ORIGINAL ARTICLE

Immunohistochemical characterization of cyclin dependent kinase-4 in different histological grades of oral leukoplakia and oral squamous cell carcinoma

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ABSTRACT

Background: Cyclin dependent kinase-4 (CDK4) encoded by CDK gene, is a heterodimer protein of cell cycle in G1-S transition. This study aimed to characterize the CDK4 immunoreactivity in different histological grades of oral leukoplakias (OLs) and oral squamous cell carcinomas (OSCCs) and also aims to discuss its probable role in the tumor biogenesis. **Materials and Methods:** Expression of CDK4 was investigated in total of 52 samples including OL (15), OSCCs (30) and normal oral tissues (07). A labeled Streptavidin-Biotin immunohistochemistry assay was performed and staining intensity was evaluated. **Results:** The staining pattern was similar in all tissues and was located in both nuclei and cytoplasm. Dysplastic epithelium displayed a progressive increase in nuclear expression of CDK4 when compared to normal tissues. Also, positive staining cytoplasm was highly evident in OSCC with loss of differentiation. **Conclusion:** Our study indicated a progressive over expression of CDK4 from normal to leukoplakias (various histological grades of dysplasias) and OSCCs.

Key words: Cyclin dependent kinase-4, leukoplakia, oral squamous cell carcinomas, tumor biogenesis

INTRODUCTION

Oral leukoplakia (OL) is a relatively common premalignant lesion, which is presented as a white patch that cannot be scrapped off. Even though all leukoplakias do not transform into malignancies, Scully and Porter showed a 2.4% malignant transformation rate at 10 years which increased to 5% at 20 years period.[1] This transformation involves many genetic alterations resulting in the inactivation of tumor suppressor genes and activation of proto-oncogenes by deletions, point mutations and gene amplification.[2] Ninety percent of cancers occurring in oral cavity are oral squamous cell carcinomas (OSCCs). The development of oral cancer often involves multiple factors, some dependent on the genetic constitution or environmental factors and others on the lifestyle of the individual. A variety of cellular processes have been shown to be deregulated in cancer cells, for example, cell cycle control, apoptosis and telomerase stability.[3] The cell cycle control may be disrupted due to abnormal functioning of growth factors, cyclin/cyclin dependent kinase (CDK) and decrease in negative regulatory factors owing to mutation in tumor suppressor genes.[4]

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CDKs and cyclins control the switches for the transitions from one phase of cell cycle to the next phase. Cyclin dependent kinase-4 (CDK4) is a heterodimer protein, which is crucial for the progression of cell cycle in G1-S transition. This forms complexes with Cyclin D and hyperphosphorylates the target protein, i.e. retinoblastoma protein (Rb), resulting in its inactivation and liberation of E2F family of transcription factors.[4] Thus, deregulation of Rb pathway clearly contributes to cancer formation. Over-expression of CDK4 occurs in lung cancers, sarcomas and melanomas.[5-7] In addition, over-expression of CDK4 has been found in OSCC and in OSCC cell lines.[8,9]

Few studies have shown an increased expression of CDK4 in premalignant lesions with dysplasia and atypia after application of carcinogens like 4-nitroquinoline 1-oxide and 7,12-dimethybenzanthracene (DMBA)

suggesting possible role of CDK4 in carcinogenesis.[10,11] Poomsawat *et al*. have found that CDK4 expression in normal mucosa was considerably lower than OL with dysplasia and OSCC suggesting that CDK4 might be implicated in the early event of carcinogenesis.[12] Piboonniyom *et al*. have found the over-expression of CDK4 in OL with mild dysplasia and OSCC and the over-expression of CDK6 in only OSCC and suggested that CDK4 might be involved in the early event of carcinogenesis of OSCC and CDK6 might play a role later.[13]

The purpose of this study was to determine and characterize the expression of CDK4 in OSCC and OL with different histological grades of dysplasia and to discuss possible role of CDK4 in tumor biogenesis.

