Research Article

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Expression of Cyclin D1 in Different Grade of Squamous Cell Carcinoma

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ABSTRACT

Background: Oral squamous cell carcinoma is a life-threatening disease of the oral cavity and the most common histopathological type of cancer accounting for 91% of oral malignancies. Oral cancer is an epithelial neoplasm generally beginning as a focal overgrowth of altered stem cells near the basement membrane, moving upward and laterally, replacing the normal epithelium. Dysregulation of the cell cycle machinery is a fundamental hallmark of cancer and this is emerging as a central theme in oral carcinogenesis. Therefore, the genes involved in cell cycle control represent privileged targets of oncogenic abnormalities among which Cyclin D1 represents the strongest case.

Material and methods: A total of 40 cases of oral squamous cell carcinoma were evaluated immunohistochemically for Cyclin D1 expression. Oral squamous cell carcinoma were subdivided by modified Anneroth et al.'s histopathological grading into Well-Differentiated, Moderately-Differentiated and Poorly-Differentiated Carcinoma and were taken up for evaluation.

Results: Positive staining was observed in both Squamous cell carcinoma and Leukoplakia, but there was variation in intensity in both these lesions.In well differentiated squamous cell carcinoma, the positive cells were observed in the periphery of the tumor islands while in moderate and poorly differentiated squamous cell carcinoma, a diffuse distribution was seen.

Conclusion: Statistically positive correlation was found between degree of expression , total grade of malignancy the pattern of invasion.

Keywords: Malignancy grading system, Squamous cell carcinoma, Cyclin D1 .

INTRODUCTION

Oral squamous cell carcinoma is a life threatening disease of the oral cavity and the most common histopathological type of cancer accounting for 91% of oral malignancies. Oral cancer is an epithelial neoplasm generally beginning as a focal overgrowth of altered stem cells near the basement membrane, moving upward and laterally, replacing the normal epithelium. Oral squamous cell carcinoma is one of the most common cancer in the world and shows marked geographic difference in occurence. Oral cancer is common where betel quid chewing, beedi smoking and alcohol consumption are high. It is a common cancer in South East Asia, where more than 100.000 new cases are reported every year.

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Development of oral squamous cell carcinoma is a multistep process requiring the accumulation of multiple genetic alterations, influenced by patient's genetic predisposition as well as by environmental factors, including tobacco, alcohol, chronic inflammation and viral infection. Understanding the pathogenesis of cancer, may well lead to improved diagnostic tests, preventive treatment and management. Every year the approach to laboratory diagnosis of cancer is becoming more complex, sophisticated and specialized. The routine diagnostic methods include histologic and cytologic methods, and the advanced diagnostic methods include immunohistochemistry, molecular diagnosis, flow cytometry and tumor markers". Histopathology is the gold standard in diagnosis although it is time taking.

Immunohistochemistry has been widely supported as a practical and reliable method to assess the protein expression in tissue specimens.[2] Numerous immunohistochemical studies have been done on oral squamous cell carcinoma with a prime objective to understand the biology, diagnosis, prognosis and treatment of this entity.[3] Rapid progress has been made in the last several years in elucidating the molecular mechanisms underlying cell cycle regulation in mammalian cells.Four phases have been identified in cell cycle i.e G1, S (DNA synthesis), G2 and M (mitotic phase).The orderly progression of the cells through the cell cycle is precisely governed by a series of proteins called “cyclins”, which exert their effect by binding and activating the Cyclin dependent kinases. This process is further regulated by phosphorylation, tumor suppressor genes and inhibited by CDK inhibitors.[3] Dysregulation of the cell cycle machinery is a fundamental hallmark of cancer and this is emerging as a central theme in oral carcinogenesis. Therefore, the genes involved in cell cycle control represent privileged targets of oncogenic abnormalities among which Cyclin D1 represents the strongest case.[3]

Studies have revealed cell cycle regulatory proteins (e.g. p63, cyclin D1, p27, Ki-67, Bcl-2) and growth factors / growth factor receptors (e.g. VEGF, EGFR) that demonstrated changes in their pattern of expression (i.e. up- or down-regulation), in terms of both intensity and area of staining, in parallel with the severity of the dysplastic changes in the oral epithelium.[4]Cyclin D1, a 45 kD protein is a part of the molecular system that regulates the cell cycle G1 to S transition. Cyclin D1, a 45 kD protein encoded by CCND1 gene located on chromosome 11q13, is a part of the molecular system that regulates the cell cycle G1 to S transition.[4] It was first isolated as PRAD1 oncogene clonally rearranged and overexpressed in parathyroid adenomas and is identical to bcl-1 protoncogene, which is translocated and overexpressed in a subset of B-cell neoplasms.[5]

