Immunohistochemical expression of Cyclin D1 in mucoepidermoid and adenoid cystic carcinoma of the salivary glands

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ABSTRACT

Background: Cyclin D1 proto-oncogene is an important regulator of (G1 to S) phase progression in many different cell types. The Aims of this study were to evaluate the immunohistochemical expression of Cyclin D1 in mucoepidermoid and adenoid cystic carcinoma of the salivary glands and to correlate the immunoexpression of this protein with the clinicopathological findings.

Materials and methods

Retrospectively, twelve of archival formalin fixed paraffin embedded tissue samples of salivary Mucoepidermoid and fourteen blocks of adenoid cystic carcinomas obtained from the archives of the department of oral pathology / college of dentistry / Baghdad university, Al-Shaheed Ghazi hospital, were included in this study. Five micrometer sections obtained and immunostained using monoclonal antibody against Cyclin D1. The immunoexpression was detected by the presence of brown stain in the nucleus of tumor cell. The proportion of cells that expressed the stain was correlated with the clinicopathological data of the patients.

Results: Cyclin D1 expression was found positive in all cases of MEC and AdCC localized in tumor cells. Non-significant statistical relation (p=0.588) was detected regarding Cyclin D1 expression in both types of tumor. Significant relation was found with stage of AdCC (p=0.04) and non-significant concerning other clinicopathological parameters in both tumor types.

Conclusion: Weak expression of Cyclin D1 in MEC and AdCC might be explained by Cyclin D1 does not represent an exclusive factor consequently; other factors might be involved in the proliferation, progression and metastasis of both tumor types.

Key words: Mucoepidermoid carcinoma, Cyclin D1, immunohistochemistry, Adenoid cystic carcinoma. (J Bagh Coll Dentistry 2015; 27(3):64-69).

INTRODUCTION

Salivary gland carcinomas constitute about 1 to 3% of all head and neck malignancies and 0.3% of all cancers [1]. The Mucoepidermoid carcinoma (MEC) is a malignant epithelial neoplasm composed of varying proportion of mucous, epidermoid, intermediate, columnar, and clear cells and often demonstrates prominent cystic growth [2]. It is one of the most common salivary gland malignancies with varying degree of gressiveness [3]. The annual incidence of MEC is 0.44 per 100,000 persons. It accounts for 12% to 29% of malignant salivary gland tumors [4]. MEC mostly occurs from the second to seventh decades of life and has a slight predilection for women (60%) [5,6]. Approximately one half of these tumors (53%–56%) arise in the major salivary glands, with 85% to 88% occurring in the parotid gland. The minor glands constitute the second most common site after parotid, especially the palate [7]. Central MEC of the jaw is rare (4% of all MECs), most often located in the mandible [3].

The adenoid cystic carcinoma (AdCC) is one of the more common and best-recognized salivary gland malignancies with a deceptively benign histologic appearance characterized by indolent, locally invasive growth with high propensity for local recurrence and distant metastasis [8]. AdCC arise from malignant transformation of the intercalated duct reverse cells and it's a malignant tumor of modified myoepithelial and ductal cells that form characteristic cribriform, tubular, and solid growth patterns and has a predilection for perineural invasion [2,5]. AdCC accounts for 1% of all malignant tumors of the oral and maxillofacial region and 23% of all salivary malignancies [9,10]. Most cases are diagnosed from the fifth to seventh decades of life and the female-to-male ratio is around 3:2. Approximately 50% to 70% of all reported cases of AdCC carcinoma occur in minor salivary glands of the head and neck, chiefly of the palate [5,6].

Cyclin D1 is a founding member of Cyclins which are group of proteins so called because of the cyclic nature of their production and degradation. They form complexes with cyclin dependent kinases (CDKs); such complexes phosphorylate crucial target proteins that drive the cell through the cell cycle [11]. It is 45 KDa protein that in human is encoded by the CCND1 gene

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located on chromosome 11q13. This is today considered a well-established human oncogene [12]. The cyclin D1 proto-oncogene is an important regulator of (G1 to S) phase progression in many different cell types. Together with its binding partners cyclin dependent kinase 4 and 6 (CDK4 and CDK6), cyclin D1 form active complexes that promote cell cycle progression by phosphorylating and inactivating the retinoblastoma protein (RB) [13]. Overexpression of cyclin D1 is known to correlate with the early onset of cancer and risk of tumor progression and metastasis[14]. However, a number of studies have shown a surprising lack of correlation between increased cyclin D1 expression and increased DNA synthesis in tumors [15]. Chromosomal translocations, gene amplification and disruption of normal intercellular trafficking and proteolysis are the procedures which lead to accumulation of cyclin D1 in tumor cell nuclei and eventually to cyclin D1 overexpression in many tumors [16]. Cyclin D1 amplification and/or overexpression have been demonstrated in a variety of human tumors, including mantle cell lymphoma, breast carcinoma, head and neck squamous cell carcinomas, and esophageal cancers. Among lymphoid neoplasms a subset of chronic lymphocytic leukemia, small lymphocytic lymphoma, and Multiple myeloma have been reported to express cyclin D1 [17].

