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Original Research

Cetuximab has an inhibitory effect on cell motility in SCC-4 oral squamous cell carcinoma cell line

L. M. Furtado[1,2], I. C. da Silveira[1], A. C. D. M. Carneiro[1], M. M. O. N. Zehuri[1], S. S. C. M. Carboni[1], B. M. Tavares-Murta[3], V. O. Crema[1*]

[1] Structural Biology Department, Institute of Natural and Biological Sciences, Federal University of TriânguloMineiro, Uberaba, MG, Brazil
[2] Department of Oral Surgery, School of Dentistry, Federal University of Uberlândia, Uberlândia, MG, Brazil
[3] Pharmacology Department, Institute of Natural and Biological Sciences, Federal University of TriânguloMineiro, Uberaba, MG, Brazil

Correspondence to: virginiacrema@icbn.uftm.edu.br

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Abstract: Cetuximab is a chimeric monoclonal antibody that acts as a competitive antagonist, by binding to EGFR. This cell signalling pathways regulates tumor progression. The oral squamous cell carcinoma undergoes to regional spreading and distant metastasis. This study aimed to evaluate the effect of treatment with Cetuximab on cell migration and invasion in OSCC cells, by using the SCC-4 cell line. Cell migration and cell invasion assay were performed and actin cytoskeleton of control and treated with Cetuximab cells were evaluated. Differences were considered significant when $p<0.05$.Cetuximab inhibited the migration of SCC-4 cells at three concentrations: 1 µg/mL, 50 µg/mL and 100 µg/mL ($p<0.0001$) in a dose-dependent manner. The number of SCC-4 treated cells with 1 µg/mL that migrated through the membrane was statistically different from 50 µg/mL ($p<0.001$) and 100 µg/mL ($p<0.0001$), and between 50 µg/mL and 100 µg/mL ($p<0.01$). Cetuximab 50 µg/mL inhibited cell invasion through the Matrigel™ compared with SCC-4 control cells ($p<0.01$). Cetuximab 50 µg/mL affected the organization of the actin cytoskeleton. Cetuximab has an inhibitory effect on actin cytoskeleton organization, cell migration and invasion, suggesting that Cetuximab treatment can be important to avoid oral squamous cell carcinoma metastasis.

Key words: Actin cytoskeleton; Cell migration; Cell invasion; Cetuximab; SCC-4.

Introduction

The squamous cell carcinoma is considered the sixth cancer more diagnosed in all over the world (1), being estimated more than300.000 new cases (2). OSCC accounts for most malignancies in the oral cavity (3). Among the oral squamous cell carcinomas of the most commonly affected is the tongue, followed by soft palate and floor of the mouth (1). Between cases of carcinoma of oral squamous cell, 36% are located and 43% have regional metastasis (involving cervical lymph nodes) and 9% with distant metastasis (4). The tongue is also the primary tumor region with the highest production invasion and metastasis because of the proximity of the lymphatic system and rich vascularity (5).

The epidermal growth factor receptor (EGFR) regulate cell-signaling pathways, contributing to tumor progression. Overexpression of EGFR has been correlated with head and neck cancer in tumor progression, resistance to conventional therapy and unfavorable prognosis (1). In more than 90% of squamous cell carcinomas of the head and neck the EGFR overexpression is associated with a worse prognosis (6).This receptor has two sites that maybe target of therapeutic inhibition, the extracellular domain where epidermal growth factor (EGF) binding occurs and the intracellular domain that exhibits tyrosine kinase activity (1).

There are two pharmacological classes that can inhibit EGFR, monoclonal antibodies (mAbs) and tyrosine kinase inhibitors (TKIs) (7). The mAbs act in the extracellular region of the receptor and the TKIs for being small and hydrophobic molecules can pass through the plasma membrane and perform on intracellular region (8). Components of the immune system have been used in cancer therapy, such as cytokines, immune system cells and monoclonal antibodies. Some monoclonal antibodies have been described in the literature for cancer treatment (9). Cetuximab is a chimeric monoclonal antibody that acts as a competitive antagonist, by binding to the extracellular domain of the EGFR (9, 10). Cetuximab has a higher binding affinity for the N-terminal domain than for endogenous ligands, furthermore, when Cetuximab binds to EGFR, this receptor is blocked from binding to endogenous binders, impairing signaling. Another effect caused by binding of EGFR Cetuximab is the receptor internalization and degradation, which generates the decrease of available receptors on the cell surface, further reducing EGFR signaling (11).

Preclinical studies have demonstrated the antitumor effect of the monoclonal antibody Cetuximab on head and neck squamous cell carcinoma (12). This preliminary study aimed to evaluate the effect of treatment with Cetuximab on cell migration and invasion in OSCC cells, by using the SCC-4 cell line from tongue.

