Immunohistochemical study of RhoC GTPase in oral squamous cell carcinoma

L. H. B. Martins¹, N. M. Pinheiro¹, A. M. B. Spina¹, A. M. R. Micheletti², S. S. C. M. Carboni¹ and V. O. Crema¹,²

¹Structural Biology Department, Institute of Natural and Biological Sciences, Federal University of Triângulo Mineiro, Uberaba, MG, Brazil
²Pathology Department, Institute of Health Sciences, Federal University of Triângulo Mineiro, Uberaba, MG, Brazil

Corresponding author: Virgínia Oliveira Crema, Structural Biology Department, Institute of Natural and Biological Sciences, Federal University of Triângulo Mineiro, Praça Manoel Terra 330, Uberaba, MG, Brazil, CEP 38025-050. E-mail: virginiacrema@icbm.uftm.edu.br

Abstract
Oral squamous cell carcinoma (OSCC) is a disease with high mortality and morbidity. Metastasis is a significant prognostic factor of the OSCC patients. The Rho GTPases are signaling proteins that controls important cellular processes in various complex mechanisms involved in carcinogenesis. This study aimed to evaluate the expression pattern of RhoC in OSCC protein by immunohistochemistry in situ. Immunohistochemical reactions were performed for RhoC by the method of avitina-biotin-peroxidase activity in samples OSCC: well differentiated (BD, n=6), moderately differentiated (MD, n=24) and poorly differentiated (PD, n=13). The morphometry was taken by QuickScore (percentage and intensity of staining) and only intensity staining. There was no statistical difference (p=0.05) through none of the modes of morphometric analysis between BD, MD and PD. And the RhoC staining was not associated with the histopathologic grading (χ² = 4.65, p>0.05). However, the morphological evaluation of immunostained for RhoC in cases BD, MD, PD OSCC, regardless of histopathologic grading. These results suggest that there is no correlation between the RhoC immunoeexpression and histopathological grading of OSCC.

Key words: Histopathological grading, oral cavity, oral squamous cell carcinoma, Rho GTPases, RhoC GTPase.

Introduction
The squamous cell carcinoma is among the ten most common malignancies in the world (1). From malignant oral cancers 90% are diagnosed with oral squamous cell carcinoma (OSCC) (2). Oral cancer may exhibit a variety of signs and symptoms, including pain, dysphagia, non-healing ulcers red and white lesions, and hardened masses (3). The most affected sites are the ventral surface of the tongue, mouth floor, lower lip, the soft palate and gums. Malignancy may be preceded by the presence of very heterogeneous precancerous lesions. Lesions may present from small and asymptomatic, to large and symptomatic lesions. There is a relationship with the size of the lesions, ulcerations, bleeding and lymphadenopathy (3, 4).

Infiltration to the underlying tissues, hardened nodules and ulcers are some standard features of malignancy OSCC. The most common is the ulcer that higher stages involve pain symptoms and less common: paraesthesia, difficulty healing after surgical procedures, dysphagia and loss of weight. Lymphadenomageal can manifest without the presence of secondary tumors. In later stages the survival rate is lower (3).

Graduations for histological classification of cancer are used, which can be considered also as prognostic factors in cancer. There is a concern these measurements to be more accurate and provide adequate information on the analyzed cancer. At least five criteria for evaluation are used: degree of keratinization, nuclear pleomorphism, number of mitoses, invasion pattern and infiltration of inflammatory cells (5, 6).

The identification of the expression of specific molecules involved with carcinogenesis provides a growing knowledge of the number of molecular markers related to the characteristics of malignancies. These specific markers for OSCC may account for individual variations in the course of the disease and also to help in the realization of better therapeutic approaches and more reliable predictions (7).

The family of Rho GTPases (RhoA, RhoB, RhoC, Rac1, Rac2, RAC3 and Cdc42) are involved in the regulation of metastatic phenotype in cancer cells located in the source organs (8, 9). Cell lines showed that significant RhoC expression promoted spontaneous metastases even without the presence of the primary tumor, acting independently (10). Considering that the protein RhoC could be an important marker for the cytodifferentiation of the cell OSCC, this study aimed to evaluate the pattern of expression of RhoC protein oral squamous cell carcinoma through immunohistochemistry in situ.