MATERIALS AND METHODS

Patients

Twenty six patients with salivary gland malignancies was randomly selected from the file records and pathologic specimens from the Maxillofacial Center in Al-Shaheed Ghazi Hospital in Baghdad, from the year 2009 through 2013, and from the archives of the department of oral diagnosis/ Collage of Dentistry/Baghdad University dated from 1972 to 2011.

Demographic and clinical data provided by the surgeon were collected from the case sheets presented with the tumor specimens, including patient's information concerning age, sex, site and clinical staging of the tumor were recorded, and staging was carried out according to (UICC) International Union against Cancer TNM classification of malignant tumors USA 2002. All clinical and histopathologic data available were analyzed to exclude cases representing secondary metastatic disease to the salivary gland or to intra-parotid lymph nodes.

Control

Five normal salivary gland tissues were used as negative external controls. At the same time by omitting of primary antibody step and addition of all other reagents were used also as negative control. Positive staining indicates a lack of specificity of the antibody and breast carcinomas were used as positive control for Cyclin D1 according to ab cam manufacturer's data sheets.

Immunohistochemistry

Sections of 5µm were de-paraffinized in xylene and rehydrated in graded alcohol. Enough drops of hydrogen peroxide block were added to slides and incubated in humid chamber at 37C° for 10 minutes, and then socked 2 times in buffer (5 minutes for each). Then tissue retrieving is done to the slides in order to uncover antigenicity because formalin or other aldehyde fixation forms protein cross-links that mask antigenic sites in tissue specimens. After that enough drops of protein block were added to slides and incubated at 37°C for 10 minutes. Then washed 2 times in buffer (5 minutes for each), finally drained and blotted gently. Then Diluted primary antibody was applied to each slide, incubated in humid chamber at 37°C. Overnight. Early in the next day, the slides were washed in buffer (4 times for each), finally drained and blotted gently as before. Next enough drops of secondary antibody reagent were added and incubated in humid chamber for 30 minutes at 37°C. After that, the slides were washed 4 times in buffer (5 minutes for each), finally drained and blotted gently. Then Streptavidine-HRP antibodies were applied on tissue and incubated for 30 minutes at 37°C. Later Diluted DAB was applied on tissue (this process was done in dark room) and incubated in humid chamber for 10 minutes at 37°C. Then slides washed carefully in tap water for 5 minutes. After that the slides were bathed in Hematoxylin counter stain for 1-2 minutes then they were rinsed with tap water for 10 minutes. Later the slides were dehydrated by immersing them in ethanol and xylene containing jars then One to two drops of DPX mounting medium were applied to the xylene wet sections and covered with cover slips and left to dry overnight. Then the results were evaluated by the Presence of brown colored end product at the site of target antigen (nucleus) was indicative of positive immunoreactivity. Percentage of IHC positive tumor cells per hotspot was calculated and the mean percentage per slide was determined.

The intensity of stain was ignored because it's subjected to individual difference during checking. The Immunoreactivity of Cyclin D1 was classified as follows: (score 0) (-ve) 0% of the tumor cells, focal (score I) (+) 1-25%, moderate (score II) (++) 26-50%, diffuse (score

III) (+++) 51-75% of positive cells, depending on counting, very diffuse (score IV) (++++) 76-100% [18].

Statistical analysis

All the clinical, histopathological and immunohistochemical relevant data so obtained was tabulated and subjected to appropriate statistical analysis using the SPSS 17 statistical software. The studied parameters were scored and considered as categorical data and presented as count and percentage.

Chi-square test used to test the relationship between categories. ANOVA test (analysis of variance) was used to detect differences for age and the marker. *P* value equal or less than 0.05 was considered to be statistically significant.

RESULTS

Cyclin D1 immunoexpression

Cyclin D1 immunoreactivity was noticed as brown staining localized in the nucleus of tumor cells. Cyclin D1 expression was found positive in all cases of MEC and AdCC in different scores (Figures 1, 2, 3, 4 and 5). The higher percentage of Cyclin D1 expression (score I) was found in 7 cases of MEC (58.3%) and in 8 cases of AdCC (57.1%).

Table 1: Cyclin D1 scores in MEC and AdCC

Cyclin D1 score	MEC	AdCC
Score I	7(58.3%)	8(57.1%)
Score II	4(33.3%)	3(21.4%)
Score III	1(8.3%)	3(21.4%)
Total	12 (100%)	14 (100%)
	p value=0.588 NS	

Non-significant statistical relation (p=0.588) was detected regarding Cyclin D1 expression in both types of tumor (table 1).Non-significant statistical relations were found concerning Cyclin D1 expression with age groups (p=0.44), gender (p=0.193), anatomical site (p=0.36), grade, and stage (p=0.207) in MEC as in tables 2, 3, 4, 5 and 6.

