Comparison of orosomucoid-1 immunoexpression and angiogenesis between oral squamous cell carcinoma cases with different histological grades

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Abstract

Background: Oral squamous cell carcinoma (OSCC) is the most common malignancy in this region, and thus, further elucidation of its tumoral mechanisms is important. One of the main roles of the acute-phase protein orosomucoid-1 (ORM1) is the promotion of angiogenesis, which is key for tumor nutrition and growth.

Aim: Our aim was to evaluate the immunohistochemical expression of ORM1 and the angiogenic activity indicated by microvascular density (MVD) in OSCC samples according to histological grade.

Materials and Methods: Formalin-fixed, paraffin-embedded sections from 45 OSCC cases were submitted to immunohistochemistry: 25 were well-differentiated OSCC, 18 were moderately differentiated OSCC and 2 were poorly differentiated OSCC. ORM1 staining was evaluated by a semiquantitative method, and CD34-positive blood vessels were quantified to calculate the MVD. The results were statically analyzed.

Results: All cases exhibited immunoexpression of ORM1 and CD34. However, no significant differences were found between the expression of both markers among the histological grades. In addition, the presence of ORM1 in inflammatory cells and in the extracellular matrix was detected in most cases.

Conclusion: These results suggest that the induction of angiogenesis is not the main role of ORM1 in OSCC and may be associated with the regulation of the immune/inflammatory response or the transport of protumoral molecules, such as sialyl-Lewis X or phorbol esters, which requires confirmation in future studies.

Keywords: Acute-phase proteins, angiogenesis, immunohistochemistry, oral cancer, orosomucoid-1 protein

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is a malignant neoplasm with squamous differentiation that arises from the oral mucosa epithelium, comprising more than 90% of oral cancers, and represents the sixth most common type of cancer worldwide. This disease occurs mainly in the fifth or sixth decade of life, and to date, smoking is...
the most important etiological factor. However, there are diverse genetic, metabolic, angiogenic and inflammatory protumoral mechanisms involved in the pathogenesis and tumor behavior of OSCC.

Despite the advances in prevention and multimodal therapies for several types of cancer in recent decades, the prognosis of patients with OSCC remains unfavorable. Therefore, further elucidation of the biological and cellular mechanisms that contribute to OSCC tumoral progression is urgently needed to improve our approach to this disease and identify new biomarkers with possible clinical and therapeutic implications.

The acute-phase response (APR) occurs due to imbalances in homeostasis caused by infection, tissue damage, immunological disorders or neoplastic growth; in this response, the concentration of a set of proteins in the plasma, called acute-phase proteins, is modified. These proteins are mainly involved in protection against invading agents, prevention of excessive cell damage through immunomodulation, induction of repair and contribution to functional restoration. Orosomucoid-1 (ORM1), also known as alpha-1-acid glycoprotein, is one of the proteins released in the APR that regulates the inflammatory response and angiogenesis in tissues. The exact functions of ORM1 are still unclear; however, ORM1 is considered an immunomodulatory and anti-inflammatory agent with a predominant role in immunosuppression that helps prevent excessive tissue damage due to the acute inflammatory response. High serological concentrations of these proteins have been reported in patients with OSCC and other malignant neoplasms, such as lung cancer, mainly in the advanced stages.

To the best of our knowledge, only one study has evaluated the expression of ORM1 in squamous cell carcinoma of the head and neck, including 15 cases of OSCC. Therefore, our study, with a larger population, will help establish the detailed expression pattern of this protein in OSCC with different histological grades.

As the CD34 and ORM1 proteins are associated with tumor behavior, the objective of this study was to establish the expression patterns of ORM1 and MVD in OSCC cases with different histological grades. The findings of this study could suggest applications for ORM1 in the study of head-and-neck neoplasms.

**MATERIALS AND METHODS**

**Sample selection**

Forty-five formalin-fixed paraffin-embedded tissue samples of OSCC obtained from the National Institute of Oncology and Radiobiology of Cuba were included in the study. The cases were classified according to histological grade: well-differentiated carcinoma (WD-OSCC), moderately differentiated carcinoma (MD-OSCC) and poorly differentiated carcinoma (PD-OSCC).

**Immunohistochemistry**

Immunohistochemistry for ORM1 (2F9-1F10, Abcam, 1:100) and CD34 (QBEnd-10, Dako, 1:50) was performed according to previously described protocols.

The evaluation of ORM1 cytoplasmic and membrane immunoreactivity was semiquantitatively scored using a four-grade scoring scale as follows: 0 (no staining); 1 (+, low): 1%–10% positive cells; 2 (++, intermediate or moderate): 11%–50% positive cells and 3 (+++, high): >50% positive cells.

The MVD was determined using the method originally described by Weidner. At low magnification, five “hot spots” with a high number of CD34-positive blood vessels were assessed. Subsequently, counting was performed in each area at ×200 field magnification. The MVD is expressed as the highest number of vessels within any ×200 field. The kappa coefficient (intraobserver concordance) was 0.886.

