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Original Article

Anti-EGFR treatment effects on laryngeal cancer stem cells

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Abstract: Laryngeal cancer (LC) is one of the common head and neck neoplasms and is characterized by resistance to conventional therapy and poor prognosis. This may result from the presence of cancer stem cells (CSCs), which form a small population in tumors with metastatic potential, high invasive capacity, self-renewal, and differentiation. This study aimed to evaluate the effectiveness of 5-fluorouracil and cisplatin individually, as well as the combination of cetuximab and paclitaxel in a CSC subpopulation separated with biomarkers related to tumoral growth (CD44, CD117, and CD133). In addition, expression of *TrkB, KRAS, HIF-1α*, and *VEGF*-A genes and proteins related to cell proliferation were evaluated in this subpopulation. The CD44, CD133, and CD117 biomarkers were used to analyze the identification and separation of both subpopulations using FACSAria Fusion. Subpopulations positive for CD44, CD133, and CD117 or lacking these biomarkers were classified as laryngeal cancer stem cells (LCSCs) or laryngeal cancer non-stem cells (non-LCSCs), respectively. Matrigel invasion and colony forming assays were performed to confirm CSC presence. Subpopulations were cultured and exposed to 5-fluorouracil, cisplatin, and cetuximab/paclitaxel drugs for 24 h. Cell proliferation was determined using MTS assay. *KRAS* and *TrkB* gene expression levels were evaluated using quantitative real time PCR with TaqMan® Assay in both subpopulations. The non-LCSC subpopulation was considered as the control for relative expression. We found that the LCSC subpopulation demonstrated more resistance to cetuximab and paclitaxel combination chemotherapy when compared with the non-LCSC subpopulation of the cell line. These LCSC subpopulations presented up-regulated expression of *KRAS, HIF-1α*, and *VEGF-A* genes and proteins and no *TrkB* gene expression, but TrkB protein expression was up-regulated in the LC cell line when compared to the non-CSC subpopulation. "In conclusion, the combination of CD44, CD133, and CD117 biomarkers has stem cell properties. Moreover, LCSCs, are capable of resisting treatment and present high *KRAS*, *HIF-1α*, and *VEGF-A* gene expression".

Keywords: Cancer stem cells, chemotherapy, head and neck neoplasms, gene expression, cell line

Introduction

Laryngeal cancer (LC) is one of the most common head and neck neoplasms, representing 2% of all malignant neoplasms [1]. Estimates show that by 2020, 9,491 new cases and 5,202 deaths may occur owing to this disease [2]. Chemotherapy with docetaxel, bleomycin, hydroxyurea, pembrolizaumab, nivolumab, methotrexate, cetuximab [3], and paclitaxel [4] drugs can be used for treating LC. Despite advances in drug therapy, individuals with LC show low survival due to the locoregional recurrence and metastasis onset [5].

A small group of cells known as cancer stem cells (CSCs) may be responsible for tumor maintenance and dissemination. These cells possess self-renewal and differentiation potential and also play an important role in tumor initiation and progression [6]. These features can be associated with poor prognosis [7] and provide tumoral resistance, leading to ineffective drug treatment [8-10]. CSCs can be identified by cell

surface biomarkers such as *CD44*, *CD117*, and *CD133* related to tumoral growth [6, 11-14].

Literature also show that genes related to the cell proliferation pathway may be associated with increase of tumoral progression and poor prognosis; for example, *tropomyosin-related kinase B* (*TrkB*), *rat sarcoma* (*RAS*), *epidermal growth factor receptor* (*EGFR*), *Hypoxia-Inducible Factor 1 alpha (HIF1-α)* and *vascular endothelial growth factor (VEGF)* genes are overexpressed in different tumor types [15-22]. Both EGFR and TrkB are cell surface receptors that are activated by binding to epidermal growth factor (EGF) and brain-derived neurotrophic factor (BDNF), respectively. These tyrosine kinase receptors are responsible for activating some downstream intracellular signals, such as the Ras-Raf-MEK-ERK pathway [16, 23].

The RAS oncogene family has three isoforms: Harvey (*HRAS*), neuroblastoma (*NRAS*), and Kirsten (*KRAS*) [17]. They encode small GTPase proteins, which have essential roles in cell proliferation, growth, survival, migration, and epithelial-mesenchymal transition (EMT), as well as important roles in tumor relapse and chemotherapeutic resistance [19, 20]. Alterations in KRAS are associated with benefits from anti-EGFR antibody therapy, consequently improving progression-free survival and overall survival [17]. Nevertheless, mutated *KRAS* can regulate the GDP-GTP process and activate Ras-Raf-MEK-ERK downstream effectors independent of EGFR and TrkB receptor activation, leading to chemotherapy resistance [17, 24].

Depending on the alterations in the *KRAS* gene, the overexpression of this gene may occur with different stimuli that activate signaling pathways with distinct impacts on the production of basal genes [25]; for example, *HIF-1α*, a nuclear transcription factor important in the hypoxia response, leads to activation of *VEGF-A [21]*, which is responsible for angiogenesis as well as preservation of blood vessels for tumors [25, 26].