Table 2: Cyclin D1 scores in relation to age groups in MEC

Cyclin D1 scores	No.	Mean	Min	Max	
Score I	7	48.42	13	62	NS
Score II	4	43	22	60	
Score III	1	25	-	-	

Table 3: Cyclin D1 scores in relation to gender in MEC

MEC Cyclin D1 Scores		Sex		Total
		M	F	
I	No.	5	2	7
II	No.	1	3	4
III	No.	0	1	1
Total	No.	6	6	12

Table 4: Cyclin D1 scores in relation to the anatomical site in MEC

Sites	Cyclin D1 scores			Total
	Score I	Score II	Score III	
Major glands	5	2	0	7
Minor glands	2	2	1	5
Total	7	4	1	12
Test	Chi square test=2; p=0.36NS			

Table 5: Cyclin D1 scores in relation to the stage in MEC

Cyclin D1 score	MEC stage			
	I	II	IV	total
Score I	1	3	3	7
Score II	3	1	0	4
Score III	1	0	0	1
Total	5	4	3	12
Test	Chi square= 5.893; p value= 0.207 NS			

Table 6: Cyclin D1 scores in relation to the stage in MEC

Cyclin D1 score	MEC Auclair grading system			
	I	II	III	total
Score I	3	1	0	4
Score II	3	1	2	6
Score III	1	0	1	2
Total	7	2	3	12
Test	Chi square= 5.893; p value= 0.207 NS			

For AdCC statistically non-significant relations were observed concerning age (p=0.1), sex (p=0.646), site (p=0.36), and grade of tumor (p=0.533). Whereas significant relation was found with stage of tumor (p=0.04) as in tables 7, 8, 9 and 10.

Table 7: Cyclin D1 scores in relation to gender in AdCC

AdCC		Sex		Total
Cyclin D1 Scores		M	F	
I	No.	2	6	8
II	No.	1	2	3
III	No.	2	1	3
Total	No.	5	9	15

Table 8: Cyclin D1 scores in relation to the anatomical site in AdCC

Sites	Cyclin D1 scores			Total
	Score I	Score II	Score III	
Major glands	3	0	1	4
Minor glands	5	3	2	10
Total	8	3	3	14
Test	Chi square test=2; p=0.36NS			

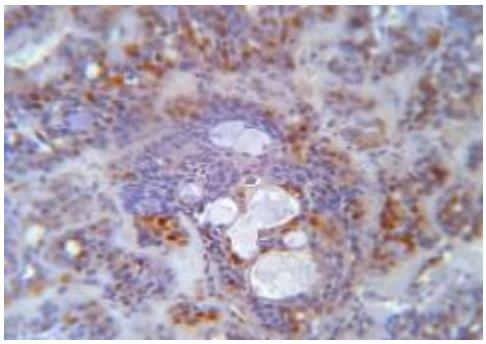

Figure 1: Cyclin D1 positive expression in low grade MEC

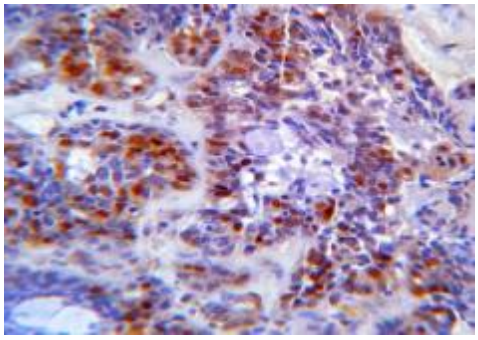

Figure 2: Cyclin D1 expression in intermediate grade MEC

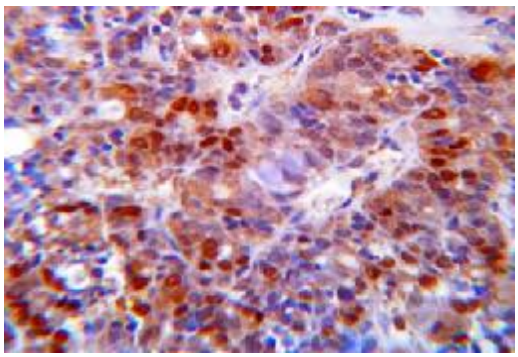

Figure 3: Cyclin D1 expression in high grade MEC

Table 9: Cyclin D1 scores in relation to the grade in AdCC

Cyclin D1 score	AdCC grade			
	I	II	III	Total
Score I	2	5	1	8
Score II	0	3	1	4
Score III	0	2	0	2
Total	2	10	2	14
Test	Chi square= 5.893; p value= 0.533 NS			

