RESEARCH ARTICLE

Immunohistochemical Assessment of E-cadherin and β-catenin in the Histological Differentiations of Oral Squamous Cell Carcinoma

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Abstract

The aim of this study was to establish the expression and localization of E-cadherin and β-catenin in oral squamous cell carcinomas (OSCC) so that we could correlate the findings with prognostic-relevant histopathological variables. E-cadherin and β-catenin expression in normal oral epithelia and in oral squamous cell carcinomas was examined immunohistochemically, and associations with histopathological differentiation and prognosis were then analyzed in 33 patients who had been operated on for OSCC. E-cadherin expression was found in (82%) of the squamous cells of well differentiated OSCC, (61%) of moderately differentiated and (39%) of poorly differentiated. E-cadherin expression was significantly associated with histological grade (p=0.000). No nuclear staining was detected. In (19.5%) of the cells E-cadherin localized in the cytoplasm, with no correlation to the histological grade (p=0.106). β-Catenin expression was found in 87% of the squamous cells of well differentiated OSCC, 67% of moderately differentiated and 43% of poorly differentiated, the expression was significantly associated with histological grade (p=0.000). the nuclear β-Catenin expression appeared in 3.3% of the cells and it was correlated to the histological grade (p=0.000). In (23.5%) of the cells β-Catenin localized in the cytoplasm, with correlation to the histological grade (p=0.002). According to this study the expression of β-catenin and E-cadherin were independent prognostic factors for histological grade. E-cadherin was closely linked to β-catenin expression in OSCC (p=0.000) and to tumor differentiation. That reflects a structural association and the role of both in tumor progression.

Keywords: Oral squamous cell carcinoma - E-cadherin - β-catenin - prognosis

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Introduction

Oral cancer is the sixth most common cancer worldwide (Shah and Gil, 2009). More than 90% of all oral cancers are squamous cell carcinomas (SCC) (Attar et al., 2010; Bagan et al., 2010) and this type of cancers composes About 95% of oral cancers in India (Krishna et al., 2014) . The most important risk factors for oral SCC are: use of tobacco or betel quid and the regular drinking of alcoholic beverages. However, infection with high-risk human papillomavirus (HPV) genotypes, and a diet low in fresh fruits and vegetables have also recently been implicated in the aetiopathogenesis of oral SCC (Petti, 2009; Shah and Gil, 2009).

In normal epithelial structures, cell-cell junctions play an important role in the maintenance, integrity and morphology of the epithelium (Giepmans and van Ijzendoorn, 2009). It has been reported that the E-cadherin/β-catenin system of adhesion molecules plays a crucial role in this processes (Weis and Nelson, 2006). The disruption of intercellular adhesions is an important component of the acquisition of invasive properties in epithelial malignancies. Alterations in the cell-cell adhesion complex, E-Cadherin/β-Catenin, have been implicated in the oncogenesis of carcinomas arising from various anatomic sites and have been correlated with adverse clinico-pathological parameters (Shen et al., 2011; Bhagat et al., 2013; Pang et al., 2013; Xu et al., 2013; Ayed-Guerfali et al., 2014; Kanczuga-Koda et al., 2014).

E-cadherin is a 120kDa calcium-dependent transmembrane glycoprotein encoded by the CDH1 gene located on chromosome 16q21, and it is expressed in most epithelial cells. (Gall and Frampton, 2013) E-cadherin has a major role in establishing cell polarity and in maintaining normal tissue architecture. The intracellular domain of E-cadherin is linked to the actin cytoskeleton through its interaction with its cytoplasmic-binding partners, the catenins (α, β, and γ-catenin) (Pannone et al., 2013). When the expression of E-cadherin is lost, the degree of tumor differentiation is decreased and the possibility of distant metastasis increases, suggesting the role of E-cadherin is inhibiting tumor invasion or metastasis (Bringuier et al.,

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1993; Deng et al., 2014), other studies demonstrated that E-cadherin expression is strongly linked with favorable survival (Taskin et al., 2012).

