Original Article

Treatment effects of the EGFR pathway drugs on head and neck cancer stem cells

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Abstract: (1) Head and neck cancer (HNC) is the sixth most common cancer worldwide and show low survival rates and drug resistance, which can be due to the presence of cancer stem cells (CSCs), a small cell population with metastatic potential, invasion and self-renewal ability. (2) Here, seven tumor cells were sorted as CD44+/CD117+/CD133+ or ALDH+, considered as HNC stem cells (HNCSCs), and as CD44-/CD117-/CD133- or ALDH-, considered non-HNCSCs after both cells sorted criteria was compared to evaluate cell migration, invasion, and colony forming assays. These subpopulations were treated with Cetuximab, Paclitaxel, or a combination of both drugs and evaluated for cell viability. Quantitative PCR and western blot were performed to evaluate EGFR, TRKB, KRAS and HIF-1α gene and protein expression. (3) HNCSCs presented more colonies and appeared to be more sensitive to the drug combination when compared with non-HNCSCs, regardless cells sorted criteria and primary tumor subsite. The EGFR, TRKB, KRAS and HIF-1α genes and proteins were upregulated in CSCs compared with non-HNCSCs, thus explaining the drug resistance. (4) This study contributes to the better development of specific therapeutic protocols based on Cetuximab and Paclitaxel drugs in the treatment of HNC in the presence of CSCs and cell proliferation biomarkers.

Keywords: Head and neck cancer, cancer stem cells, epidermal growth factor receptor, Kirsten sarcoma rat, cetuximab, paclitaxel

Introduction

Head and Neck Cancer (HNC) comprises a wide range of tumors in the lips and oral cavity (2.0%), hypopharynx (0.4%), oropharynx (0.5%), nasopharynx (0.7%), and larynx (1.0%), and is the sixth most common cancer worldwide with more than 800,000 cases [1]. The risk factors associated with HNC include smoking, alcohol consumption, human papillomavirus, and Epstein-Barr virus infections [2, 3]. HNC patients at all stages of the disease have a low five-year survival rate, and the prognosis for patients with recurrent or metastatic disease is poor [1, 2].

The standard treatment for HNC depends on the site of the primary tumor and the stage of the disease. HNC in an early stage (I/II) is usually treated with surgery or radiotherapy, while locally advanced disease (stage III/IV) requires the combination of radiotherapy, surgery, and chemotherapy with the anti-mitotic agent Paclitaxel [4]. One strategy aimed at improving the efficacy of the treatment is to add molecular target agents, such as Cetuximab, to standard chemotherapy [5]. Cetuximab is a chimeric monoclonal antibody against the epidermal growth factor receptor (EGFR) that can be safely combined with Paclitaxel in HNC treatment [6-12]. Despite the advances in drug therapy, HNC patients still present a low survival rate and high metastatic rates [13]. One hypothesis that could explain the low survival is the presence of a small group of cells named cancer stem cells (CSCs) that are present in many solid
EGFR pathway in HNCSCs

tumors, including HNC. CSCs have metastatic potential, high capacity of invasion [14-16], and the abilities of self-renewal and differentiation, as well as having a substantial function in the initiation and progression of the tumor [17-19]. These features, which may provide tumor resistance leading to treatment ineffectiveness [20], are all associated with poor prognosis [14, 15]. CSCs have been identified to express the biomarkers CD44, CD117, CD133, and ALDH, which have also been found to be overexpressed in tumors with CSCs [17, 21, 22].

CSCs are thought to arise from progenitor cells or normal stem cells showing aberrant behavior of key regulatory genes, specifically, proto-oncogenes and tumor suppressors [19]. Important proto-oncogenes that play a key role in HNC tumorigenesis are EGFR and Akt [23]. EGFR is a member of the ErbB family of receptor tyrosine kinases which is activated by epidermal growth factor (EGF) and has several known growth factor ligands that activate many downstream effectors involved in the Ras Sarcoma/raf1/mitogen-activated protein kinase pathway (RAS/raf1/MAPK pathway) [14-16, 23]. This activation leads to the expression of other proteins responsible for coordination of cell growth, promotion of tumor initiation, and disease progression [23]. EGFR is highly expressed in many cancers of epithelial origin, including head and neck squamous cell carcinoma (HNSCC), and is correlated with an increased risk of local relapse, adverse overall survival, and poor clinical outcome [2, 3, 23]. Another gene that has high expression in laryngeal cancer is the tropomyosin-related kinase B (TRKB) receptor, which can be activated by the brain-derived neurotrophic factor (BDNF) and neurotrophin 4 (NT4) [24, 25]. Cancer cells with TRKB overexpression behave as cancer stem cells [26] which shows that CSCs and high expressed TRKB may be related. The evidence has shown that BDNF/TRKB activation plays an important role in resistance to anti-EGFR treatments, such as cetuximab [24, 27].