Materials and Methods

Cell culture

The cell line of human oral carcinoma SCC-4 was obtained from American Type Culture Collection (ATCC®). These cells were cultured in medium Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) 1: 1 HAM F12 (Invitrogen, Grand Island, NY, USA), containing 400 ng/mL hydrocortisone, 10% fetal bovine serum (FBS) (Invitrogen, Grand Island, NY, USA), 100 U/mL penicillin e 100 µg/mL streptomycin in an incubator at 37°C 5% CO_2/95% air humidified.

Migration and invasion assays

$1x10^5$ SCC-4 cells per well, in triplicate, control and treated with Cetuximab (Erbitux™, Boehringer Ingelheim, Biberach, Germany) were placed in the superior chamber of the plate BD BioCoat™ 24-well plate, 8.0 µm (BD Bioscience, Bedford, MA, USA), for migration assay. Were tested three concentrations of Cetuximab: 1 µg/mL, 50 µg/mL and 100 µg/mL. For invasion assay, $1x10^5$ SCC-4 cells per well, in triplicate, control and treated with Cetuximab 50 µg/mL(the intermediate concentration of the three assays in the cell migration assay) were placed in the superior chamber of the plate BD Matrigel™ Invasion Chamber 24-well plate, 8.0 µm (BD Bioscience, Bedford, MA, USA).

The lower wells of two plates were filled with medium containing 20% FSB and the plates were maintained in an incubator for 24 h. With a cotton swab the cells that did not migrate and did not invade from the upper chamber to lower chamber were removed. Fixation of the cells that migrated through the membrane and invaded the Matrigel™ coated membrane was performed for 30 sec with methanol. Then the cells were stained with Instant Prov as recommended by the manufacturer (Newprov®, Pinhais, PR, Brazil). The cell count was performed on the entire area of the migration plate of the membrane and in the entire area of the membrane covered with Matrigel™ the cell invasion assay using a 20x objective inverted microscope (Axio Vert.A1, Zeiss®).

Evaluation of actin cytoskeleton

$1x10^5$ SCC-4 cells per well, control and cells treated with Cetuximab 50 µg/mL, were cultured for 24 h in six-well plates containing glass coverslips for later morphological analysis.

After 24 h, cells were fixed with 4% paraformaldehyde in phosphate buffer (PB) for 1 h, and incubated with 0.2% Triton X-100 for 5 min at room temperature, then incubated for 20 min with 3% serum albumin bovine. The evidenciation of the actin cytoskeleton was made for 30 min with rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR) 1:100 and the evidenciation of the nuclei was made for 15 min with 4',6-diamidino-2-phenylindole (DAPI - Sigma, St. Louis, MO, USA) 1:500. For assembly of the blades was used Vectashield® (Vector Laboratories, Burlingame, CA, USA). The fluorescence of control cells and cells treated with Cetuximab pictures were taken randomly with a confocal laser scanning microscope LSM 510 Meta (Zeiss®, Goettingen, Germany).

Statistical Analysis

SPSS software 16.0® (Chicago, IL, USA) was used for statistical analysis and GraphPad PRISM® (San Diego, CA, USA) to obtain the graphics. The homogeneity test of Levene variances, t test, ANOVA and Tukey's post-test were performed. Differences were considered significant when $p<0.05$.

Results

Migration and invasion assays

The SCC-4 cells were treated with Cetuximab in three different concentrations: 1 µg/mL, 50 µg/mL and 100 µg/mL. The Cetuximab treatment, in all concentrations studied, inhibited the number of SCC-4 cells that migrated through the membrane [$F(3,11)=131.205$, $p<0.0001$]. The reduction in the number of SCC-4 cells that migrate after treatment with Cetuximab was significant, at three concentrations studied, 1 µg/mL (376.7±47.6 cells), 50 µg/mL (214.7±20.5 cells) and 100 µg/mL (103.0±12.17 cells) when compared with control cells (539.3±22.0 cells). The number of SCC-4 treated cells that migrated at 1 µg/mL was statistically different from 50 µg/mL ($p<0.001$) and 100 µg/mL ($p<0.0001$), between 50 µg/mL and 100 µg/mL ($p<0.01$) demonstrating that the inhibition of cell migration was dose dependent manner (fig.1A).

The number of SCC-4 cells treated with Cetuximab 50 µg/mL (207.7±23.7 cells) was lower than the number of SCC-4 control cells (532.3±61.01 cells) that invaded through Matrigel™, [$t(1)=4.96$, $p<0.01$] (fig.1B) showing the inhibition of cell invasion on SCC-4 cells that received the treatment with Cetuximab.