β-catenin is a dual function protein, regulating the coordination of cell-cell adhesion and gene transcription. In humans, β-catenin is encoded by the CTNNB1 gene. (Kraus et al., 1994; MacDonald et al., 2009) β-catenin plays a role in cell-cell adhesion by controlling cadherin-mediated cell adhesion at the plasma membrane and by mediating the interplay of adherens junction molecules with the actin cytoskeleton. (Brembeck et al., 2006) β-catenin also serves as a pivot between the roles of cell adhesion and gene transcription. (Bienz, 2005) The switch between these two cellular functions is controlled by several factors, including conformation and stability of the protein, the presence of E-cadherin-mediated cell adhesion, the presence of the BCL9 family of proto-oncogenes and by the tyrosine phosphorylation/de-phosphorylation of β-catenin. (Harris and Peifer, 2005).

The role of β-catenin in oral SCC is not yet well understood, and the clinical relevance of β-catenin expression in oral SCC is controversial. Some studies have related the reduced membranous expression to poor survival and nodal metastases (Hsu et al., 2008; Lopes et al., 2009). Another studies showed that aberrant expression of β-catenin was associated with a significant increase of mortality risk (Zeng et al., 2014).

The aim of the present study was to establish the expression and localization of E-cadherin and β-catenin in the histopathological degrees of oral squamous cell carcinomas.

Materials and Methods

This laboratory-based study involved the use of buffered formalin-fixed, paraffin-embedded tissues of histopathologically diagnosed cases of OSCC, retrieved from the files of the Department of Oral Histology and Pathology at the College of Dentistry, Damascus University. The study protocol was approved by the institutional ethics committee.

A total of 33 cases were evaluated immuno histochemically for E-cadherin and β-catenin expression these include 11 cases each of well differentiated (WDSCC), moderately differentiated (MDSCC) and poorly differentiated (PDSCC) squamous cell carcinoma. The diagnosis was confirmed by two oral pathologists using sections stained with hematoxylin and eosin.

Five samples containing oral normal epithelium obtained from patients who did not have cancer, were used as a control for the immunohistochemical strainers.

Immunohistochemistry

Two or three serial sections 4μm thick were prepared and placed on silanized slides. The sections were deparaffinized and rehydrated through xylene and descending grades of alcohol. Antigen retrieval was carried out in a pressure cooker in 10 mM citrate buffer (pH 6.0) for 2 to 5min.

The sections were then incubated after covering them with 3% hydrogen peroxide for 15min to block any endogenous peroxidase activity, and then 33 slides incubated with primary anti- β-catenin monoclonal antibody (DAKO Cytomation, USA, clone β-catenin-1) for 4h at room temperature using an optimal dilution of 1:50, another 33 slides incubated with Rabbit Monoclonal antihuman E-cadherin (Bio SB 69 Santa Felicia Dr., Santa Barbara, CA 93117, USA) ready to use for 30 min, After further incubation with the secondary antibody (45 min) and streptavidin peroxidase (30min), visualization was performed using freshly prepared diaminobenzidine (DAB) chromogen for 10min. The slides were finally counterstained with Harris hematoxylin.

Five of the most invasive tumoral islands were captured and the whole epithelial cells in these islands were counted and examined. Every epithelial cell exhibited an intact membranous expression were considered as normal, and cells exhibited cytoplasmic, nuclear or imperfect membranous expression were considered as abnormal.

One-way Anova and Tukey's test were used for comparison and correlation between the different grades of OSCC. Pearson correlation coefficient was used to establish the correlation between the expressions of both strainers.

Results

Normal epithelium

The staining patterns and localization of E-cadherin and β-catenin were identical. Both markers brightly decorated the epithelium in a circumferentially membranous fashion (basolateral and glycocalyceal). No cytoplasmic or nuclear staining was noted (Figures 1 and 2). In all 5 cases, the basal and parabasal cells displayed the greatest intensity of staining, whereas the most external and differentiated layers were not stained. Also there was no stromal staining.