The high expression of these tyrosine kinases has been associated with the mutation in the Kirsten rat sarcoma virus (KRAS) gene in colorectal cancer [28]. RAS is a family of proto-oncogenes encoding proteins that are members of the small GTPases superfamily, which has essential roles in several signaling pathways controlling cell growth. KRAS is the most important gene of the family [29] because mutations in this proto-oncogene are related to independent activation of pathways associated with growth and cell survival and contribute to tumor maintenance [29-31]. Another key gene that is highly expressed in a hypoxia situation is the Hypoxia-inducible factor 1-alpha (HIF-1α). This gene activated, can initialized several transcription factors that lead to the activation of the EGFR, TRKB genes and, consequently, the mitogen activated protein kinase Mcl-1 (MPAK) pathway, which has the KRAS gene as an important gene in this pathway [32-34].

Thus, the aims of this study were: to identify and characterize two HNC cell subpopulations, namely, Head and Neck Cancer Stem Cells (HNCSCs) and Head and Neck Cancer non-Stem Cells (non-HNCSCs) in seven primary tumors of HNC patients; to compare the effectiveness of the CD44/CD117/CD133 or ALDH cell sorted criteria; the characteristics differences between oral cavity, pharynx or larynx tumor subsites; the effectiveness of Cetuximab and Paclitaxel treatment; and to evaluate EGFR, TRKB, KRAS and HIF-1α expression in both subpopulations.

Materials and methods

Sample

HNC tissues were collected from patients who underwent surgical resection at the Service of Otorhinolaryngology and Head and Neck Surgery of the Medical School of São José do Rio Preto-FAMERP. All patients signed consent letters and the study was approved by the Institutional Research Ethics Committee of the Medical School São José do Rio Preto-FAMERP, SP, Brazil (903.775). Exclusion criteria were patients that have been initiate chemo or radiotherapy treatment. Table 1 presents the clinical features and surgical staging from the patients’ seven primary tumors included in the study. Data were retrospectively obtained from medical records.

All samples were cultured in Dulbecco’s Modified Eagle Medium, (DMEM, Sigma-Aldrich Co.) supplemented with 20% Ham’s Nutrient Mixture F12 (HAMF12, Sigma-Aldrich Co.), 10% fetal bovine serum (FBS, Gibco™), 1% L-glutamine (Gibco™), 1% of penicillin, streptomycin,
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Identification and separation of CSCs were performed using the Cell Sorting BD FACS Aria Fusion flow cytometer (BD Biosciences) and specific antibodies for labeling, following manufacturers’ recommendations. Cells that were positively marked with the three antibodies together CD44-phycoerythrin (PE) (BD Biosciences), CD117-fluorescein isothiocyanate (FITC) (BD Biosciences), and CD133-allophycocyanin (APC) (Miltenyi Biotec) or only for ALDH-aldehyde dehydrogenase-bright (ALDEFLUOR™ - STEMCELL Technologies) were classified as HNCSCs (CD44+/CD117+/CD133+, or only ALDH+). Cells that were negative for labeling with the three antibodies together CD44/PE, CD133/APC, and CD117/FITC, or ALDH/FITC alone were considered non-HNCSCs (CD44-/CD117-/CD133-, or only ALDH-).

Migration and invasion assay

For the migration analysis, confluent cells grown in 2 ml of culture medium supplemented with 10% FBS within the well of a 6-well plate were “wounded” by scraping off an area using a plastic pipette tip. After the procedure, plates were incubated at 37°C for 24 h in a CO₂ incubator. Images were obtained with an inverted microscope at the beginning of the experiment and after 24 h. Six fields per well were photographed in triplicate at 40× magnification. Subsequently, the quantitative analysis was performed by measuring the invaded area at the beginning of the experiment and after 24 h using the ImageJ application. The percentage of the invaded area was calculated for each well and results subjected to statistical analysis.