**Statistical analysis**

Central tendency measures were generated for both markers: means, medians and standard deviation. Subsequently, Student’s t-test was used for determination of the significance of the immunoexpression of each protein between the groups.

**RESULTS**

After a histopathological review, the 45 OSCC cases were classified according to their histological grade as WD-OSCC (25), MD-OSCC (18) and PD-OSCC (2).

All cases showed intense and diffuse staining for ORM1, mainly cytoplasmic staining; however, in focal areas,
predominant membranous staining and/or intercellular bridges was observed. A common feature was an intense expression at certain tumor sites, while other sites, even those adjacent to stained cells, remained negative [Figure 1a-d]. Focal nuclear positivity was observed in 7 cases [Figure 1e]. In addition, another staining pattern with the appearance of secretion in the extracellular matrix (so-called “shedding”) was observed in 30 cases, [Figure 1f] with a similar distribution between histological grades [Table 1]. Stromal cells, such as endothelial cells, inflammatory cells and some fibroblasts, presented immunoeexpression of ORM-1. In addition, in foci of intravascular invasion, ORM1-positive tumoral emboli were found [Figure 1c and d].

CD34 showed positivity in the blood vessels of all cases, with a predominantly peritumoral or intratumoral arrangement [Figure 2]. The median values of MVD for each group are shown in Table 2.

The majority of the cases showed high immunostaining (+ +) of ORM1: 84% of the CBD, 55.6% of the CMD and 100% of the CPD (n = 2) [Figure 3]. The difference in the expression of CD34 was not significant between histological grades or in relation to ORM1 immunoexpression [Table 2].

**DISCUSSION**

The immunoexpression of ORM1 observed in all cases in this study suggests that this protein may have a role in the pathogenesis and tumoral mechanisms of OSCC and can be used as a biomarker for further studies. The differences in immunoeexpression of ORM1 and MVD (CD34) between histological grades were not significant, suggesting

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Table 1: Presence of stromal secretion (shedding) of orosomucoid-1 by histological group

| Histological grade | Absent, n (%) | Present, n (%) |
|--------------------|---------------|---------------|
| WD-OSCC            | 9 (36.0)      | 16 (64.0)     |
| MD-OSCC            | 6 (33.3)      | 12 (66.7)     |
| PD-OSCC            | 0             | 2 (100)       |

WD: Well differentiated, MD: Moderately differentiated, PD: Poorly differentiated, OSCC: Oral squamous cell carcinoma

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**Figure 1:** Immunostaining of orosomucoid-1 in well-differentiated (a), moderately differentiated (c and d) and poorly differentiated (b) oral squamous cell carcinoma. Note the presence of adjacent negative and positive cells exhibiting predominantly cytoplasmic staining (a-f). Perivascular invasion of negative tumor cells (arrowhead) was observed in the lumen of the affected vessels, and the presence of positive tumor emboli (asterisk) and positive endothelial and inflammatory cells was observed (arrow) (c and d). Few cases presented nuclear positivity (e). Presence of orosomucoid-1 with the appearance of secretion in the stroma (shedding) (f) (Immunohistochemistry, a, b, d, e and f: x400, c: x200)

**Figure 2:** CD34 staining of blood vessels in well-differentiated (a), moderately differentiated (b) and poorly differentiated (c) oral squamous cell carcinoma. Note the peritumoral (a) and intratumoral (b and c) distribution of blood vessels (Immunohistochemistry, a: x200, b and c: x400)
that angiogenesis may not be influenced by ORM1 in OSCC; these results were consistent with findings indicating the participation of nonangiogenic pathways for vascularization and nutrition of tumors. We suggest that the role of ORM1 in OSCC could be associated with the regulation of the immune/inflammatory response rather than angiogenesis. However, this hypothesis requires further confirmation.

In normal conditions, ORM1 is secreted by endothelial cells, participates in the regulation of capillary permeability and therefore helps to maintain homeostasis. Since angiogenically activated vessels have an increased permeability, and this modification is one of the main effects of ORM1 on endothelial cells, the increase in ORM1 is associated with angiogenic activation. However, the angiogenic modulation of ORM1 is bimodal and depends on the context: it inhibits angiogenesis induced by injury or tumor necrosis factor-alpha and stimulates developmental or vascular endothelial growth factor (VEGF)-induced angiogenesis. Irmak et al. confirmed the active role of ORM1 in angiogenesis in collaboration with VEGF-A through in vitro and in vivo tests.

ORM1 has been suggested to be necessary for the capillary permeability that allows tumors to maintain an active angiogenic process; thus, the upregulation of ORM1 has been associated with the neovascularization of malignant processes as well as with clinical parameters. For example, Yildirim et al. found that serum ORM1 levels in patients with advanced-stage lung cancer were high. Similarly, advanced stage bladder cancer has a high expression of ORM1.

