IMMUNOHISTOCHEMICAL EXPRESSION OF LIPOCALIN-2 AND MATRIX METALLOPROTEINASE-9 IN ORAL SQUAMOUS CELL CARCINOMA

Samar Hafez*, Iman Elshamy**, and Basant H. Abou Zaid***

ABSTRACT

Background: Oral squamous cell carcinoma (OSCC) is a well-known malignancy with diverse clinicopathological behavior and high mortality rate. It has great tendency to undergo lymph node metastasis. Its metastatic spread is highly regulated and entails the interplay of several factors released from cancer cells as well as stromal cells. Lipocalin-2 (LCN-2) is a secreted glycoprotein characterized by its involvement in inflammation, infection and cancer. Its role in oral cancer progression remains elusive. LCN-2 tends to form a complex with matrix metalloproteinase-9 (MMP-9), a well-known regulator of tumor microenvironment, enhancing its tumorigenic activity.

Objectives: This study aimed to investigate the immunohistochemical expression of LCN-2 and MMP-9 in OSCC.

Material and methods: The study was carried out on forty paraffin embedded archival OSCC specimens. The specimens were immunohistochemically stained against LCN-2 and MMP-9.

Results: Significant downregulation of LCN-2 expression was found in cancer cells with increased malignant criteria whereas stromal LCN-2 expression showed positive correlation with advanced histopathologic grade. Inverse correlation was observed between LCN-2 expression in both cancer and stromal with lymph node metastasis. MMP-9 expression was inversely correlated with the advanced histopathologic grades in both cancer and stromal cells.

Conclusion: The downregulation of LCN-2 and MMP-9 expressions in cancer cells with advanced OSCC histopathologic grade as well as at tumor invasive fronts propose their putative prognostic value.

KEYWORDS: Oral squamous cell carcinoma (OSCC); Oral cancer; Lipocalin-2 (LCN-2); Matrix metalloproteinase-9 (MMP-9); immunohistochemistry

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most prevalent head and neck cancer. Despite great advances in the field of research and the currently available therapeutic strategies, the overall prognosis of patients hasn’t shown a significant advance within the last few decades \(^1\). Worldwide, more than 377,713 new cases of OSCC are diagnosed annually with two thirds occurring in developing countries \(^2–6\), especially in Southeast Asia \(^7\). Incidence of OSCC is markedly influenced by demographic changes including geographic location, sex and age. Male sex tends to be involved more commonly than females, especially among old patients\(^2–4\). Tongue is the most commonly affected site followed by buccal mucosa, gingiva and floor of the mouth \(^8,9\). During cancer progression, crosstalk occurs between cancer cells and stromal components which play a major role in carcinogenesis \(^10\). Lipocalin-2 (LCN-2) is a relatively new protein entering the scene in human neoplasia \(^11\). It is a 25 kiloDalton (kDa) protein that belongs to the LCN superfamily. The 3-dimensional structure of LCN-2 reveals a central pocket enclosing an internal ligand-binding site which determines its main function \(^12\). LCN-2 was first recognized as a transport protein conveying hydrophobic substances such as prostaglandins, retinoids, arachidonic acid, hormones and fatty acids \(^12\). Its functions was further expanded to many biological processes including: growth, development and differentiation of human tissue \(^7\). Moreover, elevated LCN-2 levels have been detected in multiple human cancers including breast \(^13,14\), colorectal \(^15\), pancreas \(^16\) and OSCC \(^7\). LCN-2 was found to form a complex with matrix metalloproteinase-9 (MMP-9) sustaining its gelatinolytic effect \(^18\). MMP-9, a member of metalloproteinases family, was originally identified as a human leukocyte gelatinase and characterized as a 92-kda type V collagenase \(^7\). MMP-9 consists of hemopexin-like domain, catalytic domain, signal peptide, the hinge region and propeptide region \(^19,20\). It can degrade many extracellular (ECM) proteins in a wide spectrum of physiologic and pathologic processes encompassing tissue remodelling. MMP-9 has been widely related to cancer pathogenesis especially in the process of invasion, metastasis and angiogenesis \(^20,21\). Furthermore, MMP-9 is up-regulated in various human cancers including breast \(^22,23\), oesophageal \(^24\), lung \(^23\), pancreatic \(^25\), bladder \(^26\), colorectal cancer \(^27\) as well as OSCC\(^28\). Nevertheless, the exact role of LCN-2 and MMP-9 in OSCC has not been fully elucidated.

