Original Article

Cyclin D1 in oral premalignant lesions and oral squamous cell carcinoma: An immunohistochemical study

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Abstract

Background: Cyclin D1 is derived from PRAD1 or CCND1 gene located on chromosome 11q13 and it acts as a positive regulator of the cell cycle. In normal cells, cyclin D1 promotes progression through the G1 phase of the cell cycle. Over expression of cyclin D1 may lead to shortening of G1 phase, increased cell proliferation and reduced dependency on growth factors. Over expression of cyclin D1 has been reported in various tumors like esophageal carcinoma, hepatocellular carcinoma, lung carcinoma, and head and neck carcinoma.

Aims and Objectives: The study was carried out to evaluate and compare the expression of Cyclin D1 in premalignant lesion and different grades of oral squamous cell carcinoma.

Materials and Methods: A total 75 histopathologically diagnosed cases of oral squamous cell carcinoma and oral premalignant lesions cases were evaluated immunohistochemically for cyclin D1 expression.

Results: We found that cyclin D1 protein expression was significantly altered from epithelial dysplasia to oral squamous cell carcinomas.

Conclusion: Thus we can conclude that cyclin D1 may be a useful marker in the cases of oral precancer and cancer. It can also act as a potential target for molecular intervention studies in future.

Keywords: Cyclin D1, epithelial dysplasia, immunohistochemistry, oral squamous cell carcinomas

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INTRODUCTION

Squamous cell carcinoma (SCC) of the head and neck is the sixth most common human malignancy and the most common malignant tumor of the oral cavity. Oral SCC (OSCC) usually afflicts middle to older-aged patients who have been chronic users of tobacco and alcohol. However, there is an increased incidence of SCC in individuals with no known risk factors.[1] New biological markers might add information about the aggressiveness of tumors. Among the different cell cycle controlling proteins, one is the family of cyclins. Various research studies have correlated abundant expression of cyclin D1 in premalignant lesions and oral cancers and evaluated its role in the process of carcinogenesis.[1,2]

Over expression of cyclin D1 can be detected by various molecular methods. Immunohistochemistry has widely supported as a practical and reliable method to assess the protein expression in tissue specimens.[3] Hence, the purpose of this study is to evaluate and compare the expression of cyclin D1 in oral premalignant lesions and OSCC through immunohistochemistry.

Role of cyclin d1 in the control of cell cycle

The orderly progression of cells through the various

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phases of cell cycle is orchestrated by cyclins and cyclin-dependent kinases (CDKs) and by their inhibitors.[4,5] Cyclins are named such because they undergo a constant cycle of synthesis and degradation during cell division. They were first identified in marine invertebrates as proteins that accumulated at high levels following the fertilization of eggs and then underwent abrupt destruction during mitosis.[6,7]

When cyclins are synthesized, they act as activating protein and bind to CDKs forming a "Cyclin-CDK complex". For example, cyclin D1, D2 and D3 binds with CDK 4/6. This complex then acts as a signal for the cell to pass to the next cell cycle phase. Eventually, the cyclin degrades, deactivating the CDK, thus signaling exit from a particular phase. More than 15 cyclins such as cyclin D, E A and B and at least 8 different CDKS have been identified.[4,5,8]