The transwell invasion assay was carried out performed in duplicates in a Corning® BioCoat™ Matrigel® Invasion Chamber (Discovery Labware, Inc ©Corning Inc.). A total of 2×10⁴ cells were placed in a serum-free medium in the upper chamber, while medium containing 10% FBS was added as a chemoattractant to the lower chamber. Invading cells were fixed with 4% paraformaldehyde for 2 min followed by methanol for 20 min and stained with 0.5% crystal violet. Four fields were randomly selected and photographed under a light microscope (Olympus Microscope BX53, Olympus Life Science) at 100× magnification. The cells that invaded the inserts were counted and results statistically analyzed.

Colony forming assay

Cells were placed seeded into 6-well ultra-low plates (Ultra-Low Attachment Multiple Well Plate, Corning® Costar®) at a density of 2×10⁴ cells/well in 2 ml culture medium supplemented with 10% of FBS and incubated for 5 days. The colonies formed were counted and photo documented in an inverted microscope at 40× magnification. The procedure was performed in triplicates.

Cell viability assay

Cell viability was determined using the MTS cell proliferation kit (CellTiter 96® AQueous One Solution Cell Proliferation Assay) after treating the cells with 0.06 mg/ml of Cetuximab, 0.05 mg/ml of Paclitaxel, or Cetuximab combined with Paclitaxel (CP), and untreated cells as a control.

### Table 1. Clinical features and surgical staging characterization of the group of patients with head and neck cancer

<table>
<thead>
<tr>
<th>Sample</th>
<th>Age</th>
<th>Gender</th>
<th>Smoking Habit</th>
<th>Exposure/Time</th>
<th>Alcoholic Habit</th>
<th>Exposure/Time</th>
<th>Primary Site</th>
<th>Staging (TNM*)</th>
<th>Labeling</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNC-1</td>
<td>72</td>
<td>Male</td>
<td>Yes</td>
<td>≥1 pack/≥15 years</td>
<td>Yes</td>
<td>≥400 ml/≥35 anos</td>
<td>Oral Cavity</td>
<td>T2N0M0</td>
<td>CD44+/CD117+/CD133</td>
</tr>
<tr>
<td>HNC-2</td>
<td>57</td>
<td>Male</td>
<td>Yes</td>
<td>≥1 pack/≥15 years</td>
<td>Yes</td>
<td>≥400 ml/≥35 anos</td>
<td>Pharynx</td>
<td>T1N0M0</td>
<td>CD44+/CD117+/CD133</td>
</tr>
<tr>
<td>HNC-3</td>
<td>44</td>
<td>Male</td>
<td>Yes</td>
<td>≥1 pack/≥15 years</td>
<td>Yes</td>
<td>≥400 ml/≥35 anos</td>
<td>Oral Cavity</td>
<td>T4N0M0</td>
<td>CD44+/CD117+/CD133</td>
</tr>
<tr>
<td>HNC-4</td>
<td>68</td>
<td>Male</td>
<td>Yes</td>
<td>≥1 pack/≥15 years</td>
<td>No</td>
<td>Never</td>
<td>Larynx</td>
<td>T1N0M0</td>
<td>CD44+/CD117+/CD133</td>
</tr>
<tr>
<td>HNC-5</td>
<td>48</td>
<td>Female</td>
<td>No</td>
<td>Never</td>
<td>No</td>
<td>Never</td>
<td>Oral Cavity</td>
<td>T2N0M0</td>
<td>CD44+/CD117+/CD133</td>
</tr>
<tr>
<td>HNC-6</td>
<td>71</td>
<td>Male</td>
<td>Yes</td>
<td>≥1 pack/≥15 years</td>
<td>No</td>
<td>Never</td>
<td>Larynx</td>
<td>T3N0M0</td>
<td>ALDH+</td>
</tr>
<tr>
<td>HNC-7</td>
<td>64</td>
<td>Male</td>
<td>No</td>
<td>Never</td>
<td>No</td>
<td>Never</td>
<td>Larynx</td>
<td>T3N1M0</td>
<td>ALDH+</td>
</tr>
</tbody>
</table>

TNM* = T: size tumor; N: lymph nodes affected; M: presence of metastasis.
control. The therapeutic agent concentrations were chosen based on clinical treatments. Thus, 5×10^3 cells were resuspended in 100 μl of DMEM with no supplementation and were placed into 96-well plates. Twenty microliters of MTS were added after 24 h and absorbance measured with an ELISA plate reader (Multiskan FC, Thermo Scientific - Uniscience) at 490 nm filter.