To the best of our knowledge, only Croce et al. (2001) evaluated and described the immunohistochemical expression of ORM1 in cases of head-and-neck cancer, including 15 OSCC cases, and similar to those in our study, most of the cases presented strong and diffuse staining in the cytoplasm. Similarly, the study described the positivity between intercellular bridges and focal staining in nuclear and cytoplasmic membranes, as well as the adjacency of positive and negative tumor areas. Although this study did not report the expression of ORM1 in endothelial and inflammatory cells or extracellular matrix observed in our cases, this pattern of labeling has been described in odontogenic myxoma. These results suggested that ORM1 is an important component of the extracellular matrix of this odontogenic tumor, contributing to its characteristic viscosity, which has been associated with a high invasive capacity. In addition, the authors suggest an angiogenic regulatory role of ORM1 in conjunction with VEGFA, since both markers were expressed in endothelial cells.

Blood supply is necessary for tumor growth and spread. An angiogenic mechanism has been described in some types of carcinomas, in which tumor cells secrete growth factors to produce new vessels from the existing circulation (angiogenesis). Aggressiveness parameters have been related to the degree and pattern of angiogenesis in various malignant neoplasms. In the oral mucosa, a significant vascularity increase has been verified through CD34 and CD31 immunexpression during the transition from normal mucosa to severe dysplasia and toward invasive carcinoma. As in the present study, several authors have reported an absence of a correlation between MVD and angiogenesis with the histological grade of OSCC. In the present study, we did not find an association between the presence of ORM1 and angiogenesis through MVD. The lack of a correlation between the levels of ORM1 and MVD suggests that the induction of angiogenesis is not the main role of ORM1 in OSCC; therefore, this molecule could be related to other tumor mechanisms, such as those associated with the regulation of the immune response, which requires confirmation in future studies.
In some types of cancer, the relationship between tumor angiogenesis and clinicopathological parameters is controversial.\textsuperscript{[23,24]} In the case of OSCC, tumor angiogenesis has been related to metastasis, tumor progression and prognosis.\textsuperscript{[25]} However, this phenomenon was not associated with tumor size, degree of differentiation, invasion, metastasis, recurrence, prognosis or survival.\textsuperscript{[22]} Therefore, nonangiogenic or less angiogenesis-dependent mechanisms for OSCC cell nutrition have been suggested, which may participate in tumor progression.\textsuperscript{[13]}

There is evidence of the immunosuppressive activity of ORM1 during inflammation; however, its specific immunological or biological functions are still unclear.\textsuperscript{[12]} ORM1 may be produced by some tumors to protect themselves through immunosuppression.\textsuperscript{[24]} The immunomodulatory function of ORM1 could explain the positivity in the inflammatory infiltrate in the present study.

Studies on ORM1 immunoexpression in head-and-neck tumors are lacking, and Croce et al. (2001), who analyzed the association between ORM1 and head-and-neck cancer, is the only one that includes OSCC cases.\textsuperscript{[12]} The authors subsequently (2005) performed an immunohistochemical study of ORM1 as a possible carrier of the sialyl-Lewis X antigen in colorectal carcinoma. ORM1 and other glycoproteins have been identified as carriers of sialyl-Lewis X, whose expression is frequent in neoplastic cells, and this molecule has been strongly related to the migration of tumor cells and metastasis.\textsuperscript{[27]} Interestingly, we found areas of intravascular invasion, indicating that the clusters of tumor cells within the blood vessels showed a strong expression of ORM1, unlike those with a perivascular invasion pattern, which could be associated with the aforementioned mechanisms of cell migration, tumor spread and even with the increase of vascular permeability.

Another type of molecule carried by ORM1 are phorbol esters, which act as tumor promoters through the activation of protein kinase C,\textsuperscript{[28,29]} which has been linked to cutaneous squamous cell carcinoma caused by repeated exposures to ultraviolet radiation.\textsuperscript{[30]} Within this group, 12-O-tetradecanoylphorbol-13-acetate has been described as a potent tumor promoter, by activating the transcription of genes involved in cell proliferation, transformation and apoptosis.\textsuperscript{[31]} This finding suggests a possible additional role of ORM1 (in addition to immunomodulation) in OSCC pathogenesis.

One of the main limitations of the study was the absence of immunohistochemical tests for ORM1 in human normal oral epithelium, as there are no specific publications on this issue. Elucidation of the immunoexpression of ORM1 in healthy oral mucosa is important for comparisons with that presented in neoplastic tissues. The limited number of samples from the PD-OSCC cases ($n=2$) did not allow to include this histological grade in the statistical tests.

**CONCLUSION**

The immunoexpression of ORM1 observed in all cases in this study suggests that ORM1 can be used as a biomarker for OSCC study. The lack of a significant relationship between the expression of ORM1 and MVD among the histological grades could indicate the participation of nonangiogenic pathways, as well as a role of ORM1 in other tumor mechanisms during OSCC progression, which is probably related to immunomodulation, vascular permeability or transport of protumoral molecules such as sialyl-Lewis X or phorbol esters. This hypothesis requires additional research for confirmation.
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Conflicts of interest
There are no conflicts of interest.

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