During the G_1 phase of the cell cycle, cyclin D1 binds to and activates CDK4, forming a cyclin D1-CDK4 complex. This complex has a critical role in the cell cycle progression by phosphorylation of retinoblastoma-susceptibility protein (Rb). The phosphorylation of Rb is a molecular ON-OFF switch for the cell cycle. In its hypophosphorylated state, Rb prevents cells from replicating by forming a tight, inactive complex with the transcription factor E2F. Phosphorylation of Rb dissociates the complex and releases the inhibition on E2F transcriptional activity. Thus, phosphorylation of Rb eliminates the main barriers to cell cycle progression and promotes cell replication.[4,9,10] The molecular basis of the Rb breaking action on the cell cycle has been unraveled in elegant details. Hypophosphorylated Rb, present in quiescent cells (in G_0 or early G_1), binds to a protein complex that contains E2F and a subunit called DPI. The E2F/DPI/RB complex binds to the promoters of E2F-responsive genes. Bound to the E2F/DPI/RB complex, such genes are silent because Rb recruits histone deacetylase, an enzyme that causes compaction of chromatin and inhibition of transcription. When quiescent cells are stimulated by the growth factors, the concentrations of cyclin D and E increases, resulting in the activation of cyclin D1-CDK4 and cyclin E-CDK2 at the G_1/S restriction point and causes phosphorylation of Rb. Hypophosphorylated Rb dissociates from the complex, activating the transcription of E2F target genes that are essential for the progression through the S phase. During the M phase, the phosphate groups are removed from Rb by cellular phosphatases, thus regenerating the hypophosphorylated form of Rb. Thus again the cycle continue in orderly manner. However, in abnormal proliferation or in cases of cancers, this hyperphosphorylated state continues and complex does not get removed, causing high levels of cyclins, i.e., cyclin D1 [Figure 1].[4,5,11,12]

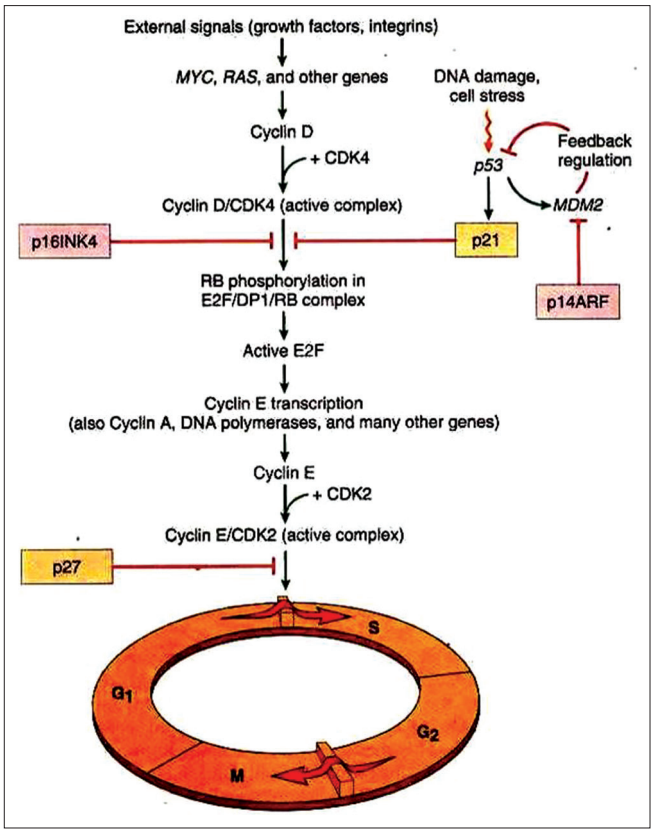

Figure 1: Role of cyclin D1 in the control of cell cycle

MATERIALS AND METHODS

A retrospective case–control study was designed. Study was approved by the Institutional Ethics Committee. The study included histopathologically diagnosed cases of OSCC and oral premalignant lesions. Samples (formalin-fixed and paraffin embedded) were retrieved from the registry of Department of Oral Pathology and Microbiology, Vidya Shikshan Prasharak Mandal's Dental College and Hospital, Nagpur from 1998 to 2010. A total 75 cases were evaluated immunohistochemically for cyclin D1 expression. These included:

- Group I --------- Oral premalignant lesions (30 cases)
- Group II --------- OSCC (30 cases)
- Group III --------- Normal mucosa (15 cases).

Two sections (4 μm) of each specimen were prepared; one section was stained with routine hematoxylin (H) and eosin (E) staining method and the other section was stained immunohistochemically to assess the expression of cyclin D1 protein.

Steps in staining

Primary antibody for Cyclin D1 (Biogenix, India) was added to sections and incubated overnight at 38°C at room temperature in a moist chamber.

