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**Immunohistochemical comparison of cyclin D1
expression in ameloblastoma, odontogenic keratocyst
and radicular cyst**

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Abstract

Objective: The present study was undertaken to compare expression of cyclin D1 in the epithelial part of odontogenic keratocysts, radicular cysts and ameloblastomas.

Material and Method: In this observational and cross-sectional study, immunohistochemical staining of cyclin D1 on formalin-fixed, paraffin-embedded tissue sections of odontogenic keratocysts (n=14), radicular cysts (n=14) and ameloblastoma (n=14) was performed by standard EnVision method. Then, slides were studied to evaluate the following parameters in epithelial part of lesions: expression, expression pattern, staining intensity and localization of expression and clinicopathological parameters: gender, mean age and site of the lesion.

Results: The data analysis showed statistically significant difference in cyclin D1 expression in studied groups ($p < 0.05$). Assessment of staining localization and staining pattern showed basal and parabasal localization and more cases of diffuse pattern in odontogenic keratocysts, but difference was not statistically significant among groups respectively ($p = 0.393, 0.465$). Considering staining intensity, cyclin D1 positive cells in odontogenic keratocysts showed most cases of strong intensity, different from radicular cysts and Ameloblastoma. The difference was statistically significant ($p = 0.002$).

Conclusion: these findings could support the theory about neoplastic origin of odontogenic keratocysts, but results may support that, these lesions are developmental cysts with neoplastic properties because of the high intrinsic growth potential.

Keywords: Cyclin D1, Odontogenic keratocyst, Radicular cyst, Ameloblastoma, Immunohistochemistry

Introduction

The classification of odontogenic jaw cysts is based on their histology, pathogenesis and presumed developmental origin. Radicular cysts have an epithelial part derived from the epithelial rests of Malassez. It is presumed that epithelia of odontogenic keratocysts originates from the odontogenic epithelium of the dental

lamina or its remnants, prior to tooth formation. However, it has also been suggested that odontogenic keratocysts could be derived from basal cells of the oral mucosa (1). Odontogenic keratocysts show a particular tendency to recur after their surgical removal that could be due to a multilocular nature or a

presence of daughter or satellite cysts that are left behind. However, it has also been hypothesized that the epithelium of these cysts could have an intrinsic potential to growth, resembling benign neoplasms (2). Ameloblastomas are tumors that arise from rests of the dental lamina. Thus, OKCs and ABs derived from the same cell population, and like ABs, OKCs may behave aggressively and can penetrate cortical bone, extending into the surrounding tissues, with also they show recurrence rates ranging from 3% to 60% (3).

Transition between different stages of cell cycle is regulated at check points. Several check points are regulated by cyclin dependent kinases (CDKs) and their activating partners, the cyclins. Cyclin D1 gene (CCDN1) is located on chromosome 11q13, encodes a critical cell cycle regulatory protein (Cyclin D1) that drives the cell cycle from G1 to S phase (4). Amplification and over expression of CCND1 has been reported in various carcinoma (5,6,7,8,9,10,11), so the study of cyclin D1 expression may improve our knowledge about the biological substrate of OKC and ameloblastomabehavior.

Based on this hypothesis, in the present study, expression of cyclin D1 in odontogenickeratocyst, radicular cyst and ameloblastoma in order to compare proliferative activity in these lesions was undertaken.

Materials and Methods

Sample collection

After reviewing clinical information and histologic findings, a total of 42 odontogenic cysts and tumors consisting OKCs (n=14), RCs (n=14) and ameloblastoma (n=14) were collected from paraffin-embedded blocks of Oral and Maxillofacial Pathology Department, Jundishapur University of Medical Sciences, Ahvaz, Iran.

Inflammatory lesions except for radicular cysts because of probable effect of inflammation on proliferative activity were excluded from the study.