This study aimed to evaluate the effectiveness of 5-fluorouracil and cisplatin individually as well as the combination of cetuximab and paclitaxel in a CSC subpopulation separated with biomarkers related to tumoral growth, CD44, CD117, and CD133. In addition, *TrkB, KRAS, HIF-1α* and *VEGF*-A gene and protein expressions related with cell proliferation were evaluated in this subpopulation.

Materials and methods

Sample

Hep2 cell line, originally established from laryngeal squamous cell carcinoma and described with HeLa cell contamination (American Type Culture Collection, ATCC, Rockville, MD, USA), was utilized in the present study. Hep2 authentication was performed using the AmpFLSTR Identifier PCR Amplification kit (Life Technologies, Carlsbad, CA, USA) at the Special Techniques Laboratory, Hospital Israelita Albert Einstein (LATE-HIAE), São Paulo, and our cell line showed 100% identify compared to the ATCC database. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Gibco™, Carlsbad, CA, USA), 1% L-glutamine (Gibco™), and 1% penicillin/streptomycin/amphotericin B (Gibco™) in a humidified 5% CO_2 atmosphere.

Cell sorting

Two Hep2 cell subpopulations were identified using the combination of three antibodies: CD44, phycoerythrin (PE; BD Biosciences, San Jose, CA, USA); CD117, fluorescein isothiocyanate (FITC; BD Biosciences); and CD133, allophyllocyanine (APC; Miltenyi Biotec, Bergisch Gladbach, Germany), and sorted by fluorescence-activated cell sorting (FACS) using FACSAria Fusion equipment (BD Biosciences) and FACSDiva Software Version 6.1.3 for analysis. Positively labeled cells ($CD44^+/CD117^+/CD133^+$) were classified as laryngeal cancer stem cells (LCSCs), and negatively labeled cells ($CD44^-/CD117^-/CD133^-$) were considered laryngeal cancer non-stem cells (non-LCSCs). Both cell subpopulations were cultured in DMEM to obtain enough cells for subsequent analysis.

Invasion assay

Quantitative analysis of invasive potential was performed using Matrigel invasion chambers with 8 μm PET membranes in 24-well plates (Corning® BioCoat™, Corning Inc., Corning, NY, USA). Cells were seeded in the upper compartment of the transwell chamber at a density of

2×10^4 cells per insert in 100 µL serum-free DMEM. Well bottoms were filled with 750 µL DMEM supplemented with 10% FBS, which acts as a chemoattractant. Cells were then incubated for 24 h at 37°C. Cells that invaded the lower membrane surface were fixed with 4% paraformaldehyde for 20 min and stained with 5% Giemsa for 10 min. Four fields were photographed from each insert at 100× magnification using an Olympus BX53 Microscope (Olympus Life Science, Waltham, MA, USA), and the cells were counted.

Sphere-forming assay

Clonogenicity characteristics were evaluated by observing the capacity of cells to generate tumor spheres. LCSC and non-LCSC cells were cultured in low-adherence 6-well plates (Ultra-low Attachment Plates, Corning) in triplicates. Then, 1×10^4 cells/well were cultured in DMEM without FBS and supplemented with 10 ng/mL EGF, 10 ng/mL fibroblast growth factor, and 1% antibiotic/antimycotic solution. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 5 days (120 h). The former colonies were counted and photographed.

Treatments and MTS assay

Cell viability was determined colorimetrically by MTS assay using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA), following the manufacturer instructions. A total of 5×10^3 cells were seeded into 96-well plates and treated with 0.37 mg/mL 5-fluorouracil, 2.0 mg/mL cisplatin, or 0.06 mg/mL cetuximab combined with 0.05 mg/mL paclitaxel. After 24 h of treatment, cell viability was determined by absorbance analysis on an ELISA plate reader (Multiskan FC; Thermo Fisher Scientific - Uniscience, São Paulo, Brazil) at 490 nm.

Gene expression

RNA was extracted from 1×10^6 cells by cell lysis with 750 µL Trizol® (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The RNA concentration was estimated using the Qubit™ RNA HS Assay Kit with the Qubit® 2.0 Fluorometer (Life Technologies). Total RNA (1 µg) was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Foster City, CA, USA). For real-time PCR, TaqMan™ (Applied Biosystems™) probes for the *TrkB* (HS00178811_m1), *KRAS* (HS00364284_g1), *HIF-1α* (HS00153153_m1), and *VEGFA* (HS-00900055_m1) genes were used in custom microplates using the TaqMan™ Universal Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and the CFX96 Touch™ Deep Well Real-Time PCR Detection System (BioRad, Hercules, CA, USA). The comparative expression level of each condition was calculated as $2^{-\Delta\Delta Ct}$ (ΔΔCt1 method). The Ct values of the samples and controls were normalized by the amount of β-actin and GAPDH.

Protein expression

Proteins were extracted using Trizol® (Invitrogen,) and the concentration was estimated using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, USA), according to the manufacturer's instructions. The expression levels of KRAS, TrkB, HIF-1α VEGF-A, and β-actin were also measured by western blot analysis.

Western blotting

Equal amounts of proteins 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; primary antibody 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 [27, 28].

Enzyme-linked immunosorbent assay (ELISA)

The ELISA sandwich assay was utilized because the western blot of *VEGF-A* protein did not present good results. Thereby, specific Quantikine™ ELISA kits used were human VEGF-A (R&D

Systems) according to protocol manufacturer's instructions. The plate was read at 450 nm. Data capture for the colorimetric ELISA assays was performed with an ELISA plate reader (Multiskan FC).

