β- catenin Expression and Its Relation to Bryne's Invasive Grading System in Oral Squamous Cell Carcinoma

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ABSTRACT

Background: Invasion in oral cancer involves alterations in cell-cell and cell-matrix interactions that accompanied by loss of cell adhesion. Catenins stabilize cellular adherence junctions by binding to E-cadherin, which further mediates cell-cell adhesion and regulates proliferation and differentiation of epithelial cells. The Wnt/β-catenin pathway is one of the major signaling pathways in cell proliferation, oncogenesis, and epithelial-mesenchymal transition.

Aims of the study: to detect immunohistochemical distribution pattern and different subcellular localization of β-catenin in oral squamous cell carcinoma and relate such expression to Bryne's invasive grading system.

Materials and Methods: This study included 30 paraffin blocks of primary oral squamous cell carcinoma. Bryne's grading performed on routein stained slides. Immunohistochemical staining for anti β-catenin was done to illustrate its pattern and subcellular localization in malignant cells. The expression correlated with the invasive grading system.

Results: β-catenin expression detected in all sample (100%). It was (23.3%) membranous, (60%) aberrant cytoplasmic and (16.7%) mixed expression. Diffuse strong homogeneous pattern was observed in (40%) of the cases. The cytoplasmic expression had significant high mean rank in score 3, diffuse strong homogeneous pattern and strong intensity. Well-differentiated carcinoma expressed great mixed membranous/cytoplasmic expression while poor-differentiated cases showed low membranous mean rank expression. The strong diffuse homogeneous pattern with strong staining was significantly frequent in well-differentiated squamous cell carcinoma.

Conclusion: Increase cytoplasmic β-catenin expression is parallel with carcinoma dedifferentiation. Suggesting maintenance of its adhesive role with the inhibition of the normal degradation of free β-catenin in the cytoplasm, which might cause accelerated tumor cell proliferation.

Keywords: β-catenin, membranous, Bryne's grading. (J Bagh Coll Dentistry 2016; 28(3):52-58).

INTRODUCTION

Invasion and metastasis of oral cancer involve complex multistep processes that lead to alterations in cell-cell and cell-matrix interactions. These changes are accompanied by loss of cell adhesion, even in the very early stages of cancer development [(1,2)]. Epithelial-mesenchymal transition (EMT) is critical for regulating embryonic development and for epithelial-derived tumors to become invasive and metastasize. Both developmental and oncogenic EMT associated with the loss of apical-basal polarity, destabilization of intercellular adhesion complexes (gap junctions, desmosomes, tight junctions and adherence junctions) and replacement of epithelial cell markers (E-cadherin and β-catenin) by mesenchymal cell markers (N-cadherin and vimentin). Thus, they lead cells to get migratory and invasive ability [(3)].

Catenins stabilize adherence junctions. They bind to E-cadherin, which mediate cell-cell adhesion, regulate proliferation and differentiation of epithelial cells [(4)]. Moreover, catenin is essential in the task of E-cadherin and brings about strongly invasive characteristics when the expression or structure of E-cadherin fails [(5)].

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The Wnt/β-catenin pathway is one of the major signaling pathways in cell proliferation, oncogenesis, and EMT. It has an independent function in cell adhesion and signal transduction. There are at least two distinct pools of β-catenin in cells. These include a cell membrane-associated pool and a pool involved in Wnt signaling and gene transcription [(6,7)]. On the other hand, nuclear β-catenin acts as a transcription factor in a complex with the HMG-box proteins of the TCF/LEF family. Thereby, they act contrarily in regulating target gene transcription, depending on the nuclear β-catenin level [(8,9)]. Nuclear localization of β-catenin has shown to involve in precancerous changes in oral leukoplakia and progression of OSCC, lymph node metastasis and cell proliferation [(10)]. Furthermore, the aberrant cytoplasmic accumulation of β-catenin induces TCF/LEF-mediated transcriptional activity, up-regulates MMP-7, and encourages EMT in oral squamous cell carcinoma (OSCC). Hence, it augments invasion and migration in OSCC [(11)].