Immunohistochemistry

IHC staining of paraffin blocks is as follow:

IHC staining was performed by standard Envision methods. After taking slices, samples were placed on slides stained with Poly-L-Lysin deployed for 24 hours at 37°C to dry. The samples were then deparaffinized in Xylene and rehydrated in varying degrees of ethanol. Consequently, in order to stop the inner peroxidase activity, samples were placed in methanol containing peroxide (H₂O₂) 0.3% for 30 minutes at room temperature and then rinsed in Phosphate buffered saline (PBS) solution PH = 7.2. Immunohistochemical staining for cyclin D1 (Leica / England / monoclonal/ Clones: P2D11F11/ Dilutions: 1:30) were performed

according to the manufacturer's recommendations. After the incubation with the primary antibody, Envision technique was used. Samples were incubated with Polymer solution (anti-mouse) for 30 minutes and washed with PBS. In the next stage, the 3,3DiaminobenzidineHydrochloride (DAB) dye that gives brown color to antigen-antibody complex was used. Samples were counterstained with hematoxylin and plates were placed on them. Finally, the immunohistochemical staining status was analyzed by optical microscope by a pathologist. In this study, Tissue sections of the oral squamous cell carcinoma were considered as positive control and or negative control PBS was used instead of specific antibody. All slides were reviewed blindly.

Staining evaluation:

We scored the cyclin D1 expression according to three categories: negative (absence of staining) and positive, and this latter into two additional categories, focal or diffuse staining (12).

Counting was carried out by graded lens. First we selected fields with high intensity of staining at x4 magnification. Then, by x40 magnification, 5 microscopic fields which showed nuclear and cytoplasmic staining were identified and the number of positive cells was divided into the total number of cells counted in every field. Then the average of all fields was calculated. The result was multiplied by 100 to find the percentage of positive cells. Because in our samples, RC did not have adequate lining tissue, we have used this method to calculate positive cells.

The intensity of staining was graded as mild, moderate and strong by objectively comparing with unstained cells. Mild staining denoting light brown color, moderate - brown color, and intense - dark brown color.

Staining localization in epithelial parts of lesions was investigated to indicate which epithelial layer(s) exhibited the most reaction: basal and parabasal, all layers.

Statistical analysis

Analysis of the data was performed using Statistical Package for Social Sciences (SPSS software) (SPSS, Inc, Chicago, IL, USA) version 18.0. The Chi-Squared test was used for all variables. Mann-Whitney test was performed for pairwise comparison of expressed cells among groups. P<0.05 was regarded as statistically significant.

Results

Out of 42 selected cases, most of them were located in mandible (n=27). Mean age in OKCs, RCs and AB was 34, 35 and 30 years respectively. Lesions were more common in male (n=26). (Table II)

All results in each group separately are as follows:

34.57% of cells were stained (Table II). Immunoreactivity in most of the cases (n=9) was restricted to the basal & parabasal with moderate intensity (n=6) and diffuse pattern (n=10).

Odontogenickeratocyst: As Table I shows, cyclin D1 expressed in 11 (78.6%) cases (Figure 1). On average

Table I: Distribution of expressed samples, staining pattern, staining intensity and staining localization of cyclin D1 and site, gender and mean age of all groups of lesions

	OKC	RC	AB	P value
	n (%)	n (%)	n (%)	
Expressed samples	11(78.6)	10(71.4)	12(85.7)	0.654
Expression pattern				0.465
Focal	1(7.1)	3(21.4)	3(21.4)	
Diffuse	10(71.4)	7(50)	9(64.3)	
Staining intensity				0.002
Mild	0(0)	0(0)	5(35.7)	
Moderate	6(42.9)	10(71.4)	4(28.6)	
Strong	5(35.7)	0(0)	3(21.4)	
Staining localization				0.393
Basal & parabasal	9(64.3)	9(64.3)	8(57.1)	
All layers	2(14.3)	1(7.1)	4(14.3)	

(RC) radicular cyst, (OKC) odontogenickeratocyst, (AB)amelobelastoma

Radicular cyst: Cyclin D1 was discernible in 10 (71.4%) cases of RCs (Figure 2) frequently in basal & parabasal layer (9 cases). Diffuse pattern of staining were observed in 7 (50%) cases of RCs. Moderate intensity was visible in all cases (Table I). As Table III shows on average 8.09% of cells were stained.

Amelobelastoma: In AB, cyclin D1 was detected in 12(85.7%) cases (Figure 3) with more diffuse patterns (n=9) of staining (Table I). On average, positive cells were 36.35% (Table II) and limited mostly in basal & parabasal. Staining intensity was mild to moderate (Table I).