Statistical analysis

Results were expressed independently as the mean ± standard deviation. Functional assays (sphere-forming and invasion assay) were compared by one-way variance analysis (ANOVA) with the Bonferroni correction, treatment and protein expressions were compared by two-way variance analysis (ANOVA) with the Bonferroni correction and gene were compared by t-test analysis. Analyses were performed using the GraphPad PRISM 8 software. Significance was set at $P<0.05$.

Results

$CD44^+/CD133^+/CD117^+$ subpopulation has cancer stem cell properties

Cells from a Hep2 cell line were sorted using the set of *CD44*, *CD133*, and *CD117* biomarkers. LCSCs were representative in 0.8% of cells, whereas non-LCSCs were representative in 4.8% of cells (**Figure 1**). The invasive potential of the LCSC and non-LCSC subpopulations was evaluated *in vitro*. **Figure 2** shows increased invasive capacity of the LCSCs when compared with the non-LCSC subpopulation after 24 h; LCSCs have a significantly higher invasive potential than non-LCSCs (P=0.0022).

The colony-forming assay was conducted for the LCSC and non-LCSC subpopulations of the Hep2 cell line (**Figure 3**). Clone formation was quantified, and LCSCs presented more colonies than non-LCSCs (p=0.0117).

LCSCs are treatment-resistant

The results showed no statistical differences between LCSCs and non-LCSCs when treated with 5-fluorouracil, but statistically significant differences were found with cisplatin (P= 0.0024) as well as cetuximab combined with paclitaxel (P=0.0069) treatments (**Figure 4A**). LCSCs had higher viability than non-LCSCs. Furthermore, cetuximab and paclitaxel combination treatment had a greater influence on subpopulation elimination than 5-fluorouracil and cisplatin treatments (**Figure 4B** and **4C**).

High KRAS, HIF-1α and VEGF-A gene expression in LCSC subpopulation

The *KRAS, HIF-1α* and *VEGF-A* genes presented up-regulation in LCSCs compared with non-LCSCs (**Figure 5**). The *TrkB* gene showed delayed expression in both subpopulations of the Hep2 cell line.

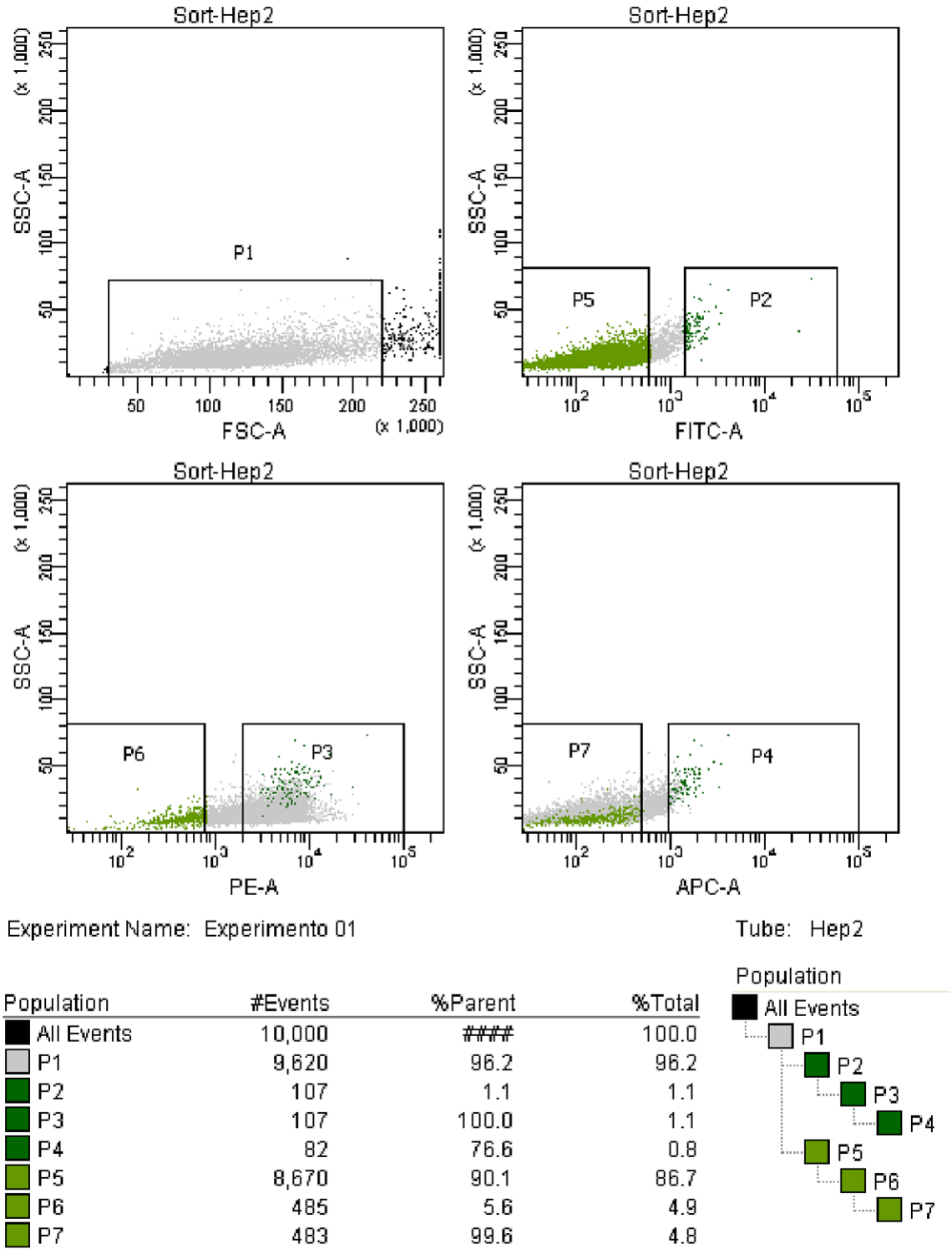
High KRAS, TrkB, HIF-1α and VEGF-A protein expression in LCSC subpopulation