**Gene expression**

Total RNA was extracted from 1×10^6 cells using TRIzol reagent (Invitrogen, ThermoFisher Scientific) according to the manufacturer’s instructions. RNA quantification was performed with the Qubit™ RNA HS Assay Kit in a Qubit® 2.0 Fluorometer (Invitrogen, ThermoFisher Scientific). Complementary DNA (cDNA) for all samples was synthesized in a 20 μl reaction tube containing 2-5 μg of total RNA, using the High-Capacity cDNA Reverse Transcription Kit (Invitrogen, ThermoFisher Scientific). The RNA concentration was 1 μg/μL. Quantification of the EGFR (HS01076090_m1), TRKB (HS00364284_g1) and HIF-1α (HS00153153_m1) gene expressions was carried out in triplicates using the TaqMan Universal Master Mix and probes (Invitrogen, ThermoFisher Scientific). Two endogenous controls, Beta Actin (MUC1) and GAPDH (FAM dye and MGB probe) were included. The relative expression of EGFR and KRAS was calculated through the 2^(-ΔΔCt) method.

**Protein expression**

Proteins were extracted using Trizol® (Invitrogen, ThermoFisher Scientific) and the concentration was estimated using PierceTM BCA Protein Assay Kit (Thermo Fisher Scientific), according to the manufacturer’s instructions. The expression levels of EGFR, TRKB, KRAS and HIF-1α and β-actin were also measured by western blot analysis.

Western blotting: a pool of proteins from the samples at 50 μg concentration were loaded on 12% SDS-PAGE gels and subsequent electrophoretic transfer was performed on iBlotR Gel Transfer Stacks PVDF, Regular (Invitrogen by Thermo Fisher Scientific). Blocking was done for 1 h in 3% BSA in 0.5% Tris buffered saline (TBS)-T. The EGFR-mouse monoclonal antibody at a 1:500 dilution (sc-373746 by Santa Cruz Biotechnology, Inc.), TRKB-mouse monoclonal antibody at a 1:1000 (8D2E8 by Novus Biological a Biotechne Brand), KRAS-mouse monoclonal antibody at a 5:1000 (H0000-3845-M5 by Abnova) and HIF-1α-rabbit monoclonal antibody at a 1:1000 (MA1-16504 by Invitrogen, ThermoFisher Scientific) was in 3% BSA in 0.5% TBS-T or PBS and incubated at 4°C overnight. Then, HRP-conjugated secondary antibodies in 3% BSA in 0.5% TBS-T were incubated at room temperature for 1 h. Enhanced chemiluminescence reagent (Invitrogen by Thermo Fisher Scientific) was used to detect immuno-reactive secondary antibodies still bound to the membrane.

These data were quantified to evaluate band intensity of mean grey values by densitometric analysis using ImageJ v4.0 software, and the relative expression levels of the samples and controls were normalized by the internal standard β-actin [35, 36].

**Statistical analysis**

The D’Agostino & Pearson test was used to assess normality. One sample t test or Wilcoxon Signed Rank Test was performed to gene expression analysis and two-way ANOVA Turkey post hoc tests were used to calculate the significance of the CSCs proprieties assays analysis, between both groups for treatments, cell sorted criteria and tumor subtypes. All data were evaluated with the GraphPad Prism version 8 software (GraphPad Software Inc., San Diego, CA, USA). A significance level of 5% was used.

**Results**

**HNCSC subpopulation has cancer stem cell properties**

The primary tumors cells were identified and sorted with CD44, CD133, and CD117, or ALDH biomarkers (Figure 1A). The CD44+/CD117+/CD133 sorted present more CSCs less non-CSCs subpopulation than ALDH sorted (Figure 1B). The oral cavity subsite presented more CSCs subpopulation than pharynx and larynx. Pharynx presented less non-CSCs subpopulation than oral cavity and larynx (Figure 1C).