Materials and methods
This study was approved by the Research Ethics Committee (CEP) of the Federal University of Triângulo Mineiro (UFTM). 43 cases of OSCC obtained from patients undergoing incisional or excisional biopsy, which were diagnosed as OSCC, were retrieved from Pathology Service of Clinical Hospital of UFTM. The mean age of OSCC patients was 59.59±13.25 years. The OSCC cases were reviewed and classified as well-differentiated (BD, n=6), moderately differentiated (MD n=24) and poorly differentiated (PD, n=13), according to the World Health Organization (6).

Immunohistochemistry reaction for GTPase RhoC
Samples were fixed in formaldehyde and paraffin blocks were performed by conventional methods. Histological sections (4 µm) were dewaxed and incubated with Citrate Buffer Antigen Retrieval, pH 6.0, by us-
ing the Decloaking Chamber Nx Gen Manual (Biocare Medical, Concord, CA) at 110°C for 15 min. The other steps of immunohistochemistry was performed as previously described (11). Samples were incubated with: H₂O₂: methanol (1:1) for 15 min, non-immune rabbit serum (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) at a 1:10 for 1 h, polyclonal primary antibody rabbit anti-RhoC, C-18 (Santa Cruz Biotechnology, Santa Cruz, CA) 1:50 for about 16 h, rabbit anti-goat (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) 1:200 for 1 h 30 minutes, avidin-biotin-peroxidase complex (Elite® ABC Kit - Vector Laboratories, Burlingame, CA, USA) for 2 h, 3,3’-Diaminobenzidine - DAB (Sigma-Aldrich, St. Louis, MO) for 15 min. (12). Then counterstaining with Harris haematoxylin was performed for 30 seg, and the slides were mounted with Entellan® (Merk KGaA, 64271 Dannstadt, Germany). As a negative control, the primary antibody was omitted. Submandibular gland of Wistar rats was used as positive control. The experiments were performed in duplicate.

**Morphometric analysis**

The samples were analyzed with two protocols by using a Axio Vert.A1® microscope (Carl Zeiss Microscopy GmbH 37081 Gottingen, Germany). A semi-quantitative evaluation of GTPase RhoC immunostaining was performed using QuickScore (13) in three fields of the samples using a 20x objective. To calculate the QuickScore (Q), they were considered: the percentage of stained cells (P) and the intensity of immunostaining (I). With the results, the QuickScore was calculated using the Excel® spreadsheet: Q = P x I.

Also, morphometric analysis was performed according to impregnate chromogen substance (14) in three fields of samples using a 20x objective. To quantify the immunostaining intensity for RhoC tagged oral squamous carcinoma cells were analyzed to the fullest extent of the histological section. The intensity of staining was considered negative (0) mark: poor (1), moderate (2) or intense (3).

**Statistical Analysis**

The results were analyzed with SPSS 16.0 software and graphics were performed with Graphpad PRISM software. The variables were analyzed with the homogeneity test of Levene variance. The χ², analysis of variance (ANOVA) and Tukey’s post-test were used. Differences were considered significant at p<0.05.

**Results**

In the semi-quantitative evaluation of immunostaining for RhoC GTPase using QuickScore (considering the percentage of stained cells and the intensity of immunostaining), there was no statistical difference [F (2,34) = 0.157, p>0.05] between cases classified as BD (18.17±13.94), MD (18.83±10.56) and PD (10.94±11.54), (Figure 1). When analyzed only the intensity of immunostaining for GTPase RhoC, there was also no statistical difference [F (2,35) = 0.831, p>0.05] between cases classified as BD (1.50±0.83), MD (1.42±0.76) and PD (1.09±0.70), (Figure 2).

RhoC staining was not associated with the histopathologic grading (χ² = 4.65, p>0.05). However, the morphological evaluation of immunostained for RhoC protein in cases BD, MD, PD and OSCC, regardless of the histopathologic grading, showed a cytoplasmic staining more intense in the intercellular bridges (Figure 3). An intense staining for RhoC in the tumor margins was not seen in our morphological evaluation. Samples did not showed greater intensity of immunostaining in mitotic cells or necrotic cells.