From Western blot assay, KRAS, TrkB, HIF-1α and VEGF-A protein expression was up-regulated in LCSCs compared with non-LCSCs (**Figure 6A**, **6B**). Moreover, ELISA assay showed that LCSCs had higher VEGF-A protein expression than non-LCSCs (**Figure 6C**).

Discussion

In our previous study, we showed that $CD44^+/CD133^+/CD117^+$ cells, classified as LCSCs and obtained from a Hep2 cell line, presented 81% more migration capacity than $CD44^-/CD13^-/CD117^-$ cells, designated as non-LCSCs [29]. In the current study, we found that $CD44^+/CD133^+/CD117^+$ has cancer stem cell properties, similar to our previous study. Furthermore, we confirmed CSC presence in subpopulation using the invasion and colony-forming assays. The results of these assays demonstrated an increased tumorigenic potential in the LCSC subpopulation of the Hep2 cell line.

Regarding treatment, we found that 5-fluorouracil was ineffective at eliminating either subpopulation. The LCSC subpopulation demonstrated greater resistance to cisplatin and the combination of cetuximab and paclitaxel compared with the non-LCSC subpopulation of the Hep2 cell line. Moreover, the cetuximab and paclitaxel combination treatment was most effective in both subpopulations compared to the other treatments, especially in the non-LCSC subpopulation. Previously, our research group demonstrated that individual cetuximab and paclitaxel treatments showed no statistical differences between LCSCs and non-LCSCs from the Hep2 cell line [29]. These drugs were chosen because they are the most commonly used to treat head and neck cancer (HNC)



Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
P1	9,620	96.2	96.2
P2	107	1.1	1.1
P3	107	100.0	1.1
P4	82	76.6	0.8
P5	8,670	90.1	86.7
P6	485	5.6	4.9
P7	483	99.6	4.8

Figure 1. Cell sorting graphics with CD44, CD117, and CD133 in FACSAria Fusion using FACSDiva Software. Cells in quadrants above 10^3 (P2, P3, and P4) were considered positive for the markers, and cells in quadrants below 10^3 (P5, P6, and P7) were considered negative for the markers. The positive cells for FITC-CD117 (P2) were selected from these cells, then those that were positive for the marker PE-CD44 (P3) were selected, and then those positive for the APC-CD133 (P4) were selected. This formed the triple cell positive group for the three tumor stem cell biomarkers. For triple cell negative group, we selected negative cells for FITC-CD117 (P5) fom the quadrants below 10^3, then selected those that were negative for PE-CD44 (P6), then those that were negative for APC-CD133 (P7).

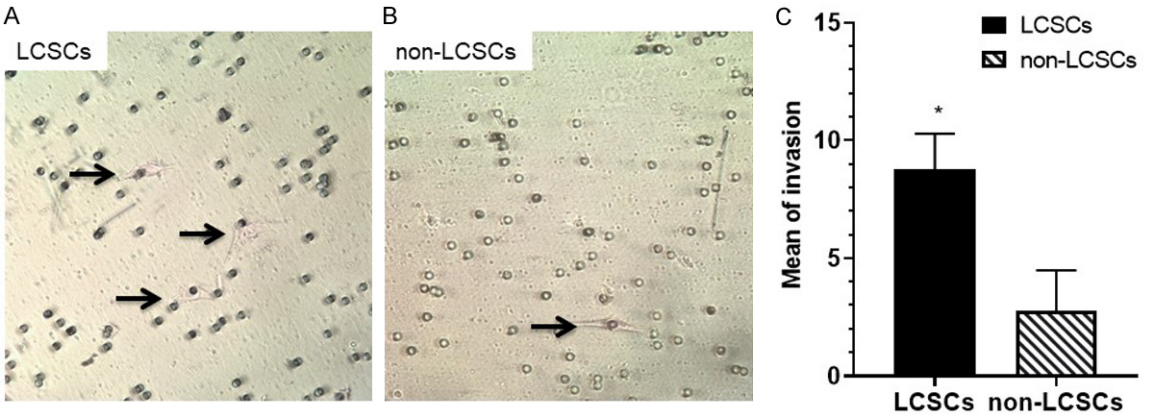


Figure 2. Cell invasion assay of LCSC and non-LCSC subpopulations of the Hep2 cell line. Cells were seeded in matrigel inserts and cultured for 24 h. A. LCSC subpopulation; B. non-LCSC subpopulation. Grayscale pictures at 24 h were observed under an optical microscope (×100). The arrows point to the cells that invaded through the matrigel insert. C. Graphic showing the comparative between LCSCs and non-LCSCs invasion. Analysis were performed in triplicate and *P<0.05 versus non-LCSCs. Statistically significant difference was determined using one-way ANOVA with Bonferroni corrections.