Our results showed that the CD44+/CD117+/CD133+, or ALDH+ HNC cell population, desig-
Figure 1. Graphs comparing the HNCSC and non-HNCSC subpopulations for all seven samples. (A) The number on the vertical axis means the mean of the percentage of the cells that was sorted; (B) comparison of the percentage of the cells sorted related to CD44/CD117/CD133 and ALDH cell sorting criteria; (C) comparison of the percentage of the cells sorted between oral cavity, pharynx and larynx primary subsites; (D) the number on the vertical axis is the mean of the number of the colonies formed compared of the seven primary tumors; (E) colony forming after 120 h in 40× magnification; (F) comparison of the colonies formed between oral cavity, pharynx and larynx primary subsites; and (G) number of the colonies formed compared between CD44/CD117/CD133 and ALDH cell sorting criteria. *P<0.01, **P<0.01, ***P<0.001 vs. to non-HNCSCs group; a = P<0.001 vs. to Pharynx in HNCSCs; b = P<0.001 vs. Larynx in HNCSCs; c = P<0.001 vs. Oral cavity in HNCSCs; d = P<0.001 vs. to Pharynx in non-HNCSCs; e = vs. Oral cavity in non-HNCSCs; f = P<0.001 vs. ALDH sorting in HNCSCs; g = P<0.001 vs. ALDH in non-HNCSCs; h = P<0.001 vs. to CD44/CD117/CD133 in HNCSCs group by two-way ANOVA statistical analysis data.
nated as HNCSCs, had a higher potential for migration, invasion, and colony formation when compared with the CD44-/CD117-/CD133-, or ALDH- population, the so-called non-HNCSCs (Figures 1, 2). The colony formation assay of the primary tumors showed more tumoral spheres in HNCSCs than in non-HNCSC subpopulations (P = 0.0013) after 120 hours, as depicted in Figure 1D, 1E. When compared colony forming between the CD44/CD117/CD133, or ALDH cell sorted criteria was showed a greater number of colonies formed in ALDH cell sorted criteria (Figure 1F) showing that the ALDH1 marker seems to be better to identify and separate CSCs than the CD44, CD133, and CD117 markers. However, a limitation of the study is that we have only one separate sample with ALDH1 being therefore necessary studies to confirm. The number of the colony forming between oral cavity, pharynx, and larynx subsites no presented significance statistical (Figure 1G).

The cell migration and invasion capacity of HNCSC and non-HNCSC subpopulations of the primary tumor were evaluated in vitro. After 24 h, HNCSCs demonstrated an increased migration and invasive potential compared with non-HNCSC subpopulations as shown in Figure 2A, 2B, 2E and 2F (P<0.0001 and P = 0.0324, respectively). To compare the difference between the cell sorting criteria were analyzed the potential of migration and invasion in this condition. The CD44/CD117/CD133 cell sorted criteria presented less cell migration ability than ALDH cell sorted criteria (Figure 2C) and no difference significant was observed to invasion potential (Figure 2D). The oral cavity subsite of the HNCSCs presented more migration ability than pharynx or larynx subsites compared to non-HNCSCs (Figure 2D) and no difference significant was observed to invasion potential between the tumor subsites of the HNC subpopulations (Figure 2H).

Thus, by migration, invasion, and colony formation assays we have confirmed that the subpopulation of HNCSCs had higher tumorigenic potential and formed spheres, a unique characteristic of non-HNCSCs, independent of the cell sorted criteria or tumor subsite.

HNCSCs are treatment resistant

Both HNCSCs and non-HNCSC subpopulations of primary tumors were treated with Cetuximab, Paclitaxel, and a combination of both drugs (CP). The viability of the two populations did not show statistical differences (P>0.05) in both CD44/CD117/CD133 and ALDH cell sorted criteria (Figure 3A-C). However, descriptive analysis suggests that HNCSCs seemed to be more sensitive to the treatment with CP. Moreover, when comparing the treatments in each subpopulation, only Cetuximab was not effective in both subpopulations, although the drug could potentiate the effects of Paclitaxel chemotherapy (Figure 3A, 3B). The literature shows that, depending on the primary site of head and neck cancer there are different responses to treatment. Then, we compared of responses to Cetuximab, Paclitaxel, Cetuximab plus Paclitaxel combination treatments and controls regarding to untreated cells HNCSCs and Non-HNCSCs by oral cavity, larynx and pharynx primary site and did not show statistical differences (P>0.05) was showed (Figure 3D). The Figure S1 shows the difference between subpopulations in each treatment for each primary site sample studied.

HNCSC subpopulations presented overexpression of EGFR, TRKB, KRAS and HIF-1α genes

EGFR, TRKB, KRAS and HIF-1α genes presented were up-regulated (mean RQ = 6.238, 6.408, 1.802 and 4.060, respectively) in HNCSCs compared with non-HNCSCs, with no significant differences between the two subpopulations (P = 0.250, P = 0.054, P = 0.253 and P = 0.153, respectively). The differential quantitative gene expression and statistical analysis are shown in Figure 4A. Because the cell signaling can be different between the CD44/CD117/CD133 and ALDH cell sorting criteria to obtain CSC and non-CSCs, we decided to compare EGFR, TRKB, KRAS and HIF-1α genes expression in these conditions. TRKB and HIF-1α genes was more expressed in the oral cavity subsite of the HNCSCs than pharynx or larynx subsites compared to non-HNCSCs (Figure 2D) and no difference significant was observed to invasion potential between the tumor subsites of the HNC subpopulations (Figure 2H).