β-catenin

β-catenin was expressed in 67.1% of the cells (87.3% of WDOSCC, 68.7% of MDOSCC and 43.5% of PDOSCC), there was a significant correlation ($p<0.05$) of

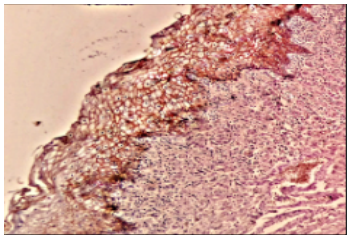

Figure 1. β-Catenin Expression in Normal Oral Epithelium

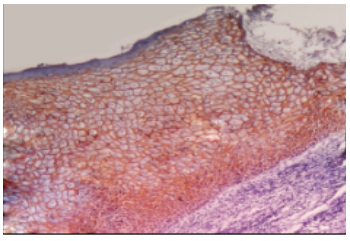

Figure 2. E-Cadherin Expression in Normal Oral Epithelium

β-catenin expression and the histological degrees.

23.5% of the cells exhibited a cytoplasmic expression (16.9% of WDOSCC, 29% of MDOSCC and 24.7% of PDOSCC), there was a significant correlation between WDOSCC and MDOSCC (p=0.001) also between WDOSCC and PDOSCC (p=0.044) and no significant correlation between MDOSCC and PDOSCC (p=0.334)

The nuclear expression for β-catenin appeared in 3.34% of the cells (0.2% of (WDOSCC, 3.4% of MDOSCC and 6.4% of PDOSCC), there was a significant correlation (p<0.05) of β-catenin expression and the histological degrees.

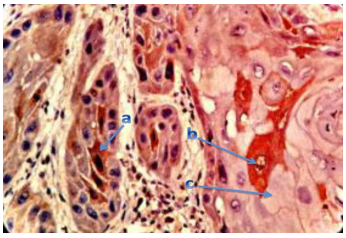

Figure 3. Aberrant Expression of β-catenin. 40x (A) cytoplasmic expression. **(B)** nuclear expression. **(C)** imperfect membranous expression

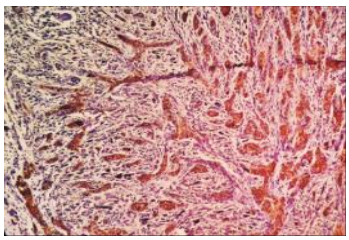

Figure 4. β-Catenin Expression in the Tumoral Islands of PDOSCC. 10x

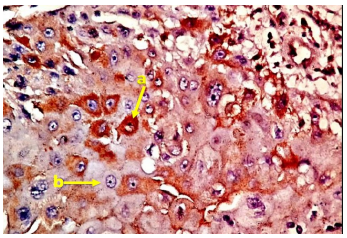

Figure 5. Aberrant Expression of E-Cadherin. 40x (A) cytoplasmic expression. **(B)** imperfect membranous expression

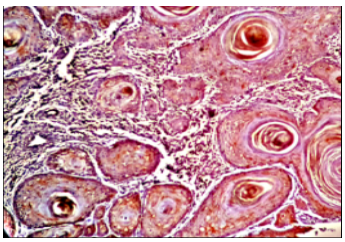

Figure 6. E-Cadherin Expression in the Tumoral Islands of WDOSCC. 10x

E-cadherin

E-cadherin was expressed in 61.2% of the cells (82.9% of WDOSCC, 61.7% of MDOSCC and 39% of PDOSCC), there was a significant correlation (p<0.05) of β-catenin expression and the histological degrees, (Table 1).

19.5% of the cells exhibited a cytoplasmic expression (14.5% of WDOSCC, 20.8% of MDOSCC and 23.2% of PDOSCC); there was a no significant relation between the cytoplasmic expression of E-cadherin and the histological degree of OSCC.

No nuclear staining was detected.

The relation between E-cadherin and β-catenin in OSCC

Pearson correlation coefficient was 0.716 (p=0.001), that indicated to a strong relation between the expression of these proteins, and when the expression of one of them increases the other protein expression increases too.