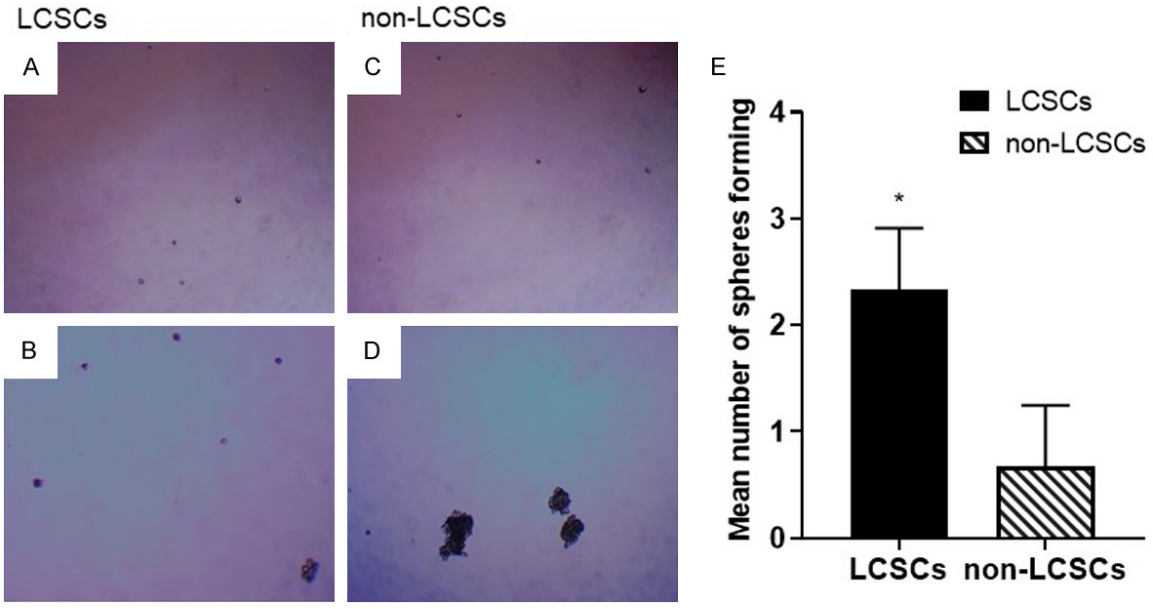


Figure 3. Colony-forming LCSC and non-LCSC subpopulations of the Hep2 cell line. The cells were seeded in ultra-low attachment surface 6-well plates and cultured for five days (120 h). (A) Non-LCSC and (C) LCSC subpopulations at 0 h; (B) Non-LCSC subpopulation after five days; and (D) colonies formed in the Hep2 LCSC subpopulation after five days. Grayscale pictures at 120 h were observed under a phase contrast microscopy (×100). (E) Graphic showing the comparative between LCSCs and non-LCSCs invasion. Analysis were performed in triplicate and *P<0.05 versus non-LCSCs. Statistically significant difference was determined using one-way ANOVA with Bonferroni corrections.

patients. Cisplatin reacts with DNA to produce crosslinks, and 5-fluorouracil is an antineoplastic antimetabolite; both drugs impair DNA replication and transcription [30, 31]. Cetuximab is a monoclonal antibody that functions by blocking EGF from binding to EGFR [18], thereby interrupting the cascade that activates KRAS [15]. Paclitaxel is a chemotherapeutic that inhibits mitotic spindle fiber dynamics [32].

Our results align with those of other studies performed in CSCs from head and neck cancers, which showed resistance to 5-fluorouracil, cisplatin, and cetuximab when used individ-