Interestingly, we found that the TRKB gene was more expressed than the KRAS gene in the ALDH cell sorted criteria (Figure 4B). The literature shows that, depending on the primary site of head and neck cancer there are different gene expression. Then, we analyzed the EGFR, TRKB, KRAS and HIF-1α genes expression in CSCs and non-CSCs by oral cavity, larynx and pharynx primary subsites and no significant differences was observed (Figure 4C).
Figure 2. Graphs comparing the HNCSC and non-HNCSC subpopulations for all seven samples. (A) The number on the vertical axis means the average percentage of the migrated area; (B) images representatives of the cell migration in 0 h and after 24 h times in 40× magnification; (C) comparison of the percentage of the cells migrated related to CD44/CD117/CD133 and ALDH cell sorting criteria; (D) comparison of the percentage of the cells migrated between oral cavity, pharynx and larynx primary subsites; (E) the number on the vertical axis means the mean of the number of the cells that went through the camera with matrigel (invasion assay); (F) images representatives of the cell invasion after 24 h in 200× magnification; and (G) comparison of the percentage of the cells invaded related to CD44/CD117/CD133 and ALDH cell sorting criteria; (H) comparison of the percentage of the cells invaded between oral cavity, pharynx and larynx primary subsites. *P<0.01 vs. to non-HNCSCs group by two-way ANOVA statistical analysis data.
Figure 3. Cell viability of HNCSCs and Non-HNCSCs subpopulations of primary tumors treated after 24 hours and control group (non-treatment cells). Comparison of responses to Cetuximab, Paclitaxel, Cetuximab plus Paclitaxel combination and controls regarding to untreated cells in (A) and (B) HNCSCs and Non-HNCSCs subpopulations of primary tumors; (C) HNCSCs and Non-HNCSCs by CD44/CD117/CD133 and ALDH cell sorting; and (D) HNCSCs and Non-HNCSCs by oral cavity, larynx, and pharynx primary site. ***P<0.001 compared to respective control group; a P<0.001 compared to respective cetuximab treatment by two-way ANOVA statistical analysis.

**HNCSC subpopulations presented high expression of EGFR, TRKB, KRAS and HIF-1α**

From Western blot assay, EGFR, TRKB, KRAS and HIF-1α protein expression was up-regulated in HNCSCs compared with non-HNCSCs (ratio of band = 3.733, 4.331, 3.094 and 1.333, respectively) (Figure 4D, 4E).

**Discussion**

We found that HNC cells with of CD44, CD133, CD117, or ALDH biomarkers showed high migration potential and invasion, and formed more and larger colonies than non-HNCSCs demonstrating the growth tumorigenic potential of the HNCSC subpopulation. The results showed that these biomarkers were effective in sorting the CSC subpopulation from the non-CSCs one. Furthermore, the migration potential, invasion, and formation of larger colonies are related to the higher aggressiveness of the HNCSCs when compared with the non-HNCSCs. In HNC cell lines was showed a great migration capacity in CSCs higher than that of the non-HNCSC subpopulation lines [37].

The literature reports the use of CD44, CD133, CD117, and ALDH genes to separate and characterize tumor stem cells. However, there is no consensus on which separation is efficient and anyone works compared this possibility. The present study demonstrated that separations with the combined CD44, CD133 and CD117 labels or with ALDH are efficient in obtaining tumor stem cells with high tumorigenic power and resistance to the studied treatments. However, our results also seem to suggest that the ALDH biomarker is more effective for obtaining CSCs. This is because ALDH is a biomarker known to be present in Tumoral Stem and Tumor cells for its involvement in differentiation, self-Renewal and self-Protection [38, 39]. For although we found few cells with the ALDH phenotype during cell sorting, when the ball formation was evaluated was higher. In this present study, first time it was compare the cell sorting criteria based on two different biomarkers in head and neck cancer. The results suggest that both CD44/CD133/CD117 and ALDH cell sorted criteria were effectiveness to obtain cancer stem cells in head and neck primary tumors.
CD133 (prominin-1) is a transmembrane glycoprotein [42] while ALDH is an intracellular cytosolic isoenzyme that converts acetaldehyde into acetate. High activity of the biomarkers has been considered as a reliable marker for CSCs. These biomarkers participate in embryogenesis, hematopoietic stem, and progenitor cells as well as carcinogenesis, and their expression is correlated with tumor progression, differentiation suppression, resistance to radio and chemotherapy, self-renewal, relapse, and metastasis [43-45].