Discussion

We investigated the immunohistochemical expressions of E-cadherin and β-catenin in SCC specimens of the oral cavity region, as well as the correlations between expression of E-cadherin and β-catenin and the histopathological differentiation of this tumor.

In this study, we demonstrate that: 1) loss of expression of both E-Cadherin and β-catenin are frequent events in oral squamous cell carcinoma of all histological differentiations, and both proteins are thus likely participants in the pathogenesis of OSCC, 2) Loss of expression of both proteins is of prognostic significance with respect to the histologic grade of OSCC.

Various clinicopathologic data provided when diagnosing various cancers have been used as important data in establishing the direction of treatment and predicting patient prognosis. However, these data are not always reliable so that researches have been focused on finding more objective and reliable diagnostic and

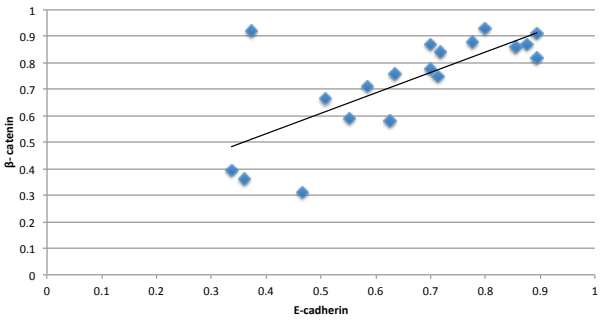

Figure 7. The Relation between the Expression of β-Catenin and E-Cadherin

Table 1. E-Cadherin Expression and Localization

	E-cadherin expression				E-cadherin cytoplasmic expression			
Grade	Highest value	Lower value	Standard deviation	median	Highest value	Lower value	Standard deviation	median
WDOSCC	82.9%	7.1%	70.0%	91.2%	14.5%	3.3%	9.5%	19.5%
MDOSCC	61.7%	8.4%	50.9%	77.1%	20.8%	9.5%	9.7%	42.2%
PDOSCC	39.0%	9.6%	5.7%	62.6%	23.1%	13.1%	11.1%	57.4%
Total	61.2%	19.9%	25.7%	91.2%	19.5%	9.9%	9.5%	57.4%

prognostic markers.(Valagussa et al., 1978).

When we could differentiate low risk- and high risk-patients according to the ideal marker, treatment outcomes could be increased drastically in high risk-patients through more aggressive treatment such as surgery combined with adjunctive radiotherapy, chemotherapy or hormone therapy and various economic and medical damages could be reduced by preventing unnecessary treatment in low risk patients (Koinuma, 2010).

Among the researches on finding reliable adjunctive prognostic markers, researches are active on adhesion molecules in solid tumors that are known to play an important role as prognostic factors related with tumor invasion and metastasis. (Ohene-Abuakwa et al., 2000; Pukkila et al., 2001; Yoshii et al., 2013).

E-cadherin is the most important mediator in maintaining the epithelial structure forming the cadherin/catenin complex within cells by binding to catenin. This function of E-cadherin was proven experimentally in which intercellular binding increases so that tumor invasiveness could be prevented when E-cadherin cDNA is transplanted in cell lines with poor prognosis having no expression of E-cadherin and tumor invasiveness could be recovered with the introduction of E-cadherin antibody (Wheelock and Jensen, 1992).

From the aspect of E-cadherin function, tumor growth shows the pattern of expansile growth, which is the growth pattern of benign tumors, without showing the growth pattern of invasive tumor even in the presence of malignant tumors when intercellular binding is maintained through normal action of E-cadherin (Birchmeier et al., 1993). Therefore, malignant transformation followed by the change in intercellular binding is essential in invasion and expansile growth, and the lack of various cell boundary structures participating in the intercellular adhesion is observed in various cancers (Lloreta-Trull et al., 2001).