MATERIALS AND METHODS

A total of 52 samples were obtained from patients consulting the Dental Clinics, Department of Surgical Oncology. This study was approved by the institutional committee on Human Rights and ethics related to Human experimentation. The tissue samples were fixed in 10% buffered formalin and were processed using conventional histopathology techniques. Then, the samples were sectioned and stained with hematoxylin and eosin. The samples were then graded by two experienced histopathologists in a double-blind manner according to the criteria of an international symposium conclusions on oral white lesions.[14] Our study included 30 cases of SCC (10 well differentiated, 10 moderately differentiated and 10 poorly differentiated), 15 cases of OL (5 mild dysplasia, 5 moderate dysplasias and 5 severe dysplasias) and 7 cases of normal mucosa used as controls. The cases were subjected to immunohistochemical staining for cell cycle protein CDK4 by using a slightly modified technique of labeled Streptavidin-Biotin immunohistochemical assay.[15] Samples of normal oral mucosa were taken from buccal mucosa of healthy volunteers and patients who have undergone minor surgical procedures.

The antibodies and reagents used for immunohistochemical technique were obtained commercially from Sigma Aldrich Company (USA) and Progen Biotechnik (Germany). According to the manufacturer, the CDK4 antibody was raised in rabbits immunized with mouse CDK4 and reacted with CDK4 from mouse, rat or humans (Mouse, monoclonal and DCS 156 clone number).

Immunohistochemical staining method

Four micron thick sections were taken onto poly-l-lysine adhesive: coated slides, dried overnight at room temperature, de waxed in xylene and hydrated through descending grades of alcohol to phosphate buffered saline (PBS). For antigen retrieval, micro-sections were immersed in 10 M sodium citrate buffer and heated twice for 5 min each in microwave oven (800 W). Sections were allowed to cool down in citrate buffer and then washed in distilled water for 5 min followed by washing thoroughly in three changes of PBS. To block endogenous peroxidase activity, micro-sections were dipped in freshly prepared 3% H_2O_2 in 18% methanol (V/V) in PBS for 10 min. Then sections were treated with 3% bovine albumin serum at room temperature for 30 min, to block non-specific protein binding sites. Sections were then incubated with prediluted primary antibody (CDK4, diluted 1:40), at room temperature for 60 min in a humid chamber, later incubated with biotinylated secondary antibody (goat-antimouse IgG) diluted 1:100, at room temperature for 30 min. Further sections were incubated with streptavidin-peroxidase conjugate, for 20-30 min. For visualization, sections were incubated with 3-amino-9-ethyl carbazole (AEC) for 10-15 min and lightly counterstained by using Mayer's hematoxylin, gently washed in running tap water and was mounted in an aqueous mounting media (Glycerol). Negative controls were carried out by omission of the primary antibody. Sections of an OSCC known to have nuclear staining for CDK4 were stained at the same time as positive controls for the antibody. All sections were processed under same conditions.

Interpretation

Assessment of antigen expressing cells was performed using light microscope at 10x and 40x magnifications. The criteria used to define CDK4 antigen positive cells were:

- Reddish brown staining in tumor cells, within the nucleus and cytoplasm
- Granular and homogenous staining within the tumor cells.

Sections of epithelial dysplasia and OSCC were evaluated for CDK4 expressing cells. In each case, four fields were selected randomly and evaluated for expression of CDK4 in the nucleus, cytoplasm and both the nucleus and the cytoplasm in the tumor cells invading the connective tissue and in the dysplastic epithelium in OSCC and OL, respectively. Slides were also evaluated for intensity of staining (dark and light) and appearance (granular and homogenous) of CDK4.

All the slides were evaluated by two qualified observers to minimize the subjective bias, the mean values of both the observers were subjected to Bonferonni '*t*' test to eliminate the inter-observer bias.

Analysis

Statistical analysis of the results was done using Bonferonni '*t*' test, Fishers *F*-test (ANOVA) and Chi-square test. For these tests, $P < 0.05$ and $P < 0.001$ was considered to be significant and very highly significant, respectively.