The altered β-catenin expression is demonstrated in a variety of human cancers [(12-14)]. In OSCC, β-catenin localization was detected in cytoplasm and nucleus in parallel with Wnt expression at the invasive front [(15)]. Moreover, an abnormal β-catenin expression is associated with poor differentiation and related to cellular proliferation in tumor progression [(16)] and lymph

node metastasis [17]. It appears that the loss of membranous expression of β-catenin and E-cadherin is a characteristic feature of OSCC. Similarly, loss of membranous β-catenin often occurs at the invasive front of poorly differentiated OSCC, which could constitute a hallmark of an aggressive biological behavior of tumor cells. Likewise, invasion and metastasis of OSCC have been shown to require methylation of E-cadherin and degradation of membranous β-catenin [18].

This study aims to detect immunohistochemical changes in both distribution pattern and subcellular localization of β-catenin in oral squamous cell carcinoma. Then correlate such expression to Bryne's invasive grading system to distinguish the role of this expression in the progression of OSCC.

MATERIALS AND METHODS

A retrospective study accomplished in School of Dentistry / University of Sulaimani, from March to the end of December 2014. The Ethical Committee of Faculty of Medical Science, University of Sulaimani approved the study. The sample included 30 formalin fixed paraffin embedded blocks previously diagnosed as primary OSCC collected from Shorsh Hospital and Private histopathological laboratories in Sulaimani. Available clinical data recorded from archive case sheets. Two serial 5µm tissue sections cut from each block.

The area of concern was the deep front and lateral invasive sites of the growth. One section stained with hematoxylin and eosin for histopathological evaluation. The other section subjected to immunohistochemical staining by keeping it in the oven (60ºC) overnight. Next day, after deparaffinized and rehydrated the sections, heat antigen retrieval performed (citrate buffer, pH 6, 15 min, 95ºC). Sections allowed cooling down to room temperature, then endogenous peroxidase activity blocked by hydrogen peroxidase (10 min). After that sections incubated with protein block (10 min) to block nonspecific background staining.

Later sections incubated with anti-rabbit -β-catenin polyclonal primary antibody (1:100 dilution, 45 min, Abcam Company) then washed three times with phosphate buffer saline. Next, the sections incubated with complement (10 min) and washed twice with phosphate buffer saline. Goat anti-rabbit HRP conjugate applied (15 min) and then washed three times. Slides stained by DAB chromogen (5 min, in the dark), and counterstained with hematoxylin. The sections dehydrated, cleared and mounted with DPX and cover slide.

Normal oral epithelium serve as a positive control [16] while applying the antibody diluents alone without primary antibody served as negative control. Hematoxylin and eosin stained slides assessed according to Bryne's grading system [19]. Immunohistochemically stained sections analyzed by Image J software at (400x) and semi-quantitatively scored as follow: score 0 = <10%, score 1 = 10 -30%, score 2 = 31-50% and score 3 = >50%.

The intensity scored as weak, moderate and strong [20]. Concerning expression pattern, sections analyzed (under 100X) into: 1=absent, 2=focal heterogeneous, 3=reduced homogeneous and 4=strong homogeneous [21]. Statistical analysis performed by using SPSS 20.0.software and applying Chi-square and Kruskal-Wallis test. The correlation of grading system to scoring, pattern, and intensity of staining tested by Somers'd correlation coefficient.

RESULTS

Bryne's grading system showed that MD-OSCC constituted the most frequent histopathological grade (13 cases; 43.3%). The strength of the relationship between the clinical data and grading system was analyzed. Sex factor had little effect (V<0.3) and the age had a medium effect (V =0.3) on the histopathological grade (Table -1).