E-cadherin expression appeared in 82.9% of the cells in WDOSCC, 61.7% of MDSCC and 39% of PDSCC. This expression was closely correlated with the histological differentiation of the OSCC, Our findings were in concordance with (Lopes et al., 2009; Zhai et al., 2010; Rosado et al., 2013), also we were in concordance with (Chan et al., 2014) whose study demonstrated a reduction of E-cadherin expression in OSCC compared to epithelium of normal oral mucosa and reactive lesions. However the results weren't in concordance with (Liu et al., 2010) who didn't find any relation, he used different staining methods and had different standards for the assessment of the expression, also (Rodrigo et al., 2007) didn't find any relation between laryngeal cancer histologic differentiation and E-cadherin expression, that maybe related to the difference in the anatomical site of the tumor.

Cytoplasmic E-cadherin expression wasn't correlated with the histological degree, also no nuclear expression was detected, that findings concord with (Fadare et al., 2005) and refer to that E-cadherin protein doesn't play any direct role in the tumor progression and its role is limited to the intercellular adhesion between cells and suppressing their proliferation.

Catenin present as α-catenin (102 kDa), β-catenin (88 kDa), and γ-catenin (80 kDa) is an anchoring protein present in cytoplasm and is essential in maintaining normal functions of E-cadherin in the cross-linkage action between actin filament and the intracellular membranous proteins, Na+/K+ adenosine triphosphatase and E-cadherin (Yu et al., 2005).

The expression of β-catenin was found in 87.3% of the cells in WDOSCC, 68.7% of MDSCC and 43.4% of PDSCC. This expression was closely correlated with the histological differentiation of the OSCC, Our findings were in concordance with (Laxmidevi et al., 2010), but not in concordance with (Fadare et al., 2005) who studied a different anatomical site-cervical carcinoma- and that may be the reason behind this difference.

cytoplasmic expression appeared in (16.9% of WDOSCC, 29% of MDOSCC and 24.7% of PDOSCC), there was a significant correlation between WDOSCC and MDOSCC ($p=0.001$) also between WDOSCC and PDOSCC ($p=0.044$) and no significant correlation between MDOSCC and PDOSCC ($p=0.334$), the cytoplasmic accumulation of β-catenin in the cytoplasm increases its chances to enter the nucleus where it play his role in activating the tumoral genes transcription. The nuclear expression was in 3.34% of the cells (0.2% of (WDOSCC, 3.4% of MDOSCC and 6.4% of PDOSCC), there was a significant correlation ($p<0.05$) of β-catenin expression and the histological degrees.

β-catenin binds directly to the cytoplasmic domain of E-cadherin molecule similar to γ-catenin. This binding is essential in stable cell-cell adhesion and is partially controlled by β-catenin. At the same time, β-catenin plays the role of an important marker in the signal transduction mediated by the proto-oncogene byproduct c-erbB2 and epidermal growth factor receptor (EGFR) (Hu and Li, 2010) (Ougolkov et al., 2003).

On the other hand, the functions of β-catenin would differ according to its location within cells. Cytoplasmic β-catenin, other than the previously mentioned membrane-bound form, is soluble, and is responsible for the Wnt/wingless signaling pathway, and is inhibited by normal adenomatous polyposis coli (APC) protein and glycogen synthetase kinase (GSK)-3-beta (Cadigan and Nusse, 1997). When β-catenin is translocated outside of the nucleus, it forms a complex with T-cell specific transcription factor (TCF)/lymphoid-enhancer-binding factor (LEF) transcription factor. Thus, nuclear β-catenin related with TCF/LEF activates the transcription of various target genes including c-myc, c-jun,fra-1, urokinase-type plasminogen activator receptor and cyclinD1, brought about by the mutation of β-catenin, deletion of APC gene, and activation of Wnt pathway (Tetsu and McCormick, 1999; Ebert et al., 2003).