The literature has been showed that the subsite of the head and neck cancers can be presented differences in the tumor aggressiveness, drug resistance and gene expression. We have found in higher percentage of the CSCs sorted in oral cavity than pharynx and larynx and in pharynx than larynx as well as the pharynx have less percentage of non-CSCs than oral cavity or larynx. Oral cavity presented high potential of the migration area than non-CSCs although no difference between others subsite was showed. However, there was no statistical difference between oral cavity, pharynx, and larynx in HNCSCs and non-HNCSC was found related colony formed or invasion potential. Then, we suggest that CSCs was presented similar in the primary tumor subsites. The literature has not showed these data about primary tumors. Nevertheless, in a previous study from our research group with HN13 (oral cavity) and HEP2 (Larynx) cell lines, the CSCs subpopulation demonstrated a migration capacity 81% higher than that of the non-HNCSC subpopulation lines [37].
We have found no statistical difference between HNCSCs and non-HNCSC subpopulations in primary tumors treated with Cetuximab, Paclitaxel, or CP, regardless of the type of cell sorting criteria or the primary subsite (oral cavity, larynx, and pharynx). Moreover, CP resulted in the most effective treatment for the HNCSC subpopulation. Cetuximab seems to potentiate the effects of Paclitaxel in the HNCSC subpopulation, however more studies are needed to validate these findings. These results are similar to our previous study with CSCs in HEP2 cell line [46]. Paclitaxel chemotherapy inhibits the fibers of the mitotic spindle and consequently interrupts cell proliferation [46]. Cetuximab binds to EGFR and inhibits the cascade of cell proliferation slowing down the disease progression and increasing the survival rate of cancer patients. KRAS is a gene encoding an intracellular signaling protein indirectly activated by EGFR, resulting in an exacerbated cellular proliferation. However, if there are changes in this cascade, such as the high expression of the KRAS gene, the signaling may not depend on the EGFR receptor activation and therefore, there is no benefit in administering Cetuximab [29, 47, 48].

Literature reports are inconclusive regarding HNCSCs treatment with Cetuximab. Studies showed that cells with CSCs features are more sensitive to Cetuximab in hypoxic conditions [16] or when they depict the CD44_{hpi}/EGFR_{low} phenotype in flow cytometry [49]. However, other contributions demonstrated that cells with CSCs features and CD44 overexpression were resistant to the Cetuximab treatment [50]. Furthermore, CSC sorted using ALDH and CD44 as biomarkers were resistant to Cetuximab and Docetaxel (similar to Paclitaxel chemotherapy) [51]. The CSC subpopulation sorted using Side Population through Hoechst exclusion, CD44^{high}, and ALDH^{high} did not show reduced proliferation when treated with Cetuximab [52].

Studies on the combination of Cetuximab with other chemotherapeutic agents such as Paclitaxel, Docetaxel, Cisplatin, and/or 5-Fluorouracil are still being performed to select the best treatment approaches [6, 7, 52-54]. Head and neck squamous cell carcinoma patients with recurrence or metastasis after platinum-based chemoradiotherapy were treated with CP and presented tolerance and a positive response to the treatment [6, 7]. Another randomized study in HNC patients found that the combination of Cetuximab with Paclitaxel and Cisplatin; or Cetuximab with Docetaxel, Cisplatin and 5-Fluorouracil increased the progression-free survival by 20% in two years compared with that of the control [55].

In the present study, we showed the high expression of EGFR, TRKB, KRAS and HIF-1α genes confirmed by high protein expression, related to more rates of the tumor proliferation, progression migration and invasion, in HNCSCs compared with that in non-HNCSCs. These results were independent of the type of cell separation criteria as well as the primary subsite (oral cavity, larynx, and pharynx). This is, to our knowledge, the first research evaluating the influence the TRKB and KRAS genes and proteins in subpopulations of stem and non-stem tumor cells in head and neck primary tumor. Both genes are related with the crosstalk or cell signaling with the CSCs marker used in this study as well as with EGFR and HIF-1α pathways.

