differentiation, while Castle et al. [23] found no correlation as in our study where there was no correlation of cyclin D1 expression and histopathological differentiation. Haas et al. [24] who found a significant correlation with a risk of recurrence in tongue Ca with cyclin D1 overexpression, in our study there was no correlation of cyclin D1 expression and site of occurrence.

Prognosis is defined as the prediction of the probable cause, duration and outcome of the disease based on the general knowledge of the pathogenesis and risk factors of the disease. Considering the biological behaviour and nature of OSCC, all parameters are interrelated, hence it is difficult to predict the prognosis independently [25]. The meta-analysis reported in the literature indicates that cyclin D1 overexpression correlates with poor clinicopathological outcome and prognosis in OSCC [1, 26], while our follow-up reported the following. Out of the 20 oral cancer cases in the study, 13 cases were managed by surgery with or without radiotherapy with or without chemotherapy, depending on the decision taken at the tumour board meeting of the institution. These patients were followed up for a period of 12 months. Of the 13 patients, two patients had a cyclin D1 score of 1 and five patients had a cyclin D1 score of 2 and three patients had a cyclin D1 score of 3 and

Fig. 4 Case with nodal recurrence

three patients had a cyclin D1 score of 4. Out of the 13 treated patients, seven patients are alive without disease who had a cyclin D1 score ranging from 1 to 4, two patients are alive with disease who had a labelling score of 3–4 and three patients with cyclin D1 score of 2–3 are dead following treatment and one was lost to follow-up (Fig. 4). The cyclin D1 expression correlation with prognosis of the disease following treatment was not found to be statistically significant (p 0.239).

Conclusion

The findings of the present study is evidence that cyclin D1 is expressed in tissues with dysplasia. Although this small sample size shows no statistically significant correlation with the treatment rendered for OSCC and the follow-up status, it opens up an opportunity for further studies on larger sample size and discussion on the role of cyclin D1 as a prognostic marker.

Compliance with Ethical Standards

Conflict of interest Reena Rachel John, C. Ravindran, N. Malathi and Rita Mary Aruna declare that they have no conflict of interest.

Ethical Approval “All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards”. Institutional ethical committee approval has been obtained.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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