Cyclin D1 protein binds and activates CDK4 and CDK6 leading to phosphorylation of retinoblastoma protein which results in release of transcriptional activator E2F, leading to transcription and activation of proteins associated with passage through G1 checkpoint and progression to the S phase.[6] Overexpression of Cyclin D1 leads to shortening of G1 phase and less dependency on growth factors resulting in abnormal cell proliferation which in turn might favors the occurrence of additional genetic lesions.[7] With this background present study was undertaken to evaluate the expression of cyclin D1 oncoprotein in oral squamous cell carcinoma and dysplastic lesions in order to determine whether this oncoprotein has a key role in early stage of tumor progressions and whether this can be used as a marker for early detection and elucidation of oral cancer.

MATERIALS & METHOD

A total of 40 cases of oral squamous cell carcinoma were evaluated immunohistochemically for Cyclin D1 expression. Oral squamous cell carcinoma were subdivided by modified Anneroth et al.’s histopathological grading into Well-Differentiated, Moderately-Differentiated and Poorly-Differentiated Carcinoma and were taken up for evaluation. Tumor was graded according to modified Anneroth’s histological malignancy grading system (1987). According to it, the tumor cell population was assessed using the criteria of degree of keratinisation, nuclear polymorphism and number of mitosis. Pattern of invasion, stage of invasion and lympho-plasmocytic infiltration were used to assess tumor host relationship.

Malignancy grading system for oral squamous cell carcinoma, Anneroth et al.(1987)

Histological grading of malignancy of tumor cell population

Morphological parameter	**1**	**2**	**3**	**4**
Degree of keratinization	Highly keratinzed (50% of cells)	Moderately keratinized (20-50% of cells)	Minimal keratinized (2-5% of cells)	No keratinisation (0-5% of cells)
Nuclear polymorphism	Little nuclear polymorphism (75% mature cells)	Moderately abundant nuclear polymorphism (50-75% mature cells)	Abundant nuclear polymorphism (25-50% mature cells)	Extreme nuclear polymorphism (0-25%mature cells)
Number of mitosis/HPF	0-1	2-3	4-5	5

Histological grading of malignancy of tumor host population

Morphological parameter	1	2	3	4
Pattern of invasion	Pushing well delineated infiltrating borders	Infiltrating, solid cords, bands and /or strands	Small groups or cords of infiltrating cells	Marked and widespread cellular dissociation in small groups of cells
Lympho-plasmocytic infiltration	Marked	Moderate	slight	None

Tumors are classified according to total score into three types:

Grade I - 5-9
Grade II - 10-15
Grade III - 16-20

Two to three serial sections of 4µm thickness were made and taken on salinized slides. The sections were deparaffinized by heating on the slide warmer at 60°C for 40-45 minutes then IHC staining procedure is done. Presence of brown colored end product at the site of target antigen was indicative of positive immunoreactivity. The negative control tissue demonstrated absence of staining. Tissue sections of breast carcinoma were taken as positive controls. Brown staining of the basal and parabasal layers showed positive reaction and was confirmed as being stained. The evaluation of study cases was done subsequently in a similar way and they were graded as positive or negative.

The positive results were assessed further for number of positive cell for p63 staining, which was graded for statistical analyses. The immunoreactivity was considered positive if 10% or more the tumor cells stained, and was graded either as weak **1** (10-25% of positive cells), mild **2** (26-50% of positive cells), moderate **3** (51-75% of positive cells) or strong **4** (76-100% of positive cells) in OSCC.

The positive results were also assessed further for intensity of staining for Cyclin D1 staining and nuclear staining was considered positive, which was graded for statistical analyses. In cases with staining heterogeneity, the expression was grouped according to the predominant staining intensity. It was graded as (**+**) mild staining, (**++**) moderate staining and (**+++**) intense staining in well, moderately and poorly differentiated oral squamous cell carcinoma.

All these observations were carried out by two more observers in order to eliminate interobserver bias.

