GLI3 knockdown decreases stemness, cell proliferation and invasion in oral squamous cell carcinoma

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Abstract. Oral squamous cell carcinoma (OSCC) is an extremely aggressive disease associated with a poor prognosis. Previous studies have established that cancer stem cells (CSCs) actively participate in OSCC development, progression and resistance to conventional treatments. Furthermore, CSCs frequently exhibit a deregulated expression of normal stem cell signalling pathways, thereby acquiring their distinctive abilities, of which self-renewal is an example. In this study, we examined the effects of GLI3 knockdown in OSCC, as well as the differentially expressed genes in CSC-like cells (CSCLCs) expressing high (CD44high) or low (CD44low) levels of CD44. The prognostic value of GLI3 in OSCC was also evaluated. The OSCC cell lines were sorted based on CD44 expression; gene expression was evaluated using a PCR array. Following this, we examined the effects of GLI3 knockdown on CD44 and ESA expression, colony and sphere formation capability, stem-related gene expression, proliferation and invasion. The overexpression of genes related to the Notch, transforming growth factor (TGF)β, FGF, Hedgehog, Wnt and pluripotency maintenance pathways was observed in the CD44high cells. GLI3 knockdown was associated with a significant decrease in different CSCLC fractions, spheres and colonies in addition to the downregulation of the CD44, Octamer-binding transcription factor 4 (OCT4; also known as POU5F1) and BMI1 genes. This downregulation was accompanied by an increase in the expression of the Involutrin (IVL) and S100A9 genes. Cellular proliferation and invasion were inhibited following GLI3 knockdown. In OSCC samples, a high GLI3 expression was associated with tumour size but not with prognosis. On the whole, the findings of this study demonstrate for the first time, at least to the best of our knowledge, that GLI3 contributes to OSCC stemness and malignant behaviour. These findings suggest the potential for the development of novel therapies, either in isolation or in combination with other drugs, based on CSCs in OSCC.

Introduction

Head and neck cancer is the seventh most common type of cancer worldwide, and oral squamous cell carcinoma (OSCC) accounts for >90% of these cases (1,2). OSCC is usually aggressive and highly invasive and is associated with a high recurrence rate, as well as with metastasis of the lymph nodes (3). As a result, the vast majority of patients with OSCC have a poor prognosis, and, despite advances in the understanding of the molecular and genetic mechanisms driving OSCC malignancy, the 5-year-survival rate has not shown any significant improvement (3,4).

Previous studies have demonstrated that OSCC, similar to other solid malignant tumours, has a small subpopulation of cells designated as cancer stem cells (CSCs). These cells are characterised by the ability to self-renew indefinitely, in addition to giving rise to transient and differentiated cells which comprise the bulk of the tumour (5-7). CSCs are therefore associated with recurrence and therapeutic resistance, and participate in OSCC metastasis due to their capability to undergo epithelial to mesenchymal transition (EMT) (8-10).

CSCs in OSCC were first identified and isolated by Prince et al based on their high expression levels of CD44, a cell surface glycoprotein that acts as a receptor for hyaluronic acid (5). Upon binding to its ligand, CD44 can activate
different signalling pathways which regulate a wide variety of cellular processes, including adhesion, proliferation, motility, apoptosis, survival and resistance to therapy (11). Subsequently, additional CSC markers were identified and used alone or in combination with CD44, including CD133 (12), epidermal growth factor receptor (EGFR) (13), ESA (14), CD24 (15) and aldehyde dehydrogenase 1 (ALDH1) (16).

Most importantly, recent studies on CSC plasticity have demonstrated that this subpopulation exists in more than one phenotype; the association of CD44 with different markers has permitted the identification of distinct subtypes of CSCs. Biddle et al (2011) demonstrated that cells expressing high levels of CD44 (CD44high) can be separated, based on epithelial-cell adhesion molecule (EpCAM)/ESA levels, into two cellular phenotypes. These phenotypes present significant differences in proliferation rates, cell motility and morphology in addition to colony- and sphere-forming ability (14). CD44high/ESAlow cells exhibit an epithelial morphology and an increased proliferative ability, while CD44high/ESAdlow cells are migratory and undergo EMT.

Signalling pathways that control stem cell self-renewal and differentiation are aberrantly activated in CSCs and include the Notch, Sonic Hedgehog (SHH) and Wnt pathways. All these pathways frequently interact with other cellular signalling pathways closely related to tumour development and progression, such as nuclear factor-κB (NF-κB), mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K) and epidermal growth factor (EGF) (17). Thus, the identification of the crucial pathways necessary for CSC maintenance represents an important therapeutic target with may be used to block CSC proliferation and self-renewal and, consequently, tumour progression.