RESULTS AND OBSERVATIONS

A total of 20 fields were observed in each of mild, moderate and severe epithelial dysplasias (histologically confirmed OLs). The intensity of the cells expressing CDK4 was evaluated and scored

as dark and light. When the appearance of CDK4 expressing cells was evaluated in different grades of dysplasias, it was found to be granular in all the fields of dysplasias. The staining intensity within the different grades of epithelial dysplasias was found to be very highly significant. On evaluating cells expressing CDK4 in the nucleus among different grades of dysplasias, a significant increase from mild to severe dysplasias was observed [Table 1, Figure 1]. Cells expressing CDK4 in both the nucleus and cytoplasm showed a gradual increase from mild to severe dysplasias [Table 1]. In different histological grades of OSCCs, there was a gradual decrease in nuclear CDK4 expressing tumor cells from well to poorly differentiated OSCCs [Table 2, Figure 2]. Significant results were obtained showing a gradual increase in cytoplasmic CDK4 expressing cells from well to poorly differentiated OSCCs. Staining intensity of CDK4 expression was found to be gradually decreasing from well to poorly differentiated OSCCs and gradually increasing from mild to severe dysplasia [Table 3]. However, the appearance of CDK4 expression was predominantly granular in well and moderately differentiated OSCCs and predominantly homogenous in poorly differentiated OSCCs.

Table 1: Expression of CDK4 within the dysplastic cells in different grades of epithelial dysplasia

Parameter	Histological grade of epithelial dysplasia	Mean no. of cells	Significance
Nucleus	Mild dysplasia	13.45	*F*=45.45
	Moderate dysplasia	14.50	
	Severe dysplasia	18.25	*P*=0.001 VHS
Cytoplasm	Mild dysplasia	1.25	*F*=2.62
	Moderate dysplasia	1.60	
	Severe dysplasia	1.80	*P*=0.082 NS
Nucleus and cytoplasm	Mild dysplasia	2.95	*F*=10.25
	Moderate dysplasia	4.50	
	Severe dysplasia	4.65	*P*=0.001 VHS

Fishers *F* test (ANOVA), the results were subjected to Fishers *F* test and significance was elicited. VHS: Very highly significant, NS: Nonsignificant, CDK4: Cyclin dependent kinase-4

Table 2: Expression of CDK4 within the tumor cells in different grades of OSCC

Parameter	Histologic grade of OSCC	Mean no. of cells	Significance
Nucleus	Well differentiated	21.12	*F*=126.8
	Moderately differentiated	18.95	
	Poorly differentiated	14.82	*P*=0.000 VHS
Cytoplasm	Well differentiated	1.00	*F*=41.38
	Moderately differentiated	1.80	
	Poorly differentiated	2.77	*P*=0.000 VHS
Nuclear and cytoplasm	Well differentiated	2.87	*F*=110.5
	Moderately differentiated	4.02	
	Poorly differentiated	7.42	*P*=0.000 VHS

Fishers *F* test (ANOVA), the results were subjected to Fishers *F* test and significance was elicited. VHS: Very highly significant, CDK4: Cyclin dependent kinase-4, OSCC: Oral squamous cell carcinoma

Table 3: Staining intensity of CDK4 expressing cells in different grades of epithelial dysplasia and OSCCs

Histologic grading	No. of cases	Intensity of CDK4 expressing cells in four selected fields		
		Dark	Light	Total
Mild dysplasia	5	1	19	20
Moderate dysplasia	5	15	5	20
Severe dysplasia	5	20	-	20
Well differentiated OSCC	10	37	3	40
Moderately differentiated OSCC	10	19	21	40
Poorly differentiated OSCC	10	8	32	40
Total	45	100	80	180

Chi-square test; $\chi 2$=40.417; *P*=0.001 (VHS); Chi-square test; χ^2=43.058; *P*=0.001 (VHS). VHS: Very highly significant, CDK4: Cyclin dependent kinase-4, OSCC: Oral squamous cell carcinoma

DISCUSSION

Tumor biogenesis is a complicated multistage process involving various genetic alterations. However, advances in cell cycle research have revealed a loss of G1 phase regulation, which is being regulated by sequential activation of cyclins and their catalytic partners like cyclin dependent kinase (CDKs). Further any disruption in this regulatory machinery can trigger an intramolecular interaction that may progressively block tumor suppressor gene products, which in turn might contribute to an uncontrolled cell proliferation. However, a number of studies have been reported on expression of CDKs in human malignancies indicating its oncogenic property.[12,16,17]