RESULTS

Table I: Age and sex wise distribution of patients of oral squamous cell carcinoma.

Sr.No.	Sex	Age ranges			Total no.of patients
		20-40yrs	40-60yrs	>60yrs	
1	F	3(20%)	8 (53.33%)	4(26.33%)	15
2	M	8(32%)	10(40%)	7(28%)	25
	Total	**11**	**18**	**11**	**40**

Table II; Correlation of sex with Cyclin D1 expression.

Sex	Cyclin D1 expression		No. of patients
	Cyclin D1 +ve	Cyclin D1 -ve	
Male	19(76%)	6(24%)	25
Female	12(80%)	3(20%)	15
Total	**31**	**9**	**40**

Table III: Correlation of age and sex with Cyclin D1 positive expression.

Age group	Total	M	F	Percentage of Cyclin D1 +ve staining
20-40	11	8	3	27.5%
40-60	18	10	8	45%
>60	11	7	4	27.5%
Total	**40**	**25**	**15**	**100%**

Statistical analysis

Parameter	Pearsons correlation r value	p value	Significance
Age	r=0.084	p>0.05	NS
Sex	r=-0.176	p>0.05	NS

Table IV: Correlation of site with Cyclin D1 positive expression.

Site	Total	Cyclin D1 expression		Mean Percentage of Cyclin D1 +ve cells
		+ve	-ve	

Tongue	22(55%)	18	4	27.01%
Floor of mouth	3(7.5%)	3	0	28.13%
Alveolus	7(17.5%)	5	2	23.64%
Buccal mucosa	8(20%)	5	3	28.42%
Total	**40(100%)**	**-**		**-**

Statistical analysis

Table V: Cyclin D1 expression in different grades of malignancy.

Histopathological Grades	Cyclin D1 +ve	Cyclin D1 -ve	Total no.of samples
I	13(76.47%)	4(23.52%)	17
II	9(69.23%)	4(30.76%)	13
III	9(90%)	1(10%)	10
Total	31	9	40

Table VI: Comparison of individual parameters of Modified Anneroth's histological malignancy grading with Cyclin D1 expression.

Parameter of malignancy grading system	Scores	No. of patients	Cyclin D1 immunoexpression	
			+ve	-ve
Degree of keratinization	1	10(25%)	2	8
	2	21(52.5%)	16	5
	3	7(17.5%)	6	1
	4	2(5%)	2	0
Nuclear polymorphism	1	8(20%)	3	5
	2	21(52.5%)	12	9
	3	9(22.5%)	9	0
	4	2(5%)	2	0
Number of mitoses	1	3(17.5%)	2	1
	2	26(65%)	15	11
	3	8(20%)	6	2
	4	3(7.5%)	2	1
Pattern of invasion	1	6(15%)	3	3
	2	17(42.5%)	11	6
	3	14(35%)	9	5
	4	3(7.5%)	3	0
Lympho-plasmocytic infiltration	1	10(25%)	6	4
	2	16(40%)	9	7
	3	13(32.5%)	11	2
	4	1(2.5%)	0	1

Table VII: Statistical analysis for various parameters of Modified Anneroth's histological grade of malignancy.

Sr. No.	Parameter	Spearman correlation. r value	p value	Significance
1	Degree of keratinization	0.536	p<0.05	S
2	Nuclear polymorphism	0.589	p <0.05	S
3	Number of mitoses	0.439	p <0.05	S
4	Pattern of invasion	0.204	p >0.05	NS
5	Lympho-plasmocytic infiltration	0.412	p <0.05	S
6	Total malignancy grade	0.594	p <0.05	S