B-Catenin expression in normal oral epithelia was strong membranous in the basal, para-basal and prickle cell layers (Fig -1A). Similarly, OSCC showed 100% β-catenin as a membranous expression in the basal and para-basal layers of epithelial islands. Still the polyhedral cells layer in organized islands revealed cytoplasmic staining and the other unorganized carcinomatous cells-growth showed even mixed expression. Thus, in a single section, one may see more than one type of sub-cellular localization. Nevertheless, in OSCC, β-catenin was (40%) membranous expression (23.3% pure membranous and 16.7 % mixed) (Fig-1B), and (60%) only cytoplasmic expression (Fig-1C). Within the above cases, nuclear membrane localization (16.6%) and perinuclear condensation (20%) were also seen (Fig-1 D). No case had β-catenin with score one expression (positivity in 10-30% of the examined cells) and 40% of the cases had a diffuse strong homogeneous pattern. Lastly strong staining was evident in 43.3% of OSCC cases (Table 2).

Regarding mean ranks expression of subcellular localization, the membranous expression had a higher mean rank in score 0 and

3, with a focal heterogeneous pattern of expression and weak staining. While the cytoplasmic expression showed significant high mean rank in score 3, diffuse strong homogeneous pattern and strong intensity. Finally mixed (membranous + cytoplasmic) expression illustrated significant expression in both diffuse strong homogeneous pattern and strong intensity (Table 3).

WD-OSCC had a high mean rank percentage of mixed (membranous and cytoplasmic) expression. Cytoplasmic expression showed great mean rank in MD-OSCC. Only the mixed expression was significantly different (p=0.034, Table -4).

B-catenin score 3 was observed equally in WD- and MD-OSCCs (7 cases). A significant strong diffuse homogeneous pattern seen in WD-OSCCs (8 cases) (p=0.026). The grades had a medium reverse effect on the pattern and score (d value was between -0.3 and -0.5). Lastly, the strong staining expression was significantly frequent in WD-OSCCs (9 cases) (p=0.002). The grade had a strong reverse effect on the intensity (d >-0.5) so that the strongest the stain the least the grade (Table -5).

Table 1: The distribution of clinical data related to Bryne's grading systems

Clinical		Bryne's system				
		Well	**Moderate**	**Poor**	**Exact Sig.**	**Cramer's V**
Sex	**Male**	6	7	3	1.000	0.034
	Female	5	6	3		
Age groups	**< 50 years**	6	4	0	0.140	0.333
	50-69 years	4	4	3		
	> 69 years	1	5	3		
Total		11	13	6		

Table 2: Frequency and percentage distribution of different positive β-catenin expression localization, score, pattern and staining intensity in OSCC

Expression			No.	%
Total positive cases			30	100.0
Localization	Membranous		7	23.3
	Cytoplasmic		18	60
	Mixed		5	16.7
	within above cases	Nuclear	2	6.7
		Nuclear membrane	5	16.6
		Perinuclear	6	20
Score	0		7	23.3
	1		0	0
	2		8	26.8
	3		15	50
Pattern	Absent		0	0
	Focal heterogeneous		7	23.3
	Diffuse reduce homogeneous		11	36.7
	Diffuse strong homogeneous		12	40
Intensity	Negative		0	0
	Weak		8	26.7
	Moderate		9	30
	Strong		13	43.3

Table 3: β-catenin expressions percentage mean ranks in OSCCs related to the total score, pattern of expression and intensity of staining

Expression % mean ranks	Score				Pattern				Intensity			
	0	1	2	3	Absent	F.H	D.R.H	D.S.H	Negative	Weak	Moderate	Strong
Membranous	17.5	0	10.1	17.4	0	17.5	14.8	14.9	0	17.2	13.8	15.5
P value	0.075				0.749				0.691			
Cytoplasmic	5.7	0	17.7	18.8	0	7.8	15.3	20.08	0	8.8	16.4	18.9
P value	0.001				0.010				0.031			
Mixed	11.1	0	14.8	17.8	0	9	13.9	20.7	0	9	15.06	19.8
P value	0.178				0.004				0.007			
Total positive cells					0	5.2	14.1	22.6	0	7	14.4	21.4
P value					0.000				0.000			
F.H: Focal heterogeneous pattern; D.R.H: Diffuse reduced homogeneous pattern; D.S.H Diffuse strong homogeneous pattern.												