The sections were then washed with TBS three times for 10 min each, incubated with biotinylated secondary antibody for 30 min at the room temperature in a moist chamber and washed in TBS three times for 10 min.

Sections were incubated with streptavidin for 45 min at the 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 dibutyl phthalate in xylene.

Interpretation of stained sections

The immunohistochemical staining for cyclin D1 was carried out and examined under a light microscope. The counting of cyclin D1 positively stained cells was performed using computer assisted image analyzer system [Figure 2]. The positive control of goat antibody provided with the IHC kit was examined for the presence of a coloured end product (DAB chromogen, brown end product) at the site of the target antigen. The presence of brown-colored nuclear staining was interpreted as positive staining. The absence of nonspecific staining in the negative control confirmed the specificity of primary antibody which was used on slides of normal tissue. All stained nuclei were scored positive regardless of intensity of staining. Cells that lacked a clear nucleus were excluded. The tissue sections were evaluated for cyclin D1 positivity by scanning the sections at a ×100 magnification. Cell counts were made at ×400 magnification in at least 10 randomly selected fields. Minimum of 1000 cells were counted in each section.

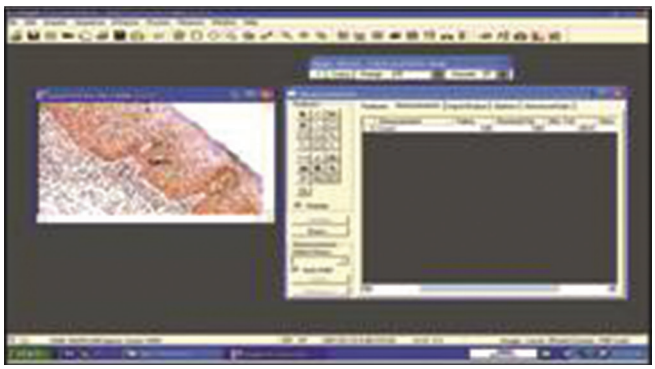

Figure 2: Output window of image-prodiscovery software showing details of measurements

The number of positively stained nuclei was expressed as a percentage of the total number counted. The cyclin D1 labeling index (LI) was calculated from the ratio of the number of tumor cells stained by cyclin D1 to the total number of tumor cells counted per section.[2]

$$\text{Cyclin D1 LI} = \frac{\text{Number of cyclin D1 positive cells}}{\text{Total number of cells observed}} \times 100$$

The pattern of cyclin D1 staining was classified as negative (−) when <10% of the cells were reactive for cyclin D1 and positive (+) when more than 10% of the cells were reactive for cyclin D1.[2] The SPSS 10.0© (Statistical Package for Social Science), online software, India was used for the statistical analysis. The relationship between protein expression and histopathological grade was analyzed by Kruskal–Wallis analysis of variance. A probability (*P* value) of <0.05 was accepted as statistically significant.

Observations

- Group I: Cyclin D1 staining was epithelial and positive cells were extended from basal layer up to prickle cell layer in most of the cases of dysplasia and in some cases throughout the thickness of the epithelium [Figures 3 and 4]
- Group II: Cyclin D1 staining was found only in epithelial cells. Cyclin D1-positive cells were found to be present throughout the tumor tissue or consisted of groups of positive cells scattered among negative cells. It was expressed mainly in the peripheral layers of tumor islands and strands. However, in some samples, positive cells were distributed in the central areas of tumor islands with loss of positivity in the most differentiated keratinized whorls. In some cases, we observed positive cells close to pearls of keratinization [Figures 5 and 6]

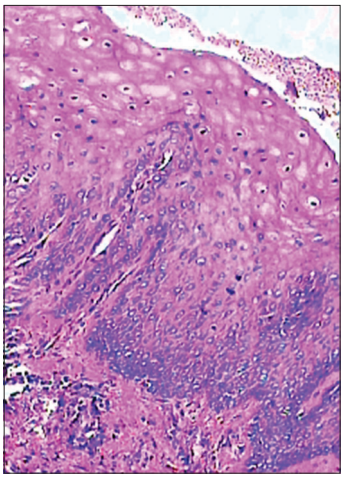

Figure 3: Hematoxylin and eosin stained section showing severe dysplasia (at ×100 magnifications)