S-significant, NS-not significant

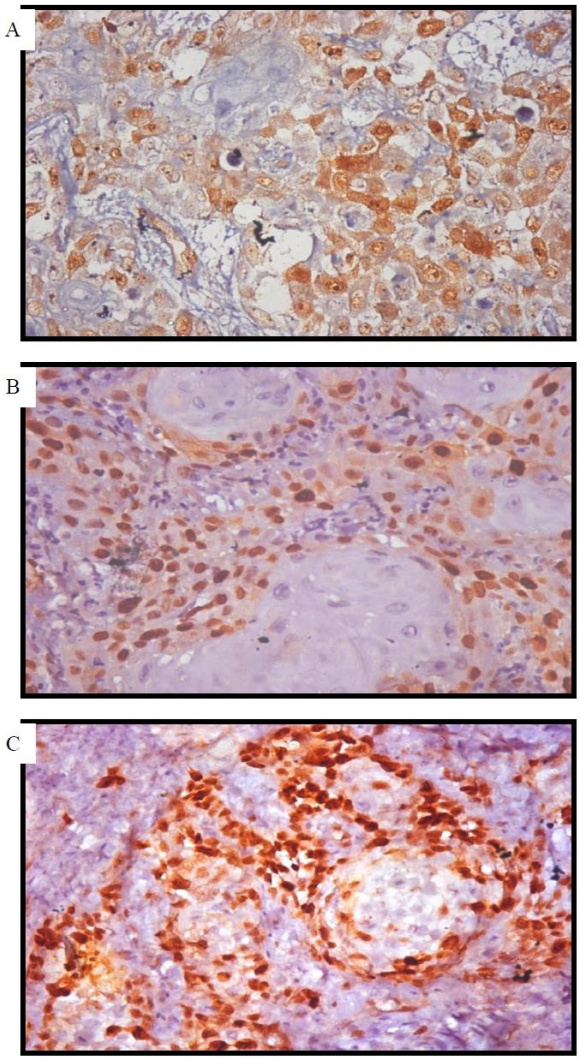

Fig 1: Photomicrograph of WDSCC showing intensity of Cyclin D1 expression. (40x objective) A: Mild, B: Moderate, C: Intense.

DISCUSSION

The cell cycle forms the basis of continuity of life and underlies the complexity of growth, renewal and repair in all organisms **(Poon RYC et al, 1997).**[10]

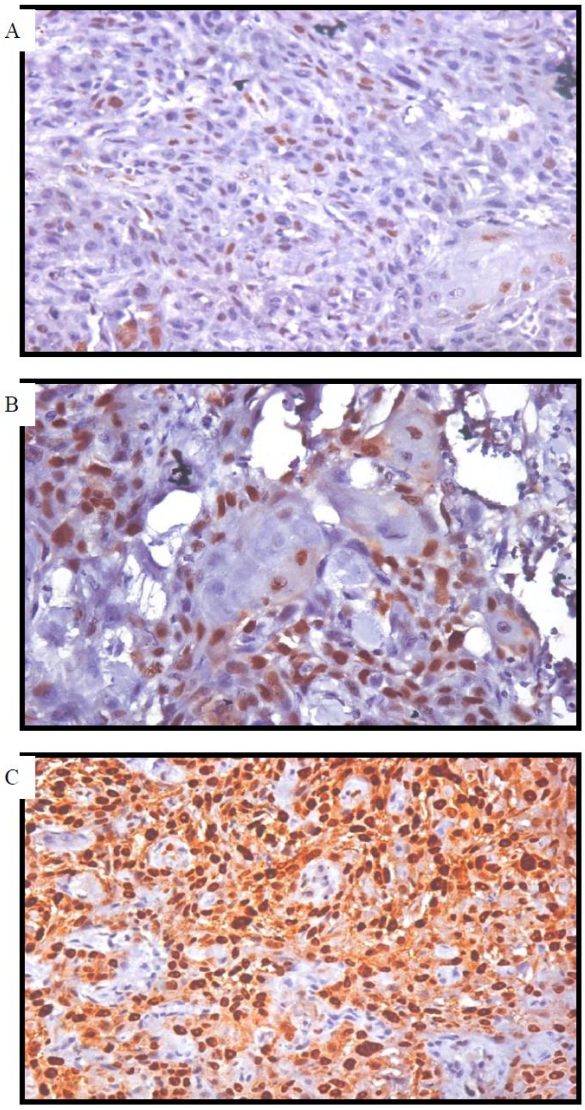

Fig 2: Photomicrograph of MDSCC showing intensity of Cyclin D1 expression. (40x objective) A: Mild, B: Moderate, C: Intense.