The adhesion molecules E-cadherin and β-catenin were stained uniformly in most normal epithelial cells around the tumor, showing normal location of adhesion molecules in the cell membrane. However, changes of E-cadherin and β-catenin expressions were seen in 58.2% and 58.6%, respectively, in OSCC. Eventually, it was presumed that the aberrant expression occurs in adhesion proteins within normal cell membrane in many cases of OSCC so that adhesion molecules become stronger or weaken to bring about the ectopic expression in the

cytoplasm or nucleus. According to the previous studies, decreased expressions of E-cadherin and β-catenin in esophagus or bladder carcinoma are significantly related with histologic differentiation and metastasis (van Oort et al., 2007). It was reported that the expressions of E-cadherin and β-catenin were negative in colonic adenocarcinoma invading the colon wall but the cases maintaining the tumor glands were frequently seen (Van Aken et al., 1993). Thus, the fact that E-cadherin and β-catenin affect differently according to organs or individuals suggests that other cadherin proteins such as OB-, P-, and R-cadherin expressed in epithelial cells at the applicable site are compensating for functions, that adhesion among cells becomes weak despite intercellular binding of tumor cells, and that the binding structure formed is not suffice so that these different effects are seen according to the degree of variables affecting (Palacios et al., 1995).

As for the relationship between E-cadherin and β-catenin in the present study, the expression of β-catenin was also aberrant when aberrant expression of E-cadherin was present, and the expression of β-catenin was also maintained when the expression of E-cadherin was normal.

Nevertheless, the pattern of β-catenin expression showed a very close relationship with the expression pattern of E-cadherin. This finding suggests some relationship between the direct down-regulation of the cadherin/catenin complex and metastasis of OSCC. The E-cadherin and β-catenin proteins are directly connected so that abnormality in these proteins suggests the adhesion of tumor cells. Nonetheless, the exact mechanism has not been determined. When even one component among the components of the E-cadherin/β-catenin complex is not functioning properly, the functions of the complex are greatly affected, affecting various histopathological indices (Bruun et al., 2014). This result agrees with the finding that the loss of cell adhesion occurred when a mutation occurs in the β-catenin gene even when the expression of E-cadherin was normal in stomach cancer cell lines (Oyama et al., 1994).

Some studies reported that the ectopic expression of β-catenin affects the expression of cyclinD1(Koay et al., 2012). Furthermore, some studies reported that the prognosis is poor when the expression of β-catenin is present within the nucleus in squamous cell carcinoma of the pharynx and hepatoblastoma (Purcell et al., 2011), and a study reported that the expression of β-catenin in the cytoplasm is a predictor of hematogenous metastasis in colorectal carcinoma (Umemura et al., 2013). Thus, the possibility of β-catenin overexpression or ectopic expression was suggested during this process since cellular adhesion is needed for tumor cells freed from blood vessels in the primary lesion to settle in a new site. Moreover, a significant correlation was present between the presence of ectopic expressions of β-catenin in the cytoplasm and nucleus, and the degree of histologic differentiation.

On the other hand, a strong membranous expression of E-cadherin and/or β-catenin was observed according to immunohistochemistry in some cancer cells compared with normal cells. In this case, abnormality occurred in the adhesion site although the expression increased according to staining so that it was possible that the binding power decreased functionally.

In summary, the degree of histologic differentiation was observed in respect to the Expressions of E-cadherin/β-catenin complex in OSCC, were analyzed through immunohistochemistry, and analyzed statistically to obtain the following results. The rate of expression was 61.2% for E-cadherin and 67.2% for β-catenin. The expression of E-cadherin and β-catenin was significantly correlated with the histological degree of OSCC. A significant correlation was seen in normal and aberrant expressions between E-cadherin and β-catenin in which an aberrant expression of one molecule was seen with aberrant expression of the other molecule. The nuclear and cytoplasmic localization of β-catenin is correlated with the histological degree, whereas the cytoplasmic localization of E-cadherin was not correlated with histological differentiation and no nuclear expression was detected.

In conclusions, the results of the present study proved that the aberrant expression of E-cadherin and the aberrant expression of β-catenin are significant factors in predicting the histological grade in patients with OSCC. Thus, we believe that analyzing the expression patterns of E-cadherin and β-catenin and determining the expression pattern of E-cadherin/β-catenin complex is very useful with other previously determined clinicopathologic indices.

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