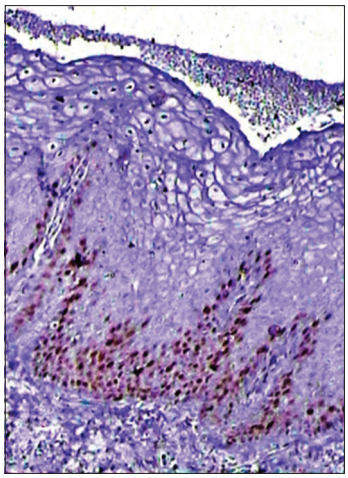

Figure 4: Immunohistochemical expression of Cyclin D1 in severe dysplasia (at ×100 magnification)

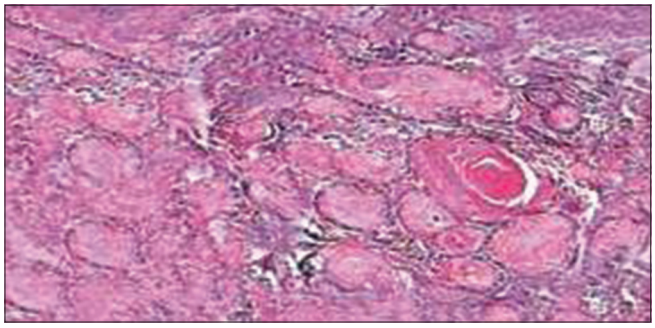

Figure 5: H&E stained section showing well-differentiated squamous cell carcinoma (at ×100 magnifications)

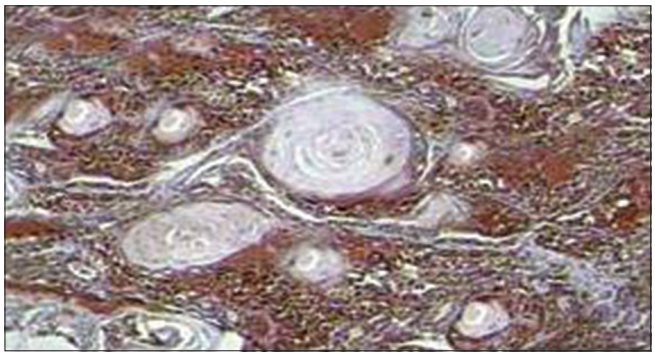

Figure 6: Cyclin D1 expression in well-differentiated squamous cell carcinoma in central areas (at ×100 magnification)

- Group III: In normal oral mucosa, cyclin D1-positive cells were present mainly in the basal cell layer, in some cases, but the percentage of expression of total cases was <10%; hence, expression was considered as negative.

RESULTS

Table 1 shows, in Group I out of premalignant cases (30), the positive cases for Cyclin D1 were 11 (36.66%) in 19 cases (63.33%) it was not expressed. Group II, out of 30 cases 20 (66.66%) cases were positive and 10 (33.33%) were negative. Group III i.e., in Normal Mucosa out of 15 cases all 15 cases were negative for expression of Cyclin D1.

Table 2 is showing the mean and range of Cyclin D1 expression in all the three groups. Expression of Cyclin D1 was higher in OSCC as compared to oral premalignant lesion and normal mucosa.

Table 3 shows that there is a statistically significant difference (P = 0.0000) for mean cyclin D1 LI between Group I, Group II and Group III.

