Expression of E-cadherin and matrix metalloproteinase-9 in oral squamous cell carcinoma and histologically negative surgical margins and association with clinicopathological parameters

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Abstract

Minimal residual cancer cells may not be detected in surgical margins of oral squamous cell carcinoma (OSCC) with routine histological examination. Using molecular markers at surgical margins can be helpful. We attempted to evaluate the MMP-9 and E-cadherin expression in OSCC samples and tumor-free surgical margins and association with clinicopathological factors. We examined E-cadherin and MMP-9 expression in 58 OSCCs including 19 grade I, 21 grade II and 18 grade III with histological tumor-free surgical margins by immunohistochemistry. Specimens were also divided in two groups: 19 samples as an early and 39 as an advanced stage. For E-cadherin in OSCCs and surgical margins, significant difference was observed between poor and moderate tumor differentiation. Different stages of OSCC demonstrated significant differences with higher expression in early stage tumors. For surgical margins, 82.1% of advanced and 84.2% of early stage samples demonstrated immunoreactivity. Both OSCC samples and surgical margins demonstrated significant differences for MMP-9 between stages with higher immunoreactivity in advanced stage, whereas there were not differences between different grades in surgical margins. E-cadherin and MMP-9 expression at histologically negative surgical margins shows the significance of these markers for prognostic values in OSCC patients with E-cadherin being the preferred predictor.

Keywords: oral squamous cell carcinoma, matrix metalloproteinase-9, E-cadherin.

Introduction

Squamous cell carcinoma (SCC) is the sixth most frequent malignant tumor of the head and neck region. The rate of the mortality and five-year survival has not been improved markedly over the last few decades [1–3]. Head and neck squamous cell cancers (HNSCC) have a high rate of local recurrence due to inadequate surgical resection [4].

Histological assessment of surgical margins leads to complete tumor excision, but minimal residual cancer cells may not be detected with routine histological examination [5]. Recurrences have been demonstrated at the primary site in histologically diagnosed tumor-free margins [6]. Using molecular markers for identification of malignant cells plays an important role in performing therapeutic options and reduces the incidence of cancer recurrences [5].

The invasion and spread of tumor cells require degradation and breakdown of the extracellular matrix (ECM) [7]. Multiple classes of ECM-degrading enzymes including matrix metalloproteinases (MMPs) are involved in migration of tumor cells [8].

E-cadherin glycoprotein is a Ca²⁺-dependent intercellular adhesion molecule in epithelial cells. It is involved in the regulation of epithelial cell-to-cell adhesion. There is a relationship between this marker and invasion of tumoral cells. Reduction the expression of E-cadherin has been shown in many malignant tumors [9, 10].

The aim of this study was to evaluate the expression of mesenchymal marker MMP-9 and epithelial marker E-cadherin in oral squamous cell carcinoma (OSCC) samples and their surgical margins with no histological evidence of malignancy and association with grade and stage of tumor. Although not the main aim of our study, the correlation between these markers and age, gender of patients, site of tumor were investigated.

Materials and Methods

The files of Department of Pathology, Omid Hospital, Mashhad University of Medical Sciences, Iran were
tumors were excluded.

The inclusion criteria of patients were based on primary disease, tumor site (oral cavity), histology of the primary tumor (squamous cell carcinoma), and histological status of surgical resection margins. All patients included in this study had a final pathology report of histologically normal resection margins, meaning none of the patients had a positive margin. Patients diagnosed with histological variants of squamous cell carcinoma (SCC) or recurrent tumors were excluded.

In our hospital for OSCC patients, the UK Royal College of Pathologists issued guideline for head and neck carcinoma in 1998 was used to define the margin status, including a histological distance from invasive carcinoma to surgical margins of more than 5 mm as clear [11]. Also for all patients, at the time of surgery, three to four margin samples (M1–M4) were taken from the surgical defect after tumor excision.

Fifty-eight OSCC samples including 19 grade I, 21 grade II, 18 grade III with histological tumor-free tissue (5 mm) at their surgical margins were retrieved and histologically evaluated by two experienced professionals.

Specimens were divided in two groups: stage I and stage II were considered as early stages and stage III, IVA and IVB as advanced stages [12]. Clinicopathological information including gender, age, and tumor location was gathered.

For IHC, four slices with a thickness of 4 μm were prepared from each paraffin-embedded section. The sections were fixed on poly-L-Lysine-coated glass slides. Deparaffinized and rehydrated slides were incubated for 30 minutes in 3% hydrogen peroxide/methanol to stop endogenous peroxidase activity and then irrigated with phosphate-buffered saline (PBS) for 20 minutes. For antigen retrieval, the sections were microwaved in citrate solution (0.01 M, pH 6.0) for 35 minutes. Specimens were incubated with the primary antibodies for one hour at room temperature. The sections were rinsed three times with PBS at room temperature. The secondary antibody was applied, and immune complexes were identified by Streptavidin peroxidase (Novo Link Polymer detection system). After three times washing with PBS, the immune reactivity was visualized by 3,3’-Diaminobenzidine and hydrogen peroxide. Finally, slides were counterstained with Hematoxylin and cover slipped with a synthetic mounting media. The immunohistochemical staining kit was the Novo Link Polymer detection system (RE7140-K 250T), which is an updated version of Biotin Labeled Streptavidin (LSAB). MMP-9 antibody (Novocastra Laboratories Ltd., 152W, dilution 1:20) and E-cadherin antibody (NCL-E-Cad, Novocastra Laboratories Ltd.; dilution 1:50) were used according to the manufacturer’s instruction (Novoceastra).