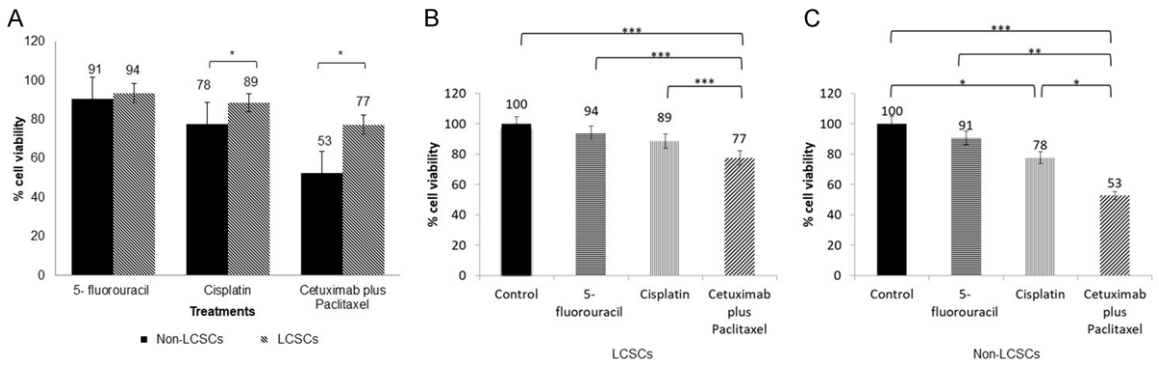


Figure 4. Cell viability after 24 h in Hep2. (A) LCSC and non-LCSC subpopulations treated with 5-fluorouracil, cisplatin, and the combination of cetuximab and paclitaxel. Comparison of responses to 5-fluorouracil, cisplatin, and the combination of cetuximab and paclitaxel in Hep2 LCSC and non-LCSC subpopulations *P≤0.05 versus non-LCSCs. Data and *p*-values are shown for the comparison between treatments with others in (B) LCSCs. ***P≤0.0001 comparison one treatment with others. Data and *p*-values are shown for the comparison between treatments with others in (C) non-LCSCs *p≤0.05; ** P≤0.001; ***P≤0.0001 comparison one treatment with others. Statistically significant difference was determined using one-way ANOVA with Bonferroni corrections.

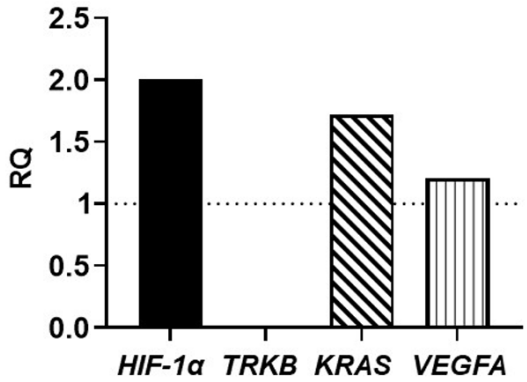


Figure 5. Graph showing the relative values of differential expression of the *KRAS*, *TRKB*, *HIF1α* and *VEGFA* genes, comparing LCSC with control. Statistically significant was determined using one sample t-test analysis compared with a hypothetical mean (1).

ually [33-36]. Grau et al. [33] observed cisplatin and cetuximab resistance in CSCs from head and neck carcinoma squamous cell (HNCSC) lines that had high expression of the CD44 biomarker. It has also been shown that CSCs from HNCSC cell lines, which used Aldehyde dehydrogenases (ALDH) as a biomarker, were resistant to 5-fluorouracil, cisplatin, and etoposide [35]. Other studies in HNCSC cell lines, which were conducted with FACS to isolate CSCs using both CD44 and ALDH biomarkers, also showed resistance to docetaxel, cetuximab, and PI3K inhibitor (ZSTK474 and PX-866) in these subpopulations, in addition to radiation, photon irradiation (2 Gy/min), and carbon ion irradiation (75MeV/n) resistance [34, 36]. In contrast, CSCs from HNCSC cell lines sorted with $CD44^{high}/EGFR^{low}$ presented sensitivity to cisplatin, cetuximab, gefitinib, and radiation compared to $CD44^{high}/EGFR^{high}$ cells [37].

The combined therapy with cetuximab and paclitaxel has been evaluated in head neck cancer clinical studies, with a better response found in oral cancer patients [38]. Furthermore, improved progression-free survival and overall survival have been observed in patients with head and neck cancer [39] mainly after failure of platinum therapy [40].

To our knowledge, to date, there are no studies on combination therapy in LCSCs. Herein, we hypothesized that the combined action of cetuximab and paclitaxel drugs may contribute to eliminating LCSCs, consequently reducing tumor aggressiveness and recurrence. Cetuximab does not have apoptosis-inducing activity; similar to our study, other researchers also observed that cetuximab might be acting as an enhancer of the paclitaxel possibly by induced apoptosis [41]. However, the precise action mechanism of cetuximab and paclitaxel combined treatment responsible for the antitumor effects is still not clear [41].

Similar to our findings, other studies using *in vitro* and *in vivo* models have demonstrated that drug combinations, related to the EGFR inhibition pathway combined with other treat-

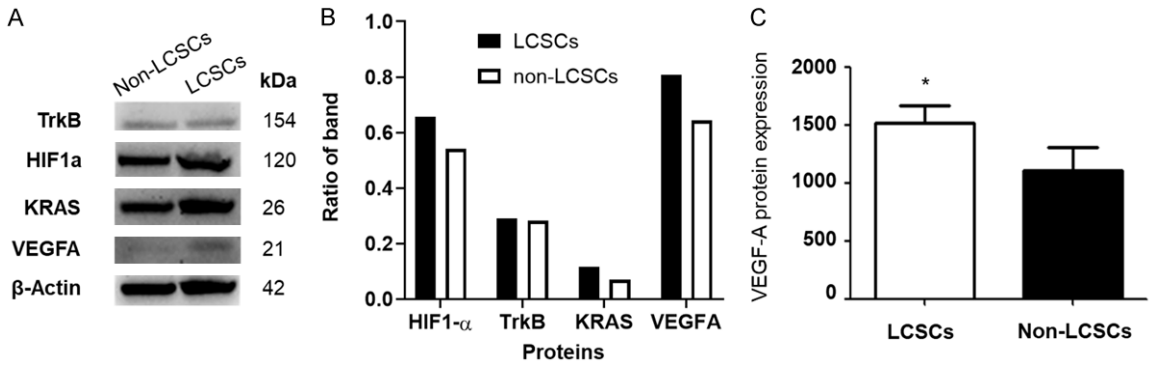


Figure 6. Protein expression data. (A) Subjected to western blot analysis of TrkB, HIF-1α, KRAS, VEGF-A and β-actin expression (B) Histogram showing quantitative fold change in protein expression normalized to β-actin expression by Image J analysis. (C) ELISA assay graph showing VEGF-A protein expression levels in both cell subpopulations in triplicate. *P<0.05 versus non-LCSCs using 2 way ANOVA with Bonferroni corretions.