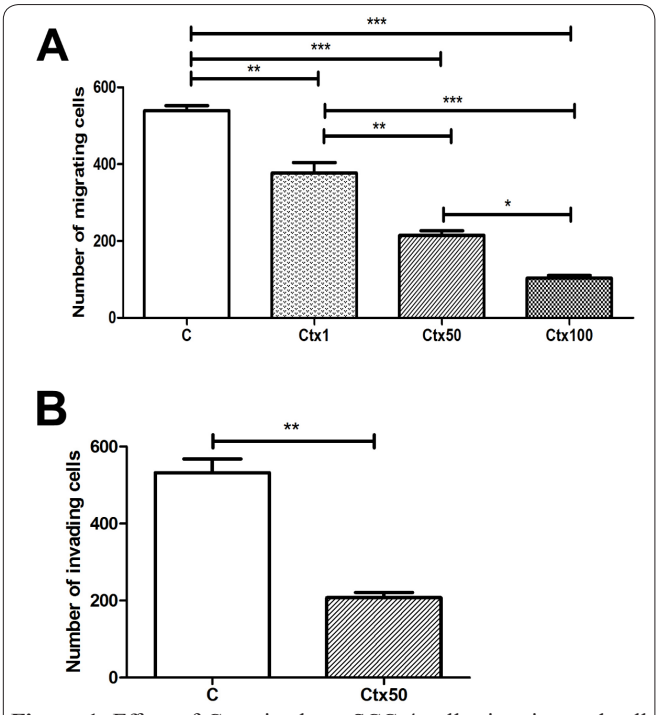

Figure 1. Effect of Cetuximab on SCC-4 cell migration and cell invasion. (A) Effect of Cetuximab on the migration: SCC-4 control cells (C) and SCC-4 cells treated with Cetuximab: 1 µg/mL (Ctx1), 50 µg/mL (Ctx50) and 100 µg/mL (Ctx100). (B) Effect of Cetuximab on the cell invasion: control cells (C) and SCC-4 cells treated with Cetuximab 50 µg/mL (Ctx50). Data were expressed as mean±standard deviation, *$p<0.05$, ** $p<0.001$ and ***$p<0.0001$.

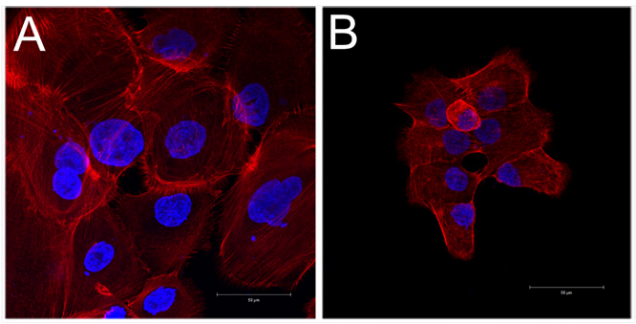

Figure 2. Effect of Cetuximab on actin cytoskeleton organization SCC-4 cells. Confocal analysis of the actin cytoskeleton and nuclei are DAPI stain. (A) control cells and (B) cells treated with Cetuximab 50 µg/mL for 24 h.

Evaluation of actin cytoskeleton

The SCC-4 control cells showed polyhedral morphology, abundant cytoplasm, organized actin cytoskeleton distributed throughout the cytoplasm, clear cell cortex, and spherical nuclei with dense chromatin (fig.2A). After treatment with Cetuximab 50 µg/mL cells maintained polyhedral morphology, but the cytoplasm was saw with reduced size, F-actin was disorganized and less quantity. However, cells showed cell cortex and nuclei preserved (fig.2B).

Discussion

This study demonstrated that Cetuximab has an inhibitory effect on actin cytoskeleton organization, cell migration and invasion, suggesting that inhibition of the EGFR pathway to treatment with Cetuximab can be important for the prevention of metastases in OSCC. Given the progress of studies on tumor behavior some clinical and biological parameters were established in order to indicate the extent, behavior and disease prognosis. The occurrence of metastatic lymph nodes reduces survival by 50% and the larger the number of metastatic lymph nodes, makes the prognosis worse (13).

In this study, SCC-4 cells from moderately differentiated tongue squamous cell carcinoma were used to demonstrate the effect of Cetuximab on the migration, invasion and dynamics of the actin cytoskeleton. The use of cell lines as models of study in research is important to elucidate molecular mechanisms, to identify cancer diagnostic markers and prognostic (14).

Tumor progression is multifactorial and requires markers to make an accurate diagnosis (13). The expression of molecules such as p53 suppressor gene and the EGFR are important because they can help to clarify relationship with environmental factors and lymph node metastasis for example (15). Several other proteins have an important influence on the diagnostic and prognostic value of OSCC and advances in molecular detection of these markers are justified in precancerous changes, so there is early detection of hazards for the development of OSCC (16). The EGFR signaling pathway is important in the process of carcinogenesis, prognosis and progression of squamous cell carcinoma of head and neck, since this receptor is overexpressed in 80 to 90% of cases of this type of tumor (17).