MATERIAL AND METHODS

This study was approved by Tanta University, Faculty of Dentistry, Research Ethics Committee. Forty paraffin embedded archival OSCC specimens were retrieved from archives of Oral Pathology Department, Faculty of dentistry, Tanta University and Tanta Cancer Institute. Serial sections were cut from each block at thickness of 4 um. One of the paraffin sections of each specimen was mounted on ordinary glass slide, dewaxed in incubator, cleared with xylene, rehydrated in descending grades of ethyl alcohol, then routinely stained with H&E stain, cover-slipped and examined under light microscope to confirm their diagnosis. The studied cases were graded histopathologically according to WHO criteria into well differentiated, moderately differentiated and poorly differentiated OSCC \(^29\). The other sections were employed in the immunohistochemical (IHC) staining procedures.

Immunohistochemistry:

The other sections were mounted on positive charged glass slides and immunohistochemically stained using the Streptavidin-Biotin Complex Universal Kit (Neomarkers, Fremont, CA, USA) according to manufacturer instructions. Human polyclonal antibody against LCN-2 (diluted at 1:10) was purchased from Quartett (Germany) and a human polyclonal antibody against MMP-9 (diluted at 1:25) was purchased from Thermo Scientific (UK). Normal-appearing oral mucosa adjacent to OSCC tissue was considered as internal normal control.

Evaluation of immunohistochemical results:

The slides were scanned at X20 and X40 magnification to evaluate immunostaining in tumor
epithelial cells as well as surrounding stromal cells. Immunoreactivity in each section was assessed semi-quantitatively according to the percentage of positive cells and the intensity of the immune staining. LCN-2 and MMP-9 expressions were scored according to the following scale as modified from Monisha et al. Staining intensity was scored as: 0, no staining, 1, weak staining, 2, moderate staining, and 3, strong staining. Percentage of positive cells was scored as: 0, (0% of positive cells), 1, (1–25% of positive cells), 2, (26–50% of positive cells), 3, (51–75% of positive cells), 4, (76–100% of positive cells).

Statistical analysis:

Data were collected, tabulated, and then statistically analysed using IBM SPSS for Windows, version 25.0 (SPSS Inc., Chicago, Illinois, USA). Changes in LCN-2 and MMP-9 expression versus histopathological grade and metastatic state were tested by using Chi-square test and Fisher’s exact test. Kappa coefficient was employed to assess the correlation between the two markers. P-value equal or less than 0.05 was considered significant.

RESULTS

Immunoreactivity in normal internal control tissue

Within normal mucosa adjacent to OSCC, there was no evidence for LCN-2 and MMP-9 in the surface epithelium. However, sporadic positive expression was noticed among the inflammatory cells found in some specimens.

LCN-2 immunohistochemical expression in OSCC specimens

Twenty two out of 40 specimens (55%) showed positive LCN-2 immunostaining in the form of diffuse cytoplasmic expression (Table 1). In well-differentiated OSCC, cancer cells exhibited weak staining intensity in 45.4% of specimens (Fig.1.A). Whereas stromal cells showed moderate immunostaining in 45.5% of specimens (Fig.1.B). Furthermore, the majority of moderately differentiated OSCC (66.67%) showed weak cytoplasmic immunostaining among cancer cells and weak stromal reaction in 44.4% of specimens (Fig.1.C and D). On the other hand, most tumor cells in poorly differentiated OSCC specimens (75%) showed negative LCN-2 immunostaining (Fig.1.E), whereas stromal cells exhibited moderate staining intensity in 40% of specimens (Fig.1.F).

MMP-9 immunohistochemical expression in OSCC specimens

MMP-9 expression was evident in 31 out of 40 specimens (77.5%) in the form of granular cytoplasmic immunostaining (Table 2). In well-differentiated OSCC, strong immunostaining was observed in both cancer and stromal cells (54.5% and 81.8% respectively) (Fig.2. A and B). In addition, more than half of the moderately differentiated OSCC exhibited strong MMP-9 immunostaining in the cancer and stromal cells (55.6% and 88.9% respectively) (Fig.2. C and D). Poorly differentiated OSCC exhibited moderate immunostaining of MMP-9 in cancer cells in 45% of the specimens (Fig.2.F), while stromal cells showed strong expression in 80% of specimens (Fig.2. E and F).

Expression of LCN-2 and MMP-9 at advancing tumor front

Most of the specimens (47.8%) exhibited weak LCN-2 staining intensity in cancer cells. Moderate immunostaining was noticed in stromal cells in 43.5% of specimens. Generally, immunostaining of LCN-2 was decreased at the invasive front as compared to intratumoral region (Fig.3. A and B). In contrast, MMP-9 immunoreactivity showed no difference between the invasive front and intratumor region in both cancer and stromal cells (Fig.3. C and D).