Each stained slide was evaluated by at least two independent investigators. For each marker expression, quantification of the number of positive cells was counted under light microscopy in five microscopic fields with a magnification of 400×.

MMP-9-stained slides were evaluated according to cytoplasmic positivity of epithelial cells. The positive cells were graded as 0: no positive staining; 1: <10%; 2: 11–50%; 3: 51–80%; 4: >80% [13].

E-cadherin stained slides were investigated according to cell membrane positivity (preserved), cell membrane and nuclear positivity (altered) [14].

Intensity of E-cadherin expression was quantified using Intensity Reactivity Score (IRS), where staining intensity (SI) was assessed to be negative (=0), weak (=1), moderate (=2) or strong (=3) and reactivity was determined by percentage of positive cells (PP). IRS was calculated by multiplying SI with PP [15, 16].

The staining pattern was evaluated by comparing the cellular localization of immuno-staining with those of normal tissue as an external positive control.

All analyses were performed using data processing program SPSS/PC version 16.0 for Windows (SPSS Inc., Chicago, IL, USA). Wilcoxon, Mann–Whitney, Kruskal–Wallis, Spearman’s rank correlation coefficient test and Fisher’s exact tests were used to assess the statistical significant differences between E-cadherin and MMP-9 expression and clinicopathological parameters in the studied groups, with p<0.05 being considered statistically significant.

### Results

The association of E-cadherin expression with tumor stage and differentiation grade

Evaluation of IRS for E-cadherin expression in OSCC sections and surgical margins by Wilcoxon test revealed statistically significant difference between poor tumor differentiation (stage IV) and moderate (stage III) tumor differentiation, while well differentiated OSCC (grade I) did not show significant difference (p>0.05) (Table 1).

<table>
<thead>
<tr>
<th>P-value</th>
<th>n</th>
<th>Studied groups</th>
</tr>
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<tbody>
<tr>
<td>0.041</td>
<td>19</td>
<td>Tumor (MMP-9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21 II</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>III</td>
</tr>
<tr>
<td>0.357</td>
<td>19</td>
<td>Surgical margin (MMP-9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21 II</td>
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<tr>
<td></td>
<td>18</td>
<td>III</td>
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<tr>
<td>0.083</td>
<td>19</td>
<td>Tumor and surgical margin (E-cadherin)</td>
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<tr>
<td></td>
<td></td>
<td>21 II</td>
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<tr>
<td>0.026</td>
<td>18</td>
<td>III</td>
</tr>
</tbody>
</table>

Altered E-cadherin expression was detected mainly in different grades of OSCC samples (Figure 1). In surgical margins of OSCC grade II and III altered immunoexpression was observed, whereas surgical margins of OSCC grade I retained preserved reactivity (Figure 2). Statistically significant difference was detected between site of E-cadherin expression and different grades in OSCC samples (p=0.01) and surgical margins (p=0.02).

According to Mann–Whitney test, different stages of OSCC (advanced and early stages) demonstrated significant difference (p<0.000) with higher E-cadherin expression in early stage tumors.

In OSCC specimens, the majority of the advanced stage cases (92.3%) showed altered E-cadherin expression while in early stage tumors both altered and preserved reactivity were detected equally.
For surgical margins, 82.1% of advanced stage and 84.2% of early stage samples demonstrated altered and preserved immunoexpression respectively. Fisher’s exact test revealed a significant difference between stage of tumor and location of E-cadherin expression both in surgical margins ($p=0.000$) and OSCC samples ($p=0.000$) (Table 2).

### Table 2 – Correlation between E-cadherin and MMP-9 expression and tumor stage in OSCC sections and surgical margins

<table>
<thead>
<tr>
<th>$p$-value</th>
<th>Mean rank</th>
<th>$n$</th>
<th>Studied groups</th>
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<tbody>
<tr>
<td>0.000</td>
<td>18.43</td>
<td>19</td>
<td>Early Tumor (E-cadherin)</td>
</tr>
<tr>
<td>0.000</td>
<td>83.22</td>
<td>39</td>
<td>Advanced Surgical margin (E-cadherin)</td>
</tr>
<tr>
<td>0.001</td>
<td>79.18</td>
<td>19</td>
<td>Early Tumor (MMP-9)</td>
</tr>
<tr>
<td></td>
<td>72.34</td>
<td>39</td>
<td>Advanced</td>
</tr>
</tbody>
</table>

The association of MMP-9 expression with tumor stage and differentiation grade

According to Kruskal–Wallis test, OSCC grade I showed a significant difference with grade II and III ($p=0.04$), whereas there was not any significant difference between different grades in surgical margins of OSCC ($p>0.05$) (Table 1).