DISCUSSION

A fundamental concept of cancer is that tumors arise from the accumulation of a number of discrete genetic events that summate to form an invasive cancer. These changes include the activation of oncogenes and inactivation of tumor-suppressor genes leading to uncontrolled cell proliferation.[13]

Cyclins are a group of proteins responsible for the activation of the main cell division transitional points. Cyclins regulate the activity of kinases, which for this reason have been named CDKs. The activation of specific cyclin-Cdk complex results in a cascade of protein phosphorylation that is required for passage through the specific stages of cell cycle.[4,11]

The transition from G1 to S phase is believed to be an extremely crucial point in the cell cycle and is guarded by the retinoblastoma protein. Cyclin D1 along with its CDK partners, CDK 4 and 6, acts on the E2F transcription factor, thereby allowing the cells to enter the S phase.[11]

Overexpression of cyclin D1 may lead to shortening of G1 phase, increased cell proliferation and reduced dependency on growth factors.[14] This may contribute to disturbance in the normal cell cycle control and mitogenic signaling pathways enhancing the cell transformation and tumorogenecity.[11] Thus, overexpression of cyclin D1 is thought to provide the tumor cells with a selective growth advantage.[15] Immunohistochemical methods offer a high sensitivity and specificity. The principle underlying assessment of cell proliferation by immunohistochemical methods is that there are cell cycle associated alterations in the amount or distribution of cellular proteins or other molecules that are recognized as antigens.

Immunohistochemical overexpression of cyclin D1 has been reported in various tumors such as esophageal carcinoma,[16] hepatocellular carcinomas,[9] breast carcinomas,[17,18] lung carcinoma,[3] colon carcinomas,[19] endometrial carcinoma,[7]

Table 1: Comparison of Cyclin D1 positive and negative cases in three groups

Groups	*n*	Group details	Cyclin D1 positive cases (%)	Cyclin D1 negative cases (%)
Group I	30	Mild dysplasia (13)	4 (30.76)	9 (69.23)
		Moderate dysplasia (9)	4 (44.44)	5 (55.55)
		Severe dysplasia (8)	3 (37.50)	5 (62.50)
		Total	11 (36.66)	19 (63.33)
Group II	30	Well-differentiated squamous cell carcinoma (14)	9 (64.28)	5 (35.71)
		Moderately differentiated squamous cell carcinoma (9)	6 (66.66)	3 (33.33)
		Poorly differentiated squamous cell carcinoma (7)	5 (71.42)	2 (28.57)
		Total	20 (66.66)	10 (33.33)
Group III	15	Normal mucosa (15)	0 (0)	15 (100)

Table 2: Descriptive statistics for mean Cyclin D1 labeling index

Group	Group details	Mean	Range
Group I	Mild dysplasia (13)	8.27	1.3-20.24
	Moderate dysplasia (9)	28.34	8-57.14
	Severe dysplasia (8)	26.37	6.8-68.20
	Total	20.99 (average)	
Group II	Well-differentiated squamous cell carcinoma (14)	29.39	3.4-52.16
	Moderately differentiated squamous cell carcinoma (9)	49.94	8.2-78.22
	Poorly differentiated squamous cell carcinoma (7)	65.43	9.4-90.20
	Total	48.25 (average)	
Group III	Normal mucosa (15)	5.73	3-8

Table 3: Comparison of mean cyclin D1 labeling index between Group I, Group II, and Group III

Groups	*n*	Mean LI	SD	Kruskal-Wallis test statistics	*P*	Result
Group I	30	20.99	22.05	20.537	0.0000	Significant
Group II	30	48.25	31.86			
Group III	15	5.73	1.38			

SD: Standard deviation, LI: Labeling Index

retroperitoneal soft-tissue sarcoma,[6] and head and neck carcinoma.[14,20] In various studies, its overexpression has been correlated with poor prognosis,[10,12] hypopharyngeal site,[21] poor histological grade,[10] increased risk of lymph node metastasis,[22] decrease in 5-year survival rate,[10,12,22] increased risk of recurrence,[23] advanced clinical stage,[1] and current or previous smoking history.[10] However, some authors found no clinical co-relation.[24,25] Expression of cyclin D1 was seen to be positively correlating with cell cycle regulatory proteins such as p21, p63, CDK 4, E2F1,PCNA,[10] p53,[25] Ki-67,[3,26] Topo II alpha and histone H3 mRNA.[27] It was found to be inversely correlating with the expression of bc1-2,[10] pRb protein.[28-30]

In our study, Group I had the mean cyclin D1 LI and percentage expression of severe dysplasia was more as compared to the mild dysplasia but was less than moderate dysplasia. The discrepancies in the over expression of cyclin D1 in different grades of dysplasia may reflect the general lack of objective quantification methods to assess protein expression since some studies use semi quantitative scoring methods or it may be due to subjective assessment of staining intensities. Furthermore, subjective nature of grading of dysplasia may be an additional factor.