Correlation between LCN-2 and MMP-9 expression and different OSCC grades:

Within the invading tumor cells, expression of LCN-2 was inversely correlated with advancing
tumor grade. Such correlation showed a highly significant statistical difference ($P = 0.0003$). Whereas within the stromal cells, LCN-2 expression revealed to be slightly upregulated with advanced histological grades. At the advancing tumor front, LCN-2 expression appeared to gradually fade out in cancer cells and increase among stromal cells. Regarding MMP-9, the overall expression in cancer and stromal cells showed inverse correlation with advancing tumor grade. Furthermore, Our results showed slight agreement (Kappa coefficient < 0.2) between the expression of either markers in cancer cells and absence of agreement between their expressions in the stromal cells.

**TABLE (1):** LCN-2 expression scores in cancer and tumor cells as observed in different OSCC grades

<table>
<thead>
<tr>
<th>Histo-pathological grade</th>
<th>Intensity</th>
<th>Percentage score</th>
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<tbody>
<tr>
<td></td>
<td>Nil</td>
<td>Weak</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Moderate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Strong</td>
</tr>
<tr>
<td>Well diff.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$n = 11$</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>27.3%</td>
<td>45.4%</td>
</tr>
<tr>
<td>Moderately diff.</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>$n = 9$</td>
<td>11.1%</td>
<td>66.7%</td>
</tr>
<tr>
<td>Poorly diff.</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>$n = 20$</td>
<td>75%</td>
<td>15%</td>
</tr>
</tbody>
</table>

**TABLE (2):** MMP-9 expression scores in cancer and tumor cells as observed in different OSCC grades

<table>
<thead>
<tr>
<th>Histo-pathological grade</th>
<th>Intensity</th>
<th>Percentage score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weak</td>
<td>Moderate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Strong</td>
</tr>
<tr>
<td>Well diff.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$n = 11$</td>
<td>0%</td>
<td>4</td>
</tr>
<tr>
<td>Moderately diff.</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>$n = 9$</td>
<td>22.2%</td>
<td>22.2%</td>
</tr>
<tr>
<td>Poorly diff.</td>
<td>5%</td>
<td>45%</td>
</tr>
<tr>
<td>$n = 20$</td>
<td>0%</td>
<td>15%</td>
</tr>
</tbody>
</table>
Fig. (1) Photomicrographs of LCN-2 immunostaining in different OSCC grades. Well differentiated OSCC showing weak cytoplasmic expression in cancer cells (A) and moderate staining in stromal cells (B). Moderately differentiated OSCC showing weak cytoplasmic staining in tumor cells (C) and stromal cells (D). Poorly differentiated OSCC exhibiting negative immunostaining in tumor cells (E) and (F) and moderate staining in stromal cells (F). (Immunohistochemical staining, original magnification 400×).
Fig. (2): Photomicrographs of MMP-9 immunostaining in different OSCC grades. Well differentiated OSCC showing strong cytoplasmic expression in cancer and stromal cells (A) and (B). Moderately differentiated OSCC showing strong cytoplasmic staining in tumor and stromal cells (C) and (D). Poorly differentiated OSCC exhibiting strong immunostaining in tumor and stromal cells (E). Another poorly differentiated OSCC showing moderate expression in cancer cells and strong immunostaining in stromal cells (F). (Immunohistochemical staining, original magnification 400×).
DISCUSSION

OSCC is a well-known malignancy with diverse clinicopathologic behavior and high mortality rate (30). Despite great advances in the field of oral cancer research and the modern therapeutic strategies, the overall prognosis of patients hasn’t shown significant improvement (1,31). This motivates investigating factors with prognostic value in order to better tailor the individual treatment strategies of OSCC patients (32). Much attention has been focused on the role of LCN-2 as a selective and an early biomarker in cancers such as pancreatic (16), ovarian (33), endometrial cancers (34). However, its expression and role in oral cancer development have not been studied thoroughly. The current results showed that LCN-2 immunohistochemical reaction in the adjacent normal appearing mucosa was absent with sporadic positive expression within the underlying CT stroma. This goes in parallel with findings of Shinriki et al. (35) who reported the absence of LCN-2 expression in normal oral epithelium with a few associated stromal cells expression. Our results showed that invasive OSCC cells attained positive LCN-2 expression with different cytoplasmic intensities in 52.5% of the studied specimens. This is consistent with observations of Candido et al. (36) who noticed that LCN-2 was higher in solid tumors in respect to their normal counterparts. In addition, Maier et al. (37) reported a similar finding in his study on colorectal cancer and found that LCN-2 expression was significantly higher in carcinomas than in normal gut mucosa. Furthermore, we found...
significant downregulation of LCN-2 expression in relation to advancing OSCC histopathologic grade. Such diminished role of LCN-2 in higher OSCC grades was explained by Lin et al. who suggested that LCN-2 overexpression is linked to declined cancer cell biological behavior including migration, invasion, tumor growth and metastatic capacity. This is also supported by the work of Tong et al. on pancreatic cancer who found that well to moderately differentiated tumor cells expressed high levels of LCN-2 whereas moderately to poorly differentiated cells expressed undetectable LCN-2 levels.