ments such as tyrosine kinase inhibition, immunotherapy or radiation, have higher therapeutic effectiveness in cancer stem cells of the head and neck cancer [35, 42]. Studies using *in vivo* models have shown that the tyrosine kinase receptors crosstalk with each other and the ligands are able to bind with other receptors to activate the signaling pathways [35, 42] that can activate the *KRAS* gene, resulting in tumor relapse and chemotherapeutic resistance [19, 20]. Further studies of combination therapy related to surface biomarkers are required to better understand the therapy response in LCSCs in *in vivo* models to improve clinical outcomes.

This is the first study to evaluate *TrkB* and *KRAS* gene expression in CSC and non-CSC subpopulations of head and neck cancer. Considering the role of these two genes in cell proliferation, we expected that both genes would be overexpressed in the Hep2 cell line, especially in the CSC subpopulation; however, the *TrkB* gene was not expressed. Recently, TrkB and BDNF were found to be expressed in 30-50% of human HNCSCs [43-45]. One limitation in our study is that only one cell line was assessed; hence, results may not be representative. Therefore, studies with a larger sample size are needed, since *TrkB* activation has been associated with cell migration, invasion, EMT, cisplatin resistance, and poor prognosis *in vivo* [43, 44, 46-48]. Indeed, some studies in head and neck cancer have shown that *TrkB* inhibition can suppress tumor growth, cell proliferation, and migration, as well as sensitize cells to cisplatin [43, 49-52]. In the present study, *KRAS* gene and protein presented high expression, which may be explained by EGFR-mediated signaling responsible for phosphorylating and activating KRAS, as shown in **Figure 7** (adapted from [24, 53]). In our previous study, we observed *EGFR* gene overexpression in LCSCs from the Hep2 cell line [29]; therefore, we suggested that this CSC subpopulation may contribute via EGFR-signaling to promote tumor cell growth, chemotherapy resistance, invasion, and migration, resulting in head and neck cancer progression.

In the present study, we showed that the *KRAS* gene leads to activation of the *HIF-1α* and *VEGF-A* genes; all genes and proteins were upregulated in the LCSC subpopulation. These high expressions are related to CSC features, such as more migration, invasion, colony forming, chemotherapy resistance, and angiogenesis, which lead to metastasis and poor prognosis. The molecular mechanism for explaining this relationship is still unclear, but one limitation of our study is that we evaluated only gene and protein expression and not mutations in the KRAS gene. However, it has been found that different *KRAS* alterations can be activated to signaling pathways with distinct impacts [25]. The ASP13 mutation in the *KRAS* gene leads to increased expression of the *VEGF-A* gene even in the absence or low expression of the *HIF-1α* gene [25, 54]. The underlying molecular mechanisms responsible for the differential overexpression of *VEGF-A* may be mediated by a distinct activation of the Raf-ERKs pathway and AP2/Sp1 elements in the proximal VEGF-A promoter [25] mainly induced by EGF [55].