In our study, the immunoreactivity of CDK4 was observed in all 45 tissue samples of epithelial dysplasias and OSCCs, the expression being in both the nuclei and cytoplasm. These findings are in accordance with the study of Chen e*t* a*l.* on OSCC.[16] Further among the 10 cases of well differentiated SCC, most of the cancer cell lines revealed an increased expression of CDK4 protein.[16] This suggests genetic rearrangement and point mutation in the gene site of CDK4, i.e. 12q13, presumably by catalyzing the phosphorylation and inactivation of Rb proteins, allowing the release of E2F family of transcriptional factors and permitting the cells to pass through G1 to S phase check point with resultant cell proliferation. Though the tumor cells were more differentiated, the CDK4 protein expressing cells were abundant, probably indicating an accumulation of aberrant protein in these cells. Whereas in moderately and poorly differentiated SCC, though there was a constant down regulation in nuclear expression of CDK4 protein, there was a noticeable amount of accumulation of the protein in the cytoplasm. Probably here as the tumor cells are losing their differentiation, there may be either an over expression of certain inhibitory proteins like p16 or other inhibitory proteins, possibly through certain extrinsic or intrinsic factors which in turn may decrease functional phosphorylation of Rb protein and a decrease in CDK4 proteins. This knockout mechanism on CDK4 is in agreement with findings of An *et al*. on DMBA induced carcinogenesis.[18]

However, in the present study, as a component of nuclear protein, CDK4 should have been limited to the nuclei, but antigen was evident both in nucleus and cytoplasm in all grades of SCC. This feature was also observed in studies done by other researchers.[19-21] Although the significance of cytoplasmic CDK4 expression is unclear, this might probably be due to a nonspecific reaction. This was also evidenced by Poomsawat e*t* a*l.* in their study on endometrial carcinomas who concluded it as having a different "functional status" or a "resting status" of CDK4.[12]

A number of studies have suggested an inactivation of CDK inhibitors, involved in the development of epithelial dysplasias and oral SCCs.[22-26] However, in the present study, a comparison of CDK4 staining showed a progressive increase in expression of this antigen from normal to different grades of epithelial dysplasia, being maximum in severe dysplasia and mainly localized to nuclei. This probably could be due to an amplification and translocation of 12q13, a stimulation of tumor biogenetic factors indicating an early event in progression from normal to dysplasia.[12] Furthermore, in the present study all the 15 cases of epithelial dysplasias exhibited the habit of smoking and chewing tobacco. Probably, the carcinogens might have induced the amplification of CDK4 which is one of the genetic alteration, supporting the study of Sgambato *et al*. on carcinogen inducing rat mammary tumors.[27]

In addition, to these observations in the current study, the staining intensity showed a progressive tendency for over-expression from normal to different grades of dysplasia and then in different grades of OSCC. However, among the dysplasias, severe dysplasias showed a dark staining ($P = 0.001$) [Table 3] compared to moderate and mild dysplasias and predominantly in the nuclei than the cytoplasm alone with a granular pattern. While in different grades of OSCC the staining intensity of CDK4 was greater in well differentiated SCC ($P = 0.001$) [Table 3] than in moderately and poorly differentiated SCC, with a heterotypic pattern and abundant nuclear staining. Further in well differentiated SCC, these positive cells were located mostly in the center of the epithelial islands than at the periphery [Figure 3]. While in poorly differentiated SCC the staining intensity was weak in majority of the cancer cell lines. One possible explanation for dark staining intensity could be decelerated catabolism of certain inhibitory proteins in the cancer cells which can further be related to tumor cell proliferation and later to metastasis of the tumor cells. The weak staining intensity in poorly differentiated SCC can be hypothesized as because of increased synthesis of inhibitory protein through unknown mechanism or might be due to mutant type of CDK4 protein. This finding further needs to be clarified, as the results of our study indicates a decline in cell proliferation protein, even though these tumor cells are proliferating and are becoming more undifferentiated which probably might be attributed to 12q13 gene alteration/aberration.