Increasing evidences indicate that the carcinogenesis process is determined by tumor cells as well as tumor microenvironment. The present study supported this concept. Within the stromal cells, LCN-2 expression was found to be upregulated with advanced histological grade. This is in accordance with the results of a study carried on breast cancer performed by Ören et al. Their results revealed that LCN-2 stromal expression enhanced their protumorogenic capacity and the malignant criteria in breast cancer. Furthermore, Shinriki et al. reported similar results and suggested that higher levels of stromal LCN-2 expression correlated with poor differentiation and bad prognosis in OSCC. The potential impact of LCN-2 on tumor differentiation would vary according to the cell type. Such notion was supported by our observations since different results were observed in LCN-2 expression amongst tumor cells and their stromal counterparts as correlated to OSCC grade.

Regarding the tumor invasive front, the present study revealed that LCN-2 expression is downregulated within cancer cells, while upregulated in stromal cells. This finding is consistent with the role played by LCN-2 in relation to its origin as stated by Shinriki et al. These findings contradicted the results of study performed on endometrial cancers carried by Mihalj et al. who revealed no difference in the expression of LCN-2 between the superficial and invasive parts of the tumor. This discrepancy in LCN-2 expression within cancer cells and stromal cells in different tumor grades as well as at the tumor invasive front might point to the diverse tumor-dependent roles played by LCN-2.

The most widely accepted hypothesis in research on LCN-2 is its ability to drive induction of the EMT via MMP-9 dependent and independent mechanisms. Binding to MMP-9 generates a macromolecular complex which prevents the endogenous degradation of MMP-9. This preserves its ability to degrade many structural molecules such as collagen, fibronectin, and laminin, enhancing the aggressiveness and invasive potential of the neoplastic cells.

This study showed absence of MMP-9 immunohistochemical reaction in the normal epithelial tissues with minimal expression in adjacent CT cells. This finding goes in line with that of Kale et al. and Fan et al. who detected lack of immunostaining in the normal oral epithelium.

In the current study, MMP-9 positive expression was observed within cancer cells in 31 (77.5%) of the studied specimens. Additionally, all specimens showed positive MMP-9 expression in the stromal cells which are consistent with other studies carried by Ondruschka et al., De et al., Henriques et al., and Kosunen et al. who reported similar result and stated that these observations would reflect the role played by MMP-9 in facilitating malignant cell infiltration into the stroma and subsequent invasion. Yorioka et al. added that the tumor-stroma crosstalk is crucial for tumor invasion and metastasis. Henriques et al. also concluded that stromal enzymes potentiate the action of MMPs produced by the parenchyma.

The present results showed that MMP-9 expression in tumor as well as stromal cells
was inversely correlated with advancing tumor grade. This is consistent with Garg et al.\textsuperscript{(48)} who suggested that MMP-9 could play a protective role and act as a tumor suppressor in colon cancer. However, controversial results have been observed in the literature such as De et al.\textsuperscript{(44)} who reported significant association of MMP-9 expression with OSCC differentiation grade. Elahi et al.\textsuperscript{(49)} and Zheng et al.\textsuperscript{(50)} also found that MMP-9 expression was not correlated with the differentiation status of colorectal tumors and of OSCC respectively. They attributed this dispute to the influence of the staining techniques used, the difference in statistical analysis in detecting correlations, as well as to variations of the antibody staining pattern in different types of cancer. In addition, the present study showed no difference in MMP-9 expression within intratumoral compartments versus tumor invasive fronts among recorded specimens. This finding goes parallel with observations of Chambers and Matrisian\textsuperscript{(51)} who found no differences in the expression of MMP-9 between intratumoral mass and margins. They suggested that expression of MMPs is a property of the whole tumor, not just a reaction of the invasive front to the host tissues.

Collectively, results of the current study would verify their function as a complex and suggest their putative role in OSCC prognosis.

CONCLUSION:

Significant downregulation of LCN-2 expression in cancer cells with advanced histopathological grade of OSCC might signal its prognostic value. Positive correlation of stromal expression of LCN-2 with the histopathologic grade of OSCC signifies the role of tumor stroma in oral cancer progression and prognosis. Absence of positive correlation between MMP-9 expression and tumor histopathologic grade as well as its indifferent expression between intratumoral regions and invading fronts would indicate its role in OSCC progression is largely based on tumor-stroma interaction rather than the sole dependence on certain cell type and its level of differentiation.

REFERENCES