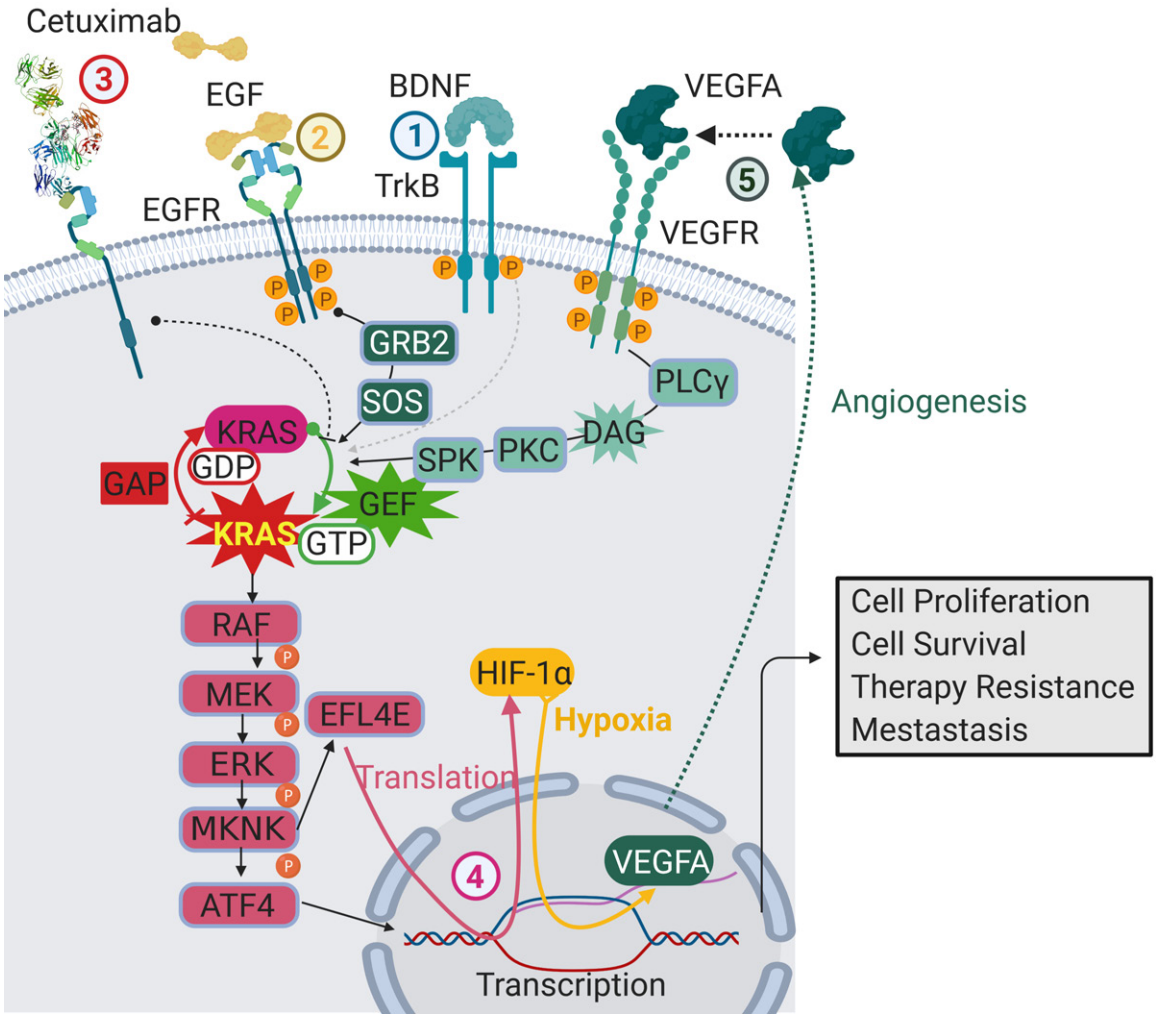


Figure 7. Summarized molecular mechanisms of the signaling pathway involving the *EGFR, TrkB, KRAS HIF-1α* and *VEGF-A* genes; adapted from [24, 53]. 1) Phosphorylation resulting from BDNF/TrkB binding can also activate *KRAS*; however, *TrkB* gene expression was not found in either subpopulation. This suggests that only *EGFR* is activating the *KRAS* gene. 2) Phosphorylation resulting from EGFR/EGF binding activates *KRAS*, which leads to cell proliferation. The results of our present and previous studies showed high *KRAS* and *EGFR* expression in the CSC subpopulation [29]. 3) Only cetuximab [29] binds with *EGFR*, which blocks EGFR/EGF binding; we suggest that this isolated treatment does not inhibit *KRAS* inactivation. 4) *KRAS* gene can active *HIF1α* and consequently *VEGF-A*. In this study, these genes were highly expressed in the CSC subpopulation. 5) VEGF-A/VEGFR binding can activate *KRAS* by the PCLy pathway leading to vascular proliferation. Created with BioRender.com.

Additionally, the CYS12 mutation in the *KRAS* gene promotes *HIF-1α*-dependent induction of glycolytic enzymes, supporting the role of *HIF-1α* in changing tumor metabolism [25, 56, 57].

The *KRAS* oncogene has been reported to increase *VEGF-A* expression in different tumor types [58]. Moreover, mutations in the *KRAS* gene have been associated with PI3k-dependent up-regulation of *VEGF-A* in colon tumors [59]. Another study did not observe any association between *KRAS* mutation status and individual expression of *VEGF*-A, but showed that up-regulation of *VEGF-A* can be associated with different types mutation in the *EGFR* gene [60]. Subsequently, a study also evaluated tumor stem cells in glioma carcinoma and found elevated levels of VEGF-A gene and protein expression under normal and hypoxia conditions compared to the non-tumor stem cell population [61]. We suggest that the up-regulation of *VEGF-A* in cancer stem cells may be associated with signaling of the *KRAS*, which may be associated with *HIF-1α*-de-

pendent *KRAS* downstream signaling by different types of *EGFR* mutations in head and neck cancer.

Our results, although limited, suggest for the first time that the combined action of cetuximab and paclitaxel drugs may be more efficient at eliminating CSC subpopulations classified by CD44, CD117, and CD133 biomarkers of a laryngeal cancer cell line than isolated therapies. We provide evidence that higher KRAS expression in LCSCs could contribute to aggressive tumor behavior and poor prognosis in LC. Thus, understanding of the molecular mechanisms that control CSCs proliferation may contribute to better strategies for treating head and neck cancer. Future clinical studies with patients with laryngeal cancer undergoing treatment with cetuximab and paclitaxel are important for further understanding our current findings. In addition, evaluating the expression and mutations of the *KRAS* gene in these patients can assist in developing specific protocols to stop tumor aggression and improve the prognosis.

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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; LC, Laryngeal cancer; LCSCs, Laryngeal cancer stem cells; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; non-CSCs, Cancer non-stem cells; non-LCSCs, Laryngeal 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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