Table II: demographic information of studied groups

	Mean age	Gender		Site	
		Male	Female	Maxilla	Mandible
OKC	34.28	10(71.4%)	4(28.6%)	3(21.4%)	11(78.6%)
RC	35.64	7(50%)	7(50%)	8(57.1%)	6(45.9%)
AB	30.64	9(64.3%)	5(35.7%)	4(28.6%)	10(71.4%)

(RC) radicular cyst, (OKC) odontogenickeratocyst, (AB)amelobelastoma

Table III: Mean percentage of expressed cells (%)

Lesions			P value
	OKC(34.57)	AB(36.35)	0.874
OKC(34.57)	RC(8.09)	0.016	
AB(36.35)	RC(8.09)	0.004	

(RC) radicular cyst, (OKC) odontogenickeratocyst, (AB)amelobelastoma

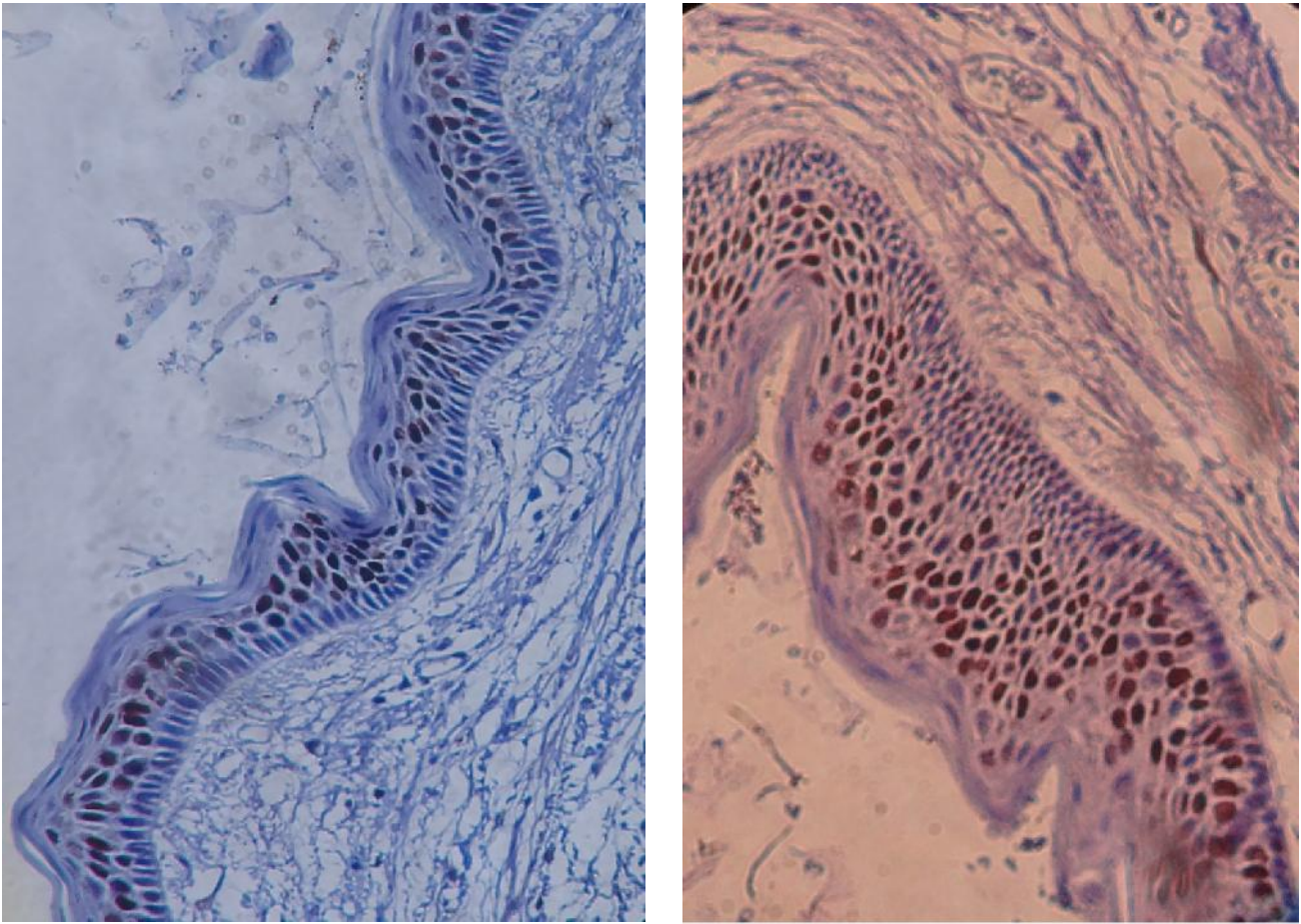


Figure 1: Cyclin D1 expression with strong intensity in odontogenic keratocyst (x40).

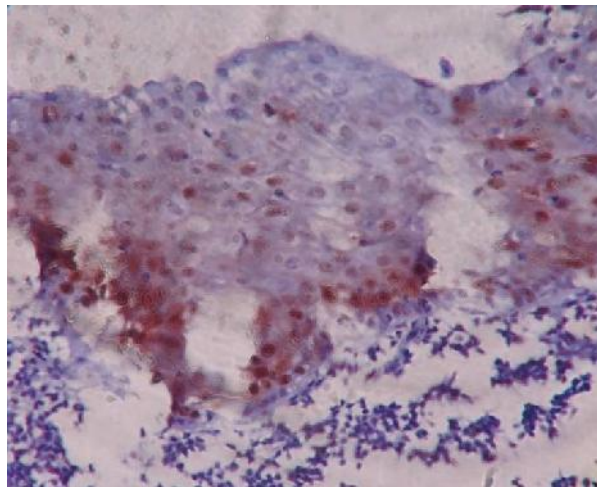


Figure 2: Cyclin D1 expression with moderate intensity in radicular cyst (x40).

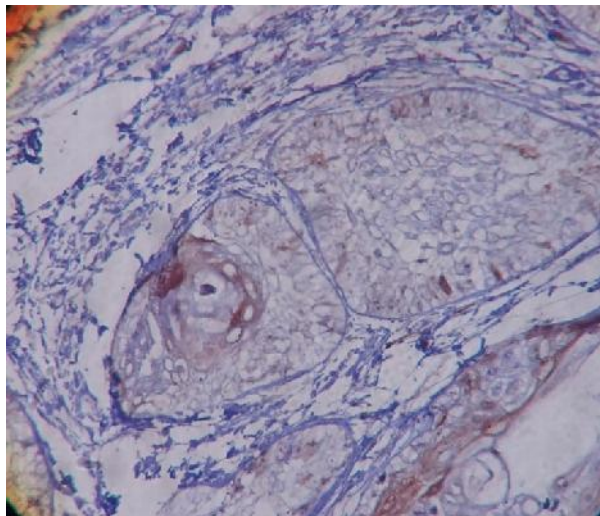


Figure 3: Cyclin D1 expression with mild intensity in ameloblastoma (x40).

The data analysis showed statistically significant difference in cyclin D1 expression in studied groups ($p < 0.05$, Mann-whitney test). Moreover, cyclin D1 expression was higher in OKC and AB than RC and that difference was statistically significant ($p < 0.05$) but the difference was not observed between OKCs and ABs ($p = 0.874$).

Statistically significant difference was also observed in staining intensity ($p = 0.002$) of cyclin D1 among three groups ($p < 0.05$, Chi-squared test). OKCs had the strongest expression in contrast to ABs which had the mildest expression among three groups. RCs showed moderate intensity. Assessment of staining pattern and staining localization using the same test showed no statistically significant difference in expression pattern ($p = 0.465$), as well as staining localization ($p = 0.393$) among all groups.

Discussion

The transition between different cell cycle stages is regulated at several checkpoints. Regulation of the G1-S transition is controlled by the Rb pathway proteins, which include, among others, the cyclin D1 gene. The overexpression of this protein shows accelerated G1 progression entering in the S-phase of cell cycle, with lower cell dependence on growth factors for proliferation. Cyclin D1 may play an important role in tumorigenesis and it has been detected in malignant tumors but also in benign neoplasms (12).