**Discussion**

Although RhoC was expected to be more intense stain in PD cases, this study demonstrated that the pattern of expression of RhoC protein was independent of histopathological grading of the OSCC, through morphological and quantitative evaluation.

Rho GTPases family participate in the regulation of various essential cellular processes. Studies have shown their involvement in many cellular processes, occurring in the carcinogenesis of several malignancies (15-17). RhoA and RhoC proteins are involved in tumorigenesis of breast cancer. The line of breast cancer cells (MCF-7) has a signaling modulator ER-alpha, which depends on the gene expression of RhoA protein, and that was not associated with modulation of RhoC protein, suggesting that RhoA and RhoC proteins play different functions in tumorigenesis (18).

**Figure 1.** Semi-quantitative evaluation of immunostained for GTPase RhoC in oral squamous cell carcinoma using QuickScore. Groups: Well differentiated (BD), moderately differentiated (MD) and poorly differentiated (PD). ANOVA and Tukey’s post-test, p>0.05.

**Figure 2.** Immunostaining intensity of evaluation for GTPase RhoC in oral squamous cell carcinoma. Groups: Well differentiated (BD), moderately differentiated (MD) and poorly differentiated (PD). ANOVA and Tukey’s post-test, p>0.05.
high histological grade and positive lymph nodes. Overexpression of RhoC was a predictor of survival in patients with breast cancer and was associated with 100% increase in the risk of death compared with patients with low expression of RhoC (22). Although it was expected intense staining for RhoC in the tumor margins, particularly in PD OSCC cases, this characteristic was not seen in our morphological evaluation.

In cell cultures derived from cervical cancer (CaSki and SiHa), RhoC showed higher expression levels suggesting its contribution to invasion and metastasis (24). RhoC is highly expressed in ovarian carcinomas, and promote more aggressive ovarian cancer cells when compared to benign ovarian tumors (25). Contrasting these results, regarding the histopathological grading in cases of OSCC, there was no RhoC overexpression in our study.

RhoC interference by siRNA results in low levels of p-p70S6K protein in cells of ovarian carcinomas, which reinforces the interrelation of RhoC and p-p70S6K, and the higher expression of both higher the degree of aggressiveness of this tumor. The abnormal expression of RhoC affects the ovarian epithelium for a malignancy therefore is considered a potential biomarker for differentiation and progression of ovarian cancer (25). The comparison of the spatio-temporal dynamics of RhoA and RhoC activity in cell culture MEF/3T3 during protrusion/cell contraction in cell migration studies. The two isoforms differ clearly in cellular activation kinetics. RhoC was activated RhoA concurrently with the edge of the cell, RhoC was preceded by activation of RhoA activation. These activations occur with different kinetics that occurs before RhoC activation events are initiated prosuasive (26).

The invasion of tumor cells is a key step in metastasis. In cell lines of breast cancer, and HCC70 SKBR-3, the analysis of the signaling pathways by controlling cancer cell invasion of ARF-1 was shown that ARF-1 appears to modulate the action of some GTPase, RhoA especially isoforms, RhoB and RhoC. The RhoA and RhoC proteins are associated with the proliferation and invasion and RhoB presenting tumor suppressor properties (27). RhoA plays a regulatory role in cellular invasion by ability to target MT1-MMP in invadopodia MDA-MB (231) and controls the RhoC coflin activities invadopodia MTLn3 cells of highly metastatic mammary adenocarcinomas in experimental models (28).