Table 4: β-catenin subcellular localization mean ranks related to Bryne's grades

Expression % mean ranks	Bryne's grading system			Kruskal-Wallis Monte Carlo sig.
	Well	Moderate	Poor	
Membranous	18.45	15.15	10.83	0.161
Cytoplasmic	14.41	17.08	14.08	0.692
Mixed	20.23	12.04	14.33	0.034
Total	18.59	15.54	9.75	0.139

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Table 5: β-catenin score, pattern of expression and intensity of the stain in Bryne's grading system

Expression		Bryne's grading system				
		Well	Moderate	Poor	Linear by Linear Sig	Somers' d
Score	0	1	3	3	0.050	-0.320
	1	0	0	0		
	2	3	3	2		
	3	7	7	1		
Pattern	Absent	0	0	0	0.026	-0.396
	F.H	1	4	2		
	D.R.H	2	6	3		
	D.S.H	8	3	1		
Intensity	Negative	0	0	0	0.002	-0.522
	Weak	0	5	3		
	Moderate	2	5	2		
	Strong	9	3	1		
F.H: Focal heterogeneous pattern; D.R.H: Diffuse reduced homogeneous pattern; D.S.H.: Diffuse strong homogeneous pattern.						

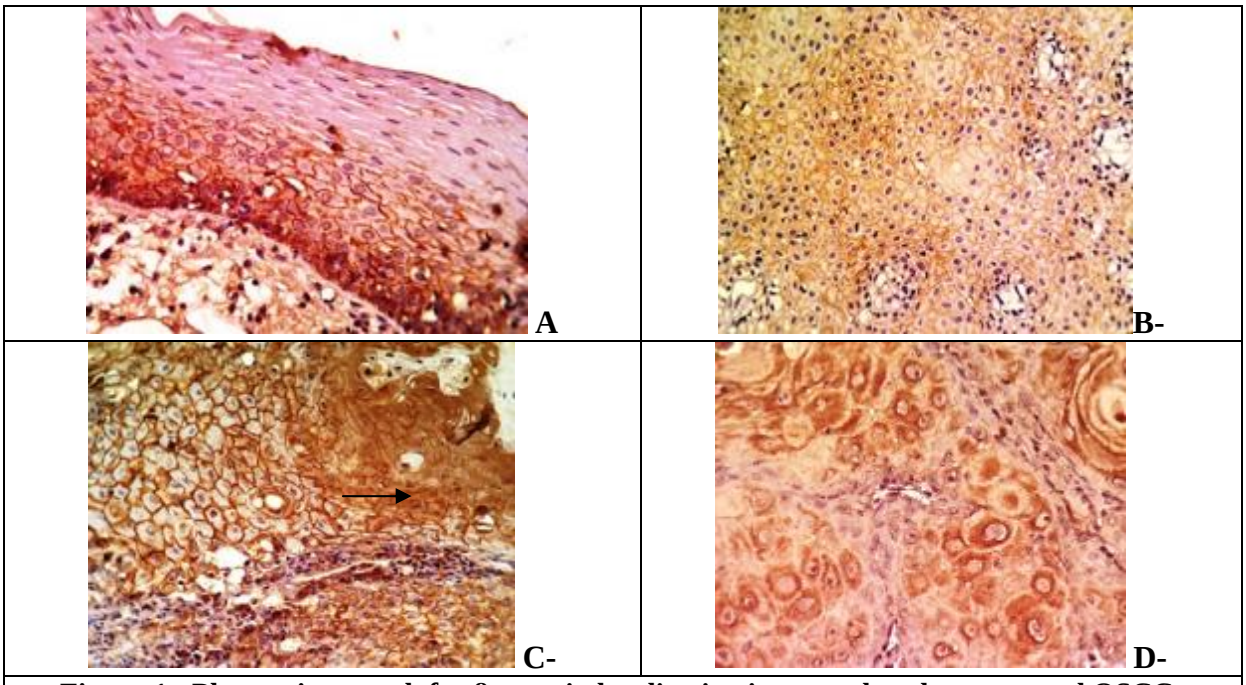

Figure-1: Photo micrograph for β-catenin localization in normal oral mucosa and OSCCs (400X). Membranous expression in normal oral mucosa (A) and OSCC (B). Cytoplasmic expression in OSCC (C) and nuclear membrane with perinuclear condensation in OSCC (D).