Mann–Whitney test showed that both OSCC samples and their surgical margins demonstrated a significant difference between early and advanced stages ($p=0.001$), with higher immunoreactivity in advanced stage (Table 2, Figures 3 and 4).

**Correlation between MMP-9 and E-cadherin**

Spearman’s rank correlation coefficient test did not show a significant correlation between MMP-9 and E-cadherin expression for different grades and stages of OSCC samples and also for surgical margins ($p>0.05$).
tumors: 1 – tongue (28); 2 – other mouth areas (30). The expression of MMP-9 and E-cadherin was positively correlated with primary site of tumor when analyzed by the Spearman’s rank correlation coefficient test. The greatest Pearson’s correlation coefficient (r) was between E-cadherin expression in OSCC samples and surgical margin in tongue specimens (r=0.81). No relationship was observed between MMP-9, E-cadherin expression and other parameters including patient gender and age (p>0.05).

**Discussion**

Squamous cell carcinoma (SCC) is the most prevalent malignant neoplasm of oral cavity [15]. Epithelial carcinogenesis is a multistep process. Progression from normal oral epithelium to cancer is believed to result from cumulative genetic alterations [17–19].

Prognostic markers that could act as a therapeutic target could play an important role in effective treatment strategies of oral cancer [20]. The prediction of oral squamous cell carcinoma (OSCC) behavior is different using conventional clinical and histopathological parameters [21]. Therefore, studies on molecular biomarkers including cell adhesion and matrix degradation markers have performed as potential tools to predict the prognosis of patients with OSCC. Immunohistochemistry (IHC) as an available tool can provide useful information about prognostic tumor markers associated with clinical outcome of OSCC [21].

Despite advances in therapeutic options for head and neck squamous cell carcinoma over the last decades, the five-year survival rate of patients has been improved moderately. This is in part due to a high percentage (10–50%) of loco-regional and distant recurrences after surgery even in patients with histopathologically negative surgical margins [6, 22].

Various studies showed the limitation of standard histopathology for evaluation of surgical margins [23]. To detection minimal residual tumor cells at histopathological normal surgical margins, molecular-based technologies have been developed [24]. It has been demonstrated that molecular investigation of apparently tumor-free margins of excised tissue plays an important role in prediction of local tumor recurrence in head and neck squamous cell carcinoma patients [25, 26]. It has been reported that the grading of epithelial dysplasia in the surgical margins may have value in assessing the risk for developing local recurrences [27].

Most of prior studies investigated head and neck squamous cell carcinomas versus general oral SCCs. Therefore, differences in the primary cancers may cause discrepancy. Therefore, in the current study only OSCC samples were evaluated. Moreover, we attempted to evaluate expression of MMP-9 as a mesenchymal marker and E-cadherin as an epithelial marker in OSCC samples and histologically negative surgical margins to show the significance of both epithelial and mesenchymal markers for prognostic values in these patients.

Miyamoto et al. [28] suggested that molecular imaging has high accuracy for complete tumor resection and improvement of head and neck cancer. They also recommended that the complexity of these new molecular approaches limit their implementation in routine clinical use.

In our study, we applied immunohistochemistry to identify residual tumor in surgical margins. Compared to histology as the gold standard this technique showed more accurate results.

As previously mentioned, E-cadherin plays an important role in intercellular adhesion in epithelial tissues. Dysfunction and reduced immunoexpression of E-cadherin is involved in malignancy and carcinogenesis.

In the current study, reduction of E-cadherin expression was significantly associated with higher stage and grade of disease. Several studies also demonstrated that reduced immunoexpression of this marker in oral squamous cell carcinoma correlates with higher stage and grade of disease, lymph node metastasis, tumor invasive behavior and carcinogenesis, which are in consistent with our study [15, 16, 29, 30].

On the other hand, Ukpo et al. [31] suggested that E-cadherin expression may not be a predictor for nodal or distant metastasis in OSCCs.

The role of various types of MMPs is important for controlled matrix remodeling. Gelatinases MMP-2 and MMP-9 are the essential parts of basement membranes. These are the only MMPs that can degrading native collagen type IV and play an important role in cell migration and invasion [32].

Here, we showed the overexpression of MMP-9 in advanced stage tumors, which is in consistent with previous studies [7, 25, 33].

**Conclusions**

Our findings suggest that E-cadherin and MMP-9 expression at histological-negative surgical margins of OSCC can detect the presence and absence of minimal residual cells in surgical margins, diagnosed as tumor-free by conventional histopathological evaluation. Therefore, they may become a helpful biomarker to identify subjects at risk of new neoplastic evolution, with E-cadherin being the preferred predictor.

**Acknowledgments**

The research results given in this paper were obtained from doctoral thesis by a Grant (No. 900088) supported from the Vice Chancellor of Mashhad University of Medical Sciences, Iran.

**References**

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Received: May 16, 2013     Accepted: January 27, 2014