In Group II (OSCC), a proportional increase in the percentage of expression of cyclin D1 with increase in the histopathological grade, i.e., from well differentiated to poorly differentiated OSCC was observed. In our study, cyclin D1 was expressed mainly in the peripheral layers of tumor islands and strands. However, in some samples, positive cells were distributed in the central areas of tumor islands with loss of positivity in the most differentiated keratinized whorls. In some cases, we observed positive cells close to the pearls of keratinization. Cells exhibiting mitosis did not show nuclear staining for cyclin D1.

This finding was expected since cyclin D1 is an activator of the cell proliferation cycle, and peripheral cells are those supposed to be the most proliferative and invasive ones. The absence of cyclin D1 in mitotic cells is probably due to the inactivation of this protein at the end of S phase, and to its short half-life. The higher expression of cyclin D1 in high-grade tumors might be explained by the fact that alterations in cyclin D1 are related to an intense proliferative activity and invasiveness capacity of the lesions. In contrast, the absence of cyclin D1 can be related to the deregulation of other proteins in the cycle.[31]

The expression of cyclin D1 in normal oral mucosa (Group III) was <10%; hence, expression was considered as negative.

The frequency of positive cells in normal oral epithelium may be tissue specific. According to Sherr *et al*.,[5] cyclin D1 is induced early in G1phase, and may be expressed throughout the cell cycle as long as a growth factor is present.

Thus, in our study, cyclin D1 protein expression was significantly altered from epithelial dysplasia to OSCCs.

There are certain limitations to our study. A quantitative prognostic relevance of cyclin D1 protein in premalignancy and OSCCs could not be established in the present study. This could be due to the small sample size of cases in individual groups and heterogeneous patient population. Homogeneity with respect to characteristics such as site, size, TNM stage and treatment of the carcinoma are required for the analysis of the relative prognostic value of each of the individual parameters of the grading system. Histologic malignancy grading systems are complicated by the heterogeneity between the subtypes of oral carcinomas and the different behavior of carcinomas arising in localized but distinct areas of the oral mucosa.[32] In dysplastic cases, subjective nature of dysplasia may also be contributing factor to this heterogeneity.[33]

Immunohistochemistry alone is not sufficient to answer the question whether these "cyclin D1-negative" tumors are really lacking cyclin Dl activity or a technical problem is present.

It is known that oral cancer is a multistep process involving alterations of various molecular markers at different levels. It is more likely that cyclin D1 could be one factor in a panel of other markers as it is observed that various markers such as Ki-67, PCNA, P21, cyclin D1, AP-1, bcl-2 etc., influence the expression of each other's in the process of malignant transformation.[3,34,35] Hence, it is difficult to state that any single protein will ever be identified as the ideal prognostic or predictive marker. Further studies should be undertaken to investigate the interrelationship between the expressions of such markers in cases of malignancies.

CONCLUSION

In conclusion, this study reports that alteration of cyclin D1 is frequent in oral precancer and OSCC. In our study, expression of cyclin D1 was significantly altered from oral epithelial dysplasia to OSCC. This indicates and supports the previous studies that over expression of cyclin D1 may be an early event in oral cancer development as oral cancer is mostly preceded by oral precancer. Our results suggest that alteration of this protein may contribute importantly to the multistep nature of oral carcinogenesis. Thus, cyclin D1 may be a useful marker in cases of oral precancer and cancer. It can also act as a potential target for molecular intervention studies in future.

Although numerous studies done to find a marker that which guides the course of the disease and the expected outcome of a treatment protocol, head and neck cancer still has high mortality rate and unexpected outcome.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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