Abbreviations: Oral squamous cell carcinoma (OSCC), well differentiated (WD), moderate differentiated (MD), poor differentiated (PD), Epithelial-mesenchymal transition (EMT)

DISCUSSION

By the application of Bryne's invasive system, OSCC were more frequently at high grades (moderate and poor differentiation).

In agreement with published literature [10,22], the membranous immunohistochemical expression of β-catenin of normal oral epithelia reflect its role as an adhesion molecule. It localized to the cell membrane of all keratinocytes layers. On the other hand, the majority of cancerous islands showed membranous basal and suprabasal staining, still the superficial areas either loose or get shifting of the β-catenin staining to the cytoplasm compartment. Therefore, β-catenin in these cells has conserving cellular adhesion and has other vital function related to differentiation and proliferation [22].

The high percentage of β-catenin aberrant cytoplasmic localization (60%) and the persistence of membranous expression in 23.3% of cases are in agreement with other studies [22, 23]. Iwai et al. indicated that cytoplasmic expression might induce TCF/LEF-mediated transcriptional activity and up-regulate MMP-7. Also it prompts Rho family member mediated reorganization of the actin cytoskeleton and redistribution of E-cadherin [11]. Accordingly this results in EMT of OSCC cells and rising cell invasion and migration. While nuclear and nuclear-membrane localization showed 23.3% expression. Here it is believed that nuclear β-catenin functions as oncogenes. It interacts with the transcription factor TCF/LEF complex, which facilitates independently the expression of c-Myc and cyclin D1, in which they had a key role in both cell cycle control and cellular proliferation [24].

The predominance of homogeneous diffuse β-catenin expression throughout the growth (36.7% diffuse reduced and 40% diffuse strong) do not fit with the heterogeneous nature of OSCC growth. Thus, biologically most cells might categorize toward gaining more neoplastic features and showing aggressive performance.

In agreement with previous reports [17, 20], β-catenin has strong staining in 43.3% of cases. This strong high intensity reflects the accumulated large amount of β-catenin within the cytoplasm. This finding may explain by the reduction in protein degradation that reduces its binding to E-cadherin.

The focal, faint, low score of β-catenin expression in cell membrane ascertain cells that lost adhesion with the neighboring surrounding cells within a mass. It is believed that these cells start to inter the cell cycle (proliferating islands) since β-catenin expression increase with proliferating basal and parabasal layers [25, 26]. Still there are focal areas that showed up-regulation of

this unstable marker. On the other hand, the diffuse strong homogeneous, with strong staining and high scoring were linked to the cytoplasmic expression. This findings suggest stabilization of β-catenin to acts as a signaling molecule and increased membranous degradation (loss of the adhesive function). Such accumulation also mediates transcriptional activity leading to up-regulation of oncogenes and increase invasion and metastasis. Thus identifying the extension and distribution pattern of β-catenin provide valuable information about cell behavior at least about different stages of cell differentiation during tumorigenesis.

The present work identified that β-catenin has a persistent membranous expression in WD-OSCC, shifting from membranous to cytoplasmic in MD-OSCC and minimum total positive expression in PD-OSCC. This altered localization was in agreement with other studies [22, 23]. They showed a decrease in membranous expression in parallel with carcinoma dedifferentiation and invasion.

In this study, it was obsereved that among the higher-grade tumor, there were reduced membranous expression and predominant weak intensity, with reduce homogenous diffuse pattern of expression. This finding possibly relates the mechanical pathway of β-catenin to the invasive front and the possible β-catenin interacting signaling to modulate the invasive ability of oral cancer.

In conclusion; β-catenin persevered membranous expression in well-differentiated cells as adhesion molecule. It augmented cytoplasmic expression in parallel with carcinoma dedifferentiation and invasion. therefore, it is suggested that β-catenin maintenane its role as a signaling molecule and the normal degradation of free β-catenin in the cytoplasm inhibited. The descending pattern and intensity of staining of this marker with high-grade tumor further support its role in detecting the progression and acceleration of oral cancer.

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