In a comparative study of cyclin D1 expression between odontogenickeratocyst, dentigerous cyst,

radicular cyst and AB, Vicente DJC et al., found diffuse expression in AB, nuclear staining in parabasal cells of odontogenickeratocyst and sporadic expression in dentigerous cyst and radicular cyst supporting the role of cyclin D1 in proliferation of these odontogenic lesions (12 <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3227254/> - ref15). Taghavi N et al., found focal expression in OKC with strong intensity in parabasal layer, equally focal and diffuse pattern and mild to moderate intensity in RC and sporadic expression in DC and GOC (13). In a comparative study of cyclin D1 expression in AB and adenomatoidodontogenic tumors, Kumar H et al., found respectively, moderate to strong expression intensity in most cases of AB, and more mild cases of adenomatoidodontogenic tumor (14).

In the present study cyclin D1 was found in 78.6% of OKCs, and the rate of its expression was significantly higher than that of radicular cysts, but it did not differ between AB and OKC. That could probably be related with the biological aggressiveness of AB and OKCs. Results of AB and OKC's relation differs from what Juan-Carlos de-Vicente et al. (12) found. This difference is likely to be explained by differences in staining process. Regarding cyclin D1 expression, Vicente et al. used the clone SP4, diluted 1:50, while we used the clone P2D11F11 at a 1:30. It can be also because of difference in histological types of AB between two studies. Furthermore, the mechanism of tumoral invasion in AB is very complex, involving a variety of adhesion molecules, MMPs, cytokines, and associated genetic changes, so it may indicate its invasive characteristic by other mechanisms(15).

The results in this study showed significant difference in staining intensity among groups but no statistically difference in staining localization and expression pattern. Conversely, Nasin Taghavi et al. (13) found significant difference in staining localization and no statistically difference in staining intensity and expression pattern. But our results are in line with Juan-Carlos de-Vicente et al. in staining pattern and localization. In our study OKC showed strongest intensity, in contrast to AB, that had the mildest intensity. All cases of RCs expressed in moderate intensity. On the other hand we observed 3 strong cases in AB while we did not have it in RC. This difference in AB may be due to retrospective sample collecting. This means that the blocks between the years 1388-1394 were used. Perhaps the type of fixation and time of it affected divisiveness. As we mentioned, complicated and various mechanisms of invasion in AB can be another cause.

As the marker was present in 100% of positive RCs with moderate intensity, this finding may reflect a high level of cell proliferation in radicular cyst epithelium and could explain the high levels of tumoral changes in the RCs. Inflammation is a major component in the radicular cyst that can lead to cell proliferation (13).

We observed the strongest intensity in OKCs, which could probably be related with the biological aggressiveness of OKCs. These results are in agreement with Taghavi N et al. (13), which found that most cases of odontogenic keratocysts were strong in intensity, but the difference was not statistically significant in previous study.

Bando et al. showed that cytokines and growth factors released by inflammatory cells present in connective tissue can stimulate epithelial proliferation in RC and more intense inflammation may cause greater proliferative activity (16). While we did not find any statistical association in staining pattern among studied groups, it's noticeable that in one case of radicular cyst cyclin D1 expression was also seen in all layers of epithelial part. Therefore, according to Bando et al. results, cyclin D1 expression in all layers may be related to the intensity of inflammation in cyst wall.

It is suggested that the expression of cyclin D1 marker be evaluated in larger sample sizes of OKCs and AB, and its expression be evaluated in other cysts and odontogenic tumors.

Conclusion

In conclusion, the results showed that cyclin D1 expression was higher in OKCs and ABs respectively comparing to RCs. Immunostaining expressed with strong intensity in most cases of OKCs whereas AB respectively had mild, moderate and strong intensity.

Also RCs showed moderate intensity in all positive cases. These findings could help us to explain neoplastic origin of OKCs and ABs. Therefore, from an immunohistochemical point of view, OKCs could be seen as odontogenic neoplasms that show similarities with odontogenic cysts or as developmental cysts with neoplastic properties because of the high intrinsic growth potential.

Acknowledgments

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