The EGFR family consists of four receptor tyrosine kinase, of which: ErbB1/human, ErbB2/HER2/neu, ErbB3/HER3 and ErbB4/HER4. This receptor contains an extracellular region or N-terminal domain which is the ligand binding region, and have a hydrophobic transmembrane region and an intracellular region or C-terminal domain with tyrosine kinase activity. The most important endogenous ligands of this receptor are the EGF and transforming growth factor α (TGF-α) (18). This receptor is activated by binding EGF or TGF-α in ErbB3 or ErbB4. This binding induces the homodimerization of the receptor or heterozimerization with other receptors, such as with ErbB2. The conformational change of the receptor that causes autophosphorylation occurs C-terminal domain. Therefore, phosphorylation of intracellular cascades are activated to promote signal transduction and cellular responses to generate (19). In human cancer, the signaling of EGFR stimulates some activities such as proliferation, migration, stromal invasion, tumor angiogenesis and resistance signals that induce cell death. Due to the large involvement of EGFR in human cancers, they are the target of therapeutic development. Preclinical studies have shown that monoclonal antibodies inhibit proliferation, has low toxicity to normal tissues and are synergistic to standard therapies (20). In this study, inhibitionof EGFR with Cetuximab resulted in decreased migration and cell invasion. Similar results were found in a study that used SAS and Ca9-22 (21) and with Tca8113 cell lines (22).

The Cetuximab has a highly specific mechanism of action and synergistic activity with current treatments. It is a valuable treatment option in patients with squamous cell carcinoma of the head and neck. The antagonist effect to EGFR occurs without any change in the pattern and severity of side effects of radiation. Thus, an increase in survival of patients in disease free survival in drug response and tumor control rate places it as a drug rather indicated in the treatment of head and neck squamous cell carcinoma (23). Despite not having been tested the effect of Cetuximab on other processes involved in tumorigenesis, such as cell proliferation and gene expression, this study showed that the Cetuximab treatment can be important for the prevention of metastasis in OSCC.

In addition, the present study demonstrated that treatment with Cetuximab affects the morphology of SCC-4 cells. Recently a correspondence was reported on a treatment with Cetuximab in animal model and OSCC cell line, demonstrating that a high sensitivity to monoclonal antibody in both models was linked to a reduction of EGFR and pEGFR expression (24). To occur the cell migration, it is essential the formation of protrusive structures such as filopodia and lamelipodia in the cell surface, due the ability to rearrange the actin cytoskeleton (25). In MKN1 cells from gastric carcinoma, EGF had an important role in cell motility, since it increased undulations in the cell membrane, also the filopodia and lamelipodia formation (26).

In this study, the cellular morphology analysis was performed by using the intermediate concentration of the three assays in the cell migration assay and confocal laser scanning microscope analyis. Two fluorophores were used, DAPI and and rhodamine conjugated to phalloidin, that has affinity for the nucleus and actin filaments, respectively. It is well known thar fluorescence microscopy is an important tool for analyzing cellular

physiology (27).

Future studies may test the Cetuximab treatment with other therapies can lead to an inhibitory effect able to prevent completely the cell migration/invasion, since this study with treatment with Cetuximab in SCC-4 cells, a reduction in the number of migrating cells. In a study using A549 cell of lung cancer, treatment with 1 nM of Cetuximab combined with 25 mM of Afatinib reduced the invasiveness of these cells (28). In a study with cell lines of OSCC the combination of Cetuximab to the non-thermal plasma at atmospheric pressure inhibited migration and invasion of cells resistant to treatment with Cetuximab (29). The HSC3 cell line of OSCC were treated with the combination of Cetuximab with Celecoxib and the proliferation, migration and invasion of this cells were decreased (30).

The clinical efficiency of Cetuximab involves several mechanisms, including inhibition of cell cycle progression, induction of apoptosis, inhibition of angiogenesis, inhibition of metastasis and ability to increase the response to chemotherapy and radiotherapy (31). Although this preliminary study was conducted with only one cell line, this study demonstrated that the Cetuximab is able to affect the organization of the actin cytoskeleton, suggesting that its action can occur in the early stages of tumorigenesis. And also inhibits cell motility, migration and invasion, suggesting that the blocking of EGFR pathway with Cetuximab treatment can lead to avoid metastasis. Futures studies should be performed *in vivo* to verify his effectiveness anti-metastaic drug to OSCC.

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Conflict of interests

The authors declare that they have no conflict of interests.

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