Literature showed that the EGFR gene can activate KRAS, one of the genes responsible for cell growth and tumor recurrence. The EGFR gene expression is controversial. Some studies showed EGFR [16, 56] and p-EGFR down-regulation [50, 56]; however, others demonstrated EGFR [23, 57] and p-EGFR overexpression. In a previous work we reported the down-regulation of the CD44 gene and the up-expression of the EGFR gene in laryngeal CSCs cell line; and the up-regulation of the CD44 gene and the down-expression of the EGFR gene in an oral CSCs cell line [37]. The KRAS gene expression was not evaluated in HNCSCs primary tumors. Thus, we decided to evaluate the expression of EGFR and KRAS genes in primary tumors and its real representativeness. Our results reinforce the relation between the KRAS pathway activated by EGFR phosphorylation and a significant role in cell proliferation, tumor progression, and resistance to chemotherapy in HNC.

Moreover, have been demonstrated in vivo that the TrkB activation has been associated with cell invasion, migration, epithelial-mesenchymal transition, drug resistance, and poor prognosis [54, 58-61] and that TrkB inhibition can suppress cell proliferation, tumor growth, migration and drug sensitization [35, 37, 38, 49, 54]. Some studies have shown a crosstalk
EGFR pathway in HNCSCs

These genes and proteins were high expression and related to CSCs features, such as more migration, invasion, colony forming, chemotherapy resistance, and angiogenesis, which lead to metastasis and poor prognosis in this sample. However, the limitation of this study is that we not evaluated mutations in the KRAS gene. Different changes in the KRAS gene can activate signaling pathways with different impacts. It was shown that the ASP13 and CYS12 mutation in the KRAS gene leads to increased expression of the HIF-1α, supporting the role of HIF-1α in tumor metabolism [64-66].

We suggest that the up-regulation of these genes in cancer stem cells may be associated with HIF-1A-dependent KRAS downstream signaling by different types of EGFR mutations and crosstalk with TRKB in head and neck cancer. It is known that gene expression is extremely variable among the tumor subsets of HNC. Limitations of our study were the small sample size and the difficulties maintaining the tumor stem cells in primary tumors due to their extreme fragility. A graphical summary of our results is shown in Figure 5. Therefore, more studies are needed on other tumor sites.

Conclusions

We identify head and neck cancer stem cells and to the first time that is compared CD44/CD117/CD133 or ALDH cell sorted criteria. Here we show that HNCSCs form more colonies, an exclusive characteristic of CSCs in the seven tumors than non-HNCSCs, and presented more aggressivity cellular features, regardless HNC primary tumor subsites (oral cavity, larynx and pharynx). The combination of the Cetuximab with Paclitaxel seems to be more
EGFR pathway in HNCSCs

beneficial in the elimination of both cellular subpopulations of HNC cells, equally in all tumor subsites. EGFR, TRKB, KRAS and HIF-1α genes and proteins are overexpress in HNCSCs mainly TRKB in separate CSCs with ALDH although not related to the tumor subsite. Therefore, this study contributes to the better development of specific therapeutic protocols based on Cetuximab and Paclitaxel in the treatment of head and neck cancer in the presence of CSCs and cell proliferation biomarkers. The combined presence of these biomarkers of CSCs and cell proliferation genes in any tumor subsite of HNC may indicate that the combined CP treatment is more suitable for the patient than the single treatment. Furthermore, the relation between the combination of the Cetuximab with Paclitaxel and the high expression of the genes may contribute to elucidate tumor resistance and progression processes. However, more studies are necessary to understand the role of these genes in the chemoresistance of CSCs.

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Disclosure of conflict of interest

None.

Abbreviations

BDNF, Brain-derived neurotrophic factor; CSCs, Cancer stem cells; DMEM, Dulbecco’s modified Eagle medium; EGF, Epidermal growth factor; EGFR, Epidermal growth factor receptor; EMT, Epithelial-mesenchymal transition; FBS, Fetal bovine serum; HRAS, Harvey rat sarcoma; KRAS, Kirsten rat sarcoma; HNC, Head and Neck cancer; HNCSCs, Head Neck Cancer Stem Cells; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; non-CSCs, Cancer non-stem cells; non-CSCs, cancer non-stem cells; non-HNCSCs, Head Neck Cancer non-Stem Cells; NRAS, Neuroblastoma rat sarcoma; RAS, Rat sarcoma; SCF, Stem cell factor; TrkB, Tropomyosin-related kinase B; HIF-1α, Hypoxia-Inducible Factor 1 alpha; VEGF-A, Vascular endothelial growth factor.

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EGFR pathway in HNCSFs


EGFR pathway in HNCSCs


Figure S1. Cell viability of HNCSCs and Non-HNCSCs subpopulations of primary tumors separated by tumor site, treated with (A) Cetuximab, (B) Paclitaxel and (C) Cetuximab plus Paclitaxel combination, after 24 hours.