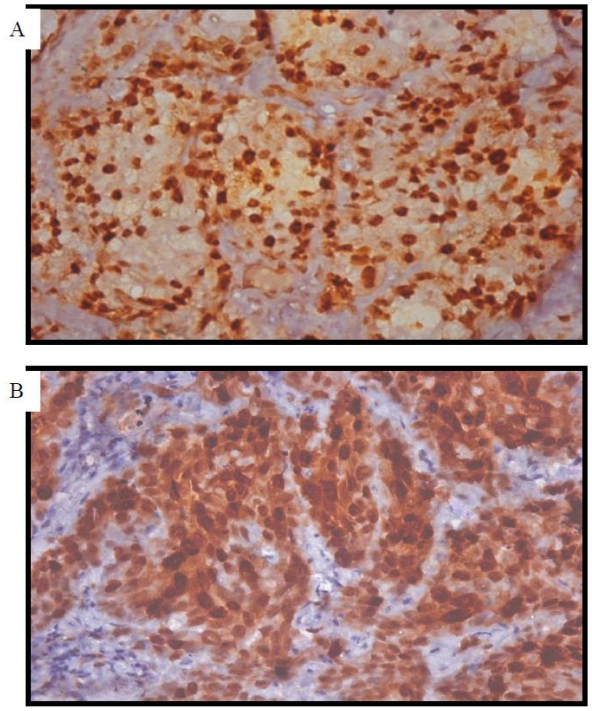

Fig 3: Photomicrograph of PDSCC showing intensity of Cyclin D1 expression. (40x objective) A: Moderate, B: Intense.

It may be visualized as a relay race consisting of G1, S, G2, M and G0 phases/laps. Each of these laps is regulated by a distinct set of "cyclins" that activate their CDK partners, and propel the cell forward in its proliferative pool. The negative control over the cell cycle is exerted by CDK inhibitors which silence the CDK's **(Kumar V et al, 2003).**[5] The present study utilized the Cyclin D1 monoclonal antibody on oral squamous cell carcinoma to elucidate the role of this key cell cycle regulator in this neoplasia. tumor types. These alterations include oncogenes that mediate cell cycle control (e.g., cyclins, cyclin-dependent kinases [CDK] and their inhibitors [CDI]), or genes that mediate stress or damage response (p53).[22] These genetic alterations provide mechanism for the development of cancer, are putative targets of therapy, and/or may serve as diagnostic and prognostic markers.[23]

In the present study, on the basis of age, it was found that the cyclin D1 expression in younger individuals (<60 years) is higher with statistically significant correlation. These results can be attributed to genetic defects and partly to difference in carcinogen exposure between the two age groups. Changing patterns in the incidence of oral cancer suggest that there may be mechanistic difference with regard to tumor progression between young and old individuals, and therefore, now the younger group of population are at a risk of developing aggressive disease.[62] No sig nificant correlation was found in relation to gender, and site distribution may be attributed to the relatively small sample size or racial differences and mig ht be due to variation in tumor biology at different sites in OSCC.[30]Overexpression of cyclin D1 is a common genetic event in head and neck squamous cell carcinoma. In the present study, overexpression of cyclin D1 was reported in 77.5% cases of OSCC. This was in accordance with previous studies done by Lam *et al*.[63] and Angadi *et al*. who have reported 63 and 70.7% positivity, respectively. Few studies conducted by Chetty *et al*. and Fracchiolla *et al*. showed a very low percentage of positivity of 29 and 39%, respectively. A very high cyclin D1 expression of 95 and 97% has been reported by Swaminathan *et al*. and Shiang *et al*., respectively. Thus, there is wide variation in the reported positive IHC reactivity in the literature. In case of normal oral epithelium, the expression was restricted to the basal and parabasal layers of epithelium, and the results were considered to be positive or negative correlating with the staining in the control epithelium. Hunter *et al*. suggested that cyclin D1 is an activator of the cell proliferation, and because peripheral cells are the most proliferating ones, they express more cyclin D1. Loss of cyclin D1 in mitotic cell is suggested to be due to the inactivation of this protein by the end of S phase and because of its short half-life.[64]

The present study suggested a positive correlation for cyclin D1 expression with lack of differentiation, which is in contrast with previously reported studies of Saawarn *et al*. and Wu *et al*. where they reported a positive correlation with the degree of differentiation. However, a similar study performed on lung carcinoma by Mate *et al*. and OSCC by Lam *et al*. stated that differentiation in addition to proliferation is a major target of cyclin D1 impingement upon mechanisms of development and neoplasia. This was supported by a report by Shapek *et al*., where a direct relationship between cyclin D1 overexpression and inhibition of myogenic differentiation in myoblasts was explained in an experimental model. This raises a possibility that cyclin D1 overexpression may play a role in inhibition of tumor cell differentiation in

some cell types, probably by binding with nonfunctional CDK. The high expression of cyclin D1 with poor cellular differentiation is related to intense proliferative activity and invasiveness of the lesion. In contrast, Wu *et al*. suggested that the absence of cyclin D1 can be related to the deregulation of other proteins in the cycle.[31]