In squamous cell carcinomas of the head and neck, the expression of some of the GTPases family proteins has been reported by several authors, participating in the regulation of cell differentiation (29), as a potential target proteins for prognostic (29-31), in the invasion (32-35) and cell migration (5, 36). In tumor of head and neck (UM-SCC-11 and 1), the RhoC protein showed high levels in its active form. When RhoC expression was inhibited, the degree of aggressiveness and invasiveness of cells were reduced, indicating that can control the formation of metastases (37). The overexpression of Rho and Rac in carcinoma suggests that these molecules can become markers of tumor progression (38-38). In this study it was not possible to associate the RhoC protein with invasion and progression of OSCC, once it was performed only immunohistochemistry asay. RhoC seems to participate in the regulation of cell

In metastatic prostate carcinomas and breast carcinomas, PKN3 the AGC family protein kinases are involved in regulating the growth of these tumors in vivo and in vitro and feature interactions with the Rho family proteins, especially RhoC. This complex PKN3 / RhoC has an important functional role in the advanced stages of these tumors. In the formation of the complex PKN3 / RhoC, the PKN3 promoted an increase in growth and invasion of tumor cells while RhoC seems to promote metastasis without affecting primary tumor growth (19).

Analysis of the percentage of stained cells and the intensity of immunostaining for RhoC were not different in cases of OSCC classified as BD, MD and PD in our study. The RhoC overexpression in more aggressive tumors induced breast cancer cell lines of stem cells, these proteins show an important regulatory metastasis. In cell lines tested showed significant expression of RhoC that promoted spontaneous metastases even without the presence of the primary tumor, acting independently (10). The RhoC overexpression seems to induce an increase in the expression of angiogenic factors by increasing tumor vascularization and the probability of metastasis in breast tumors (20). Growing interest in studies of RhoC due to their overexpression in invasive breast carcinomas and this protein is involved with the progression and invasion of these cancers. RhoA appears to prevent RhoC to stimulate cell invasion in this type of cancer, requiring research into tumors in other parts of the body (21).

Other studies showed that in induced mammary tumors in mice, the inactivation of RhoC not affect embryogenesis, normal cellular functions and immune responses. RhoC is dispensable for embryonic and postnatal development and the absence of RhoC did not affect tumor growth but reduces the motility of tumor cells and cell survival (22, 23). In patients with invasive breast carcinoma, the high expression of RhoC was associated with features of aggressive behavior, including
invasion processes when present in the nodal metastasis, and are also identified in small primary tumors with the high invasive potential. Also, it is overexpressed in squamous cell carcinomas of the head and neck metastatic. Inactivity of RhoC may be a potential target for developing new strategies for treatment of cancer, being suggested as protein marker of poor prognosis (39).

In head and neck carcinomas, tumor cells had intense staining for RhoC. However, in this study we have not seen morphological features that we could relate the intensity of immunostaining RhoC with histopathological grading in cases of OSCC studied, only the intense staining was seen in the intercellular bridges. RhoC plays an important role in the progression of head and neck cancer and metastasis. The development of specific inhibitors RhoC may be important therapeutic target or marker of prognosis (37).

In esophagus squamous cell carcinomas, the cancer cells showed high expression of RhoC and G3BP in metastatic lymph nodes. Suggesting that the G3BP protein RhoC and can promote tumor invasion and metastasis via the same route at different sites (31). In another work with esophageal cancer, the RhoA protein was observed more overexpressed in the cell cytoplasm that RhoC (40). In other malignancies, in a lineage osteosarcoma (U2-OS) study showed a correlation between MRK protein of an effector RhoC that seems to regulate the invasion of tumor cells, raising the possibility that inhibitors exert effector functions invasion-inducing molecules, and may be effective in treating metastatic tumors dependent RhoC (41).

In ovarian carcinomas, it is shown an association of RhoC expression and cell proliferation markers such as Ki-67, but are not related to age or serum levels. RhoC also appears to play a role in apoptosis (25). However, our study did not showed greater intensity of immunostaining in cells in mitosis or necrotic cells.

Metastasis has a significant impact on the OSCC’s prognosis factor. Most lymph node metastases are found in the cervical lymph nodes (42). The incidence differs according to the site of the primary lesion and can be hidden or distant metastases. Distant metastasis can be observed in 20% of cases, mostly in lung and liver (43, 44).

In conclusion, although only has been performed immunohistochemical study, the results suggest that there is no correlation between the immunoreactivity of RhoC and histopathological grading of OSCC. Future studies will compare the gene expression of RhoC protein in patients with OSCC nonmetastatic and metastatic.

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References