Table 10: Cyclin D1 scores in relation to the stage in AdCC

Cyclin D1 score	AdCC stage				
	I	II	III	IV	Total
Score I	1	3	4	0	8
Score II	1	1	0	1	3
Score III	0	0	0	3	3
Total	2	4	4	4	14
Test	X^2= 12.979; p value=0.043 S (p<0.05)				

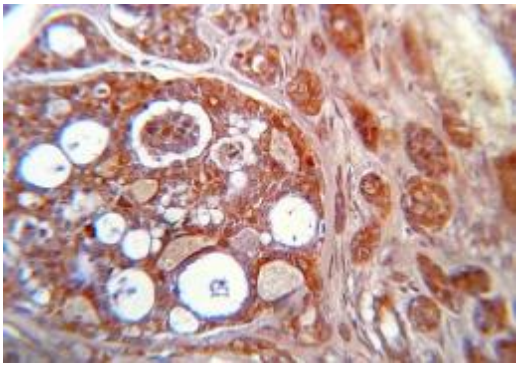

Figure 4: Cyclin D1 expression in AdCC

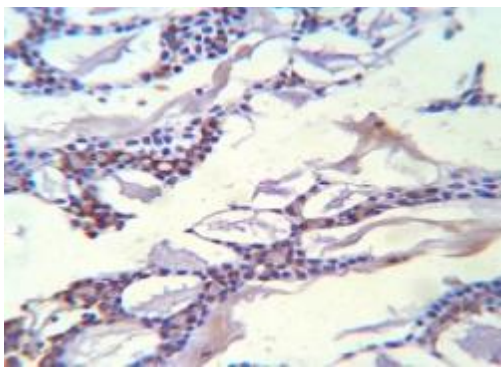

Figure 5: Strong positive immunostaining of Cyclin D1 in AdCC

DISCUSSION

Cyclin D1 is the first cyclin that accumulates after mitogenic signaling. Cyclin D1 binds to CDK4 or CDK6, forming a complex that binds to the pRb protein, phosphorylating and inactivating it and leading to the release of transcription factor E2F, thus initiating cell proliferation through the G1 phase [19]. The cyclin-CDK complex controls cell cycle progression by means of ordered activation and inactivation. Any disturbance in this mechanism may play an important role in the pathogenesis of various malignant tumors.

Some studies indicate that overexpression of cyclin D1 occurs at the beginning of tumor development and might therefore be considered an early marker of cell proliferation, whereas others have demonstrated late overexpression of this protein during the development of malignant tumors [20]. The levels of D-cyclins are controlled by the extracellular environment. It is therefore considered that D-cyclins present a link between extracellular mitogenic stimulation and core cell cycle machinery [21]. In this study all cases of MEC and AdCC showed immunoreactivity for Cyclin D1 with variable scores. The immunostaining was limited to the epidermoid cells of the MEC and luminal and abluminal cells of the AdCC. The majority of the cases were (score I) immunostaining (57.6%). These findings were in agreement with another studies conducted by Jour and his colleagues [18] and with another findings by Zhou et al. [22] and disagreed with another studies [23-25] whose detected the expression of cyclin D1 in AdCC and observed overexpression in 4 of 22 cases, 3 of them showing the solid pattern, which have the worst prognosis. One study in 2006 showed that Cyclin D1 overexpression was present in 90% (35/39) of AdCC evaluated cases [26]. Focal expression of Cyclin D1 in MEC and AdCC might be explained by Cyclin D1 does not represent an exclusive factor consequently; other factors might be involved in the proliferation, progression and metastasis of both tumor types.

At least nine classes of cyclins and seven CDK catalytic subunits have been identified in mammalian cells, two CDK subunits (CDK4 and CDK6) in combination with three D-type cyclins (D1, D2 and D3), and CDK2 in combination with cyclin E, are involved in G1/S progression and regulation and the kinase activity is inhibited by a number of specific proteins belonging to the INK4 and CIP/KIP families (p21, p27, p57) [27]. Teruyo and Hiroki [28] showed that cells over-expressing Cyclin D1 exhibited significantly increased invasiveness and the Cyclin D1 expression was associated with the increased gelatinolytic activity with the activation of proMMP-9 to the intermediate form of MMP-9. They concluded that Cyclin D1 may modulate invasive ability by increasing MMP activity and cell motility and this explain significant relation of Cyclin D1 expression with the stage in AdCC [28].

As conclusion; Cyclin D1 immunoexpression was detected in all cases of studied samples with variable scores. Weak expression of Cyclin D1 in MEC and AdCC might be explained by Cyclin D1 does not represent an exclusive factor consequently; other factors might be involved in the proliferation, progression and metastasis of both tumor types.

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