The mechanisms underlying Cyclin D1 overexpression include gene amplification, chromosomal translocation and other post translational mechanisms **(Todd R et al, 2002; Mate JL et al, 1996).**[4,12]

Cyclin D1 predominantly expresses in the nucleus, few studies in breast carcinoma and tongue carcinoma have reported both nuclear and cytoplasmic expression **(Gillett C et al, 1996)**, [13] and only cytoplasmic expression also **(Vora H.H. et al, 1999)**.[14] These were attributed to the alteration in stability of Cyclin D1 or to an alteration in gene amplification process **(Vora H.H. et al, 1999)**.[14]

In the present study, only those cases showing distinct nuclear immunoreactivity and specific nuclear expression exceeding the cytoplasmic expression were considered positive for Cyclin D1 similar to **Miller KD et al (1999)**[155] and **Zhang S Y et al, (1994)**.[16] The occasional cytoplasmic expression was attributed to poor fixation or excessive heat pretreatment **(Miller KD et al, 1999).**[15]

All the cells which expressed Cyclin D1 did not show uniform intensity but showed cell to cell variation. This variation in the intensity probably reflects the abundance of Cyclin D1 which shows characteristic cell cycle dependent variation i.e. accumulation throughout the G1 phase, peak in late G1 and decline before the onset of S phase **(Bartkova et al, 1994; Frachiolla NS et al, 1994)**.[17,8] Hence, the predominant expression intensity in each case was taken into consideration.

There is little information on the expression of this Cyclin in normal epithelia and precursor lesions **Uhlman DL (1996),**[18] **A Rousseau et al 2001,**[19] **S. Shintni et al 2002.**[20]

It has been detected in oral epithelial dysplasia **(Castle JT, 1999)**,[21] endometrial hyperplasia **(Quddus, MR, 1999)**[21] and benign lesions like parathyroid adenoma **(Arnold A, 1989)**,[22] therefore it has been implicated as an early event in tumorogenesis and may be a useful biomarker in molecular epidemiology and chemoprevention studies.

In the present study, 9 cases (23.33%) of OSCC did not showed Cyclin D1 expression. This could have been due to sustained mutations in other key cell cycle regulators like Rb, p53, Cdk4, p16 **(Gimenez Conti et al, 1996; Jie Xu et al, 1998; Bartkova et al, 1994; Koontongkaeu S et al, 2000)**[33,25,17,32] which are also capable of abrogating the cell cycle machinery resulting in unlimited proliferation.

CONCLUSION

Male (62.5%) predominance was found in oral squamous cell carcinoma. Patients of 40-60 years age range (45%) were most commonly affected.Out of 40 biopsy samples, 55% were taken from tongue, 7.5% from floor of mouth, 17.5% from alveolus, and 20% from the buccal mucosa. 77.5% biopsy samples showed positive cyclin D1 expression and remaining 22.5% were negative for cyclin D1 expression. Mean index of cyclin D1 expression was higher in males (29.27%) than that of females (22.66%). Cyclin D1 expression was not statistically correlated with sex of the patients. Highest cyclin D1 expression was found in age range of 40-60 years (66.66%). Highest cyclin D1 expression was found in buccal mucosa (28.42%), followed by floor of mouth (28.13%) tongue (27.01%), and alveolus (23.64%). Cyclin D1 expression was not statistically correlated with tumor site. Highest cyclin D1 expression was seen in grade III tumors (47.57%). Statistically positive correlation was found between total grade of malignancy and cyclin D1 expression ($p<0.05$). Statistically positive correlation was found between degree of expression ($p<0.05$). Similarly significant correlation keratinization and cyclin D1 was found for nuclear polymorphism ($p<0.05$), number of mitoses ($p<0.05$) and lympho-plasrnocytic infiltration ($p<0.05$). Statistically negative correlation was found between the pattern of invasion and cyclin D1 expression($p<0.05$)

CONFLICTS OF INTEREST

The authors declare they have no potential conflict of interests regarding this article.

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