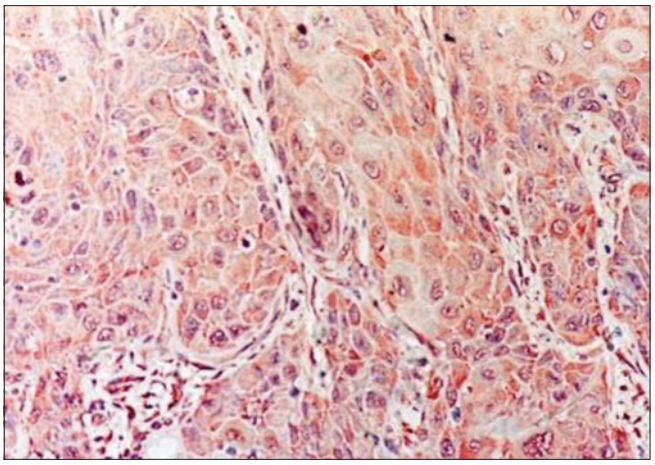

Figure 1: Severe dysplasia showing nuclear and cytoplasmic cyclin dependent kinase-4 expression - dark intensity (IHC stain, ×250)

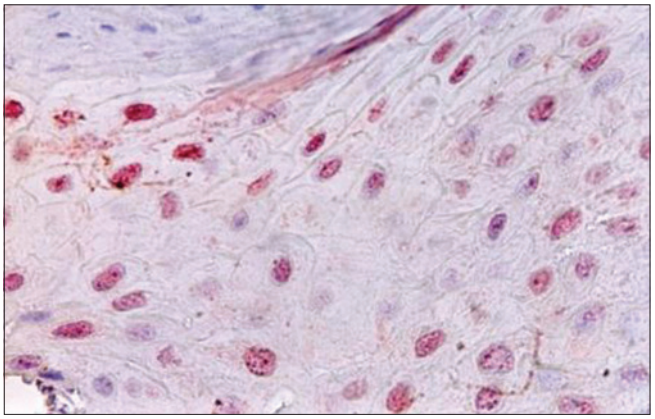

Figure 2: Well differentiated squamous cell carcinoma showing nuclear cyclin dependent kinase-4 expressing cells - dark intensity (IHC stain, ×400)

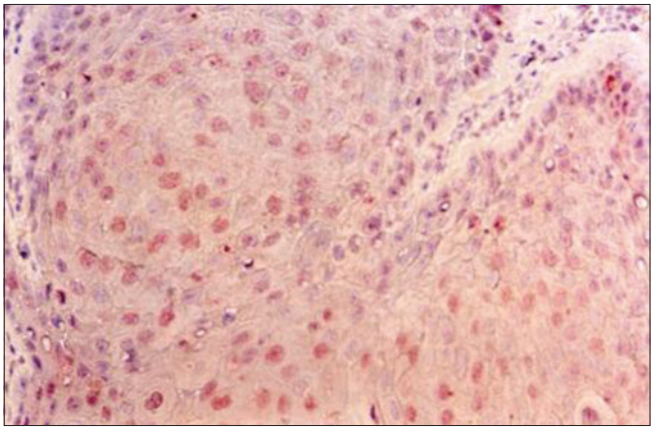

Figure 3: Moderately differentiated squamous cell carcinoma showing a tumor island with cyclin dependent kinase-4 expressing cells - darker intensity at center and lighter intensity at the periphery (IHC stain, ×400)

Various classifications have been proposed for classification of dysplasias. WHO system which was used in our study, (2005) is based on tissue architecture and cytology and it grades dysplasia in to hyperplasia (increased cell numbers without cellular atypia),

mild dysplasia (minimal architectural changes typically confined to the basal third of epithelium), moderate dysplasia (marked architectural changes seen in basal two-thirds of epithelium), severe dysplasia (marked architectural changes involving more than two-thirds of epithelium) and carcinoma *in situ* (severe form of epithelial dysplasia characterized by full thickness or almost full thickness cytological and architectural changes).[28,29]

However, further studies on mutant type of CDK4 and its inhibitory proteins are essential to clarify the exact role of CDK4 in oral carcinogenesis.

CONCLUSION

The immunoreactivity and the staining intensity showed a progressive tendency for overexpression from normal to different grades of dysplasia (OLs) and in different histological grades of OSCC. However, the intensity of CDK4 was observed to be weak (light) in poorly differentiated OSCC, when compared to well- and moderately-differentiated OSCC. The molecular mechanisms underlying the observed down regulation of CDK4 in poorly differentiated OSCC is unclear. Further studies are essential by employing mutant types of CDK4 protein and various inhibitory proteins to understand the intricate function of CDK4 in the process of oral carcinogenesis.

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