In this context, the SHH/Patched/Gli (SHH/PTCH/GLI) pathway, involved in the patterning, growth, differentiation and survival of normal stem cells also plays an important role in CSCs; it provides proliferative cues that enable the cells to accumulate oncogenic mutations that drive self-renewal, metastasis and therapeutic resistance (17,18). This signalling pathway initiates with the binding of Hedgehog proteins (Sonic, Desert and Indian HH) to the transmembrane receptor, PTCH. This receptor, in the absence of the Hedgehog ligands, inhibits signal transduction by repressing the Smoothened (SMO) transmembrane receptor (18,19), which acts as a putative pathway activator. Following HH binding, PTCH is internalised and degraded, thus allowing SMO to become phosphorylated and activated (19); this in turn triggers an intracellular signalling cascade that promotes the recruitment and activation of GLI family transcription factors (20,21).

There are three GLI proteins in mammalian cells that act in a specific manner to regulate tissue patterning, cell proliferation and survival via positive and negative feedback mechanisms depending on the context and cell-type (22,23). GLI proteins can act as activators or repressors, depending on the ratio of said proteins (24). GLI1 is a transcriptional activator. GLI2 and GLI3 genes function as either positive or negative regulators according to their post-transcriptional and post-translational modifications, e.g., via phosphorylation or acetylation (25,26).

In the absence of a Shh ligand, GLI3 is cleaved from its larger activated cytoplasmic form to a truncated repressor nuclear form, which inhibits the signalling pathway (27). In adult haematopoiesis, a progressive decrease in the Shh pathway is associated with increased hematopoietic cellular fate and the transition from an embryonic to a hematopoietic stem cell. In this context, GLI3 plays a crucial role in mediating Shh pathway inhibition (28).

In cancer, SHH deregulated activation was first described in nevoid basal cell carcinoma syndrome, where the inherited loss-of-function mutations of the PTCH1 gene was associated with tumour development (29,30). Abnormal Shh signalling is now associated with the progression and maintenance of several malignant tumours, e.g., glioblastomas, lung cancer, prostate cancer and gastric cancer (24,31-34). Additionally, it can participate in tumour development via somatic mutations in upstream pathway proteins (SMO and PTCH1), overexpression of GLI transcription factors or in a ligand-dependent manner (25).

In different cell types, HH/GLI activation leads to the transcription of genes critical to tumour initiation and maintenance. This pathway is associated with an increase in the quantity of cell cycle proteins which are responsible for G1/S and G2/M progression, mainly D-type cyclins and anti-apoptotic proteins (35-37). Additionally, it participates in the regulation of EMT by inhibiting E-cadherin and inducing N-cadherin expression (37,38). Furthermore, GLI2 overexpression is associated with a decrease in E-cadherin expression and an increase in SNAIL gene expression, as well as with an increase in matrix metalloproteinase (MMP)2 and integrin-beta-1-binding proteins (ICAP-1); all of which favour cell invasion and metastasis (39).

Some studies have demonstrated that the Shh signalling pathway is upregulated in CSCs. The activation of these pathways in breast CSCs increases GLI expression and leads to an enhanced SOX2 and OCT4 expression, favouring CSC maintenance (40). In gastric cancer cell lines and tissues, CSCs identified by the CD44+CD24+ phenotype have demonstrated an overexpression of the SHH, PTCH1 and GLI3 genes (41). Zhang et al demonstrated that in liver cancer, CD90+ CSCs with GLI1 or GLI3 knockdown, exhibited low proliferation rates, and decreased migratory ability and sphere formation capacity, as well as a decrease in tumour formation in vivo; indicating that both genes are relevant to the maintenance of the stem cell properties observed in CD90+ cancer cells (42).

In oral cancer, the increased expression of GLI1, PTCl, SMO and SHH has been observed in OSCC samples when compared to non-tumour oral mucosa (43,44). Moreover, both SHH and GLI1 have been shown to be associated with lymph node metastasis, but only GLI1 expression has been shown to be associated with tumour recurrence, clinical stage and lower 5-year survival rates (39). Schneider et al also observed the overexpression of all Hh signalling pathway proteins in OSCC and found that a high Shh expression was also associated with a poor overall survival (45). Concordant with this, an in vitro study demonstrated that the inhibition of SHH was associated with a significant decrease in tumour growth, as well as in angiogenesis and osteoclastic activity in a mouse model of OSCC bone invasion (46). GLI1 or GLI2 loss of function increases apoptosis and DNA fragmentation and also promotes keratin 17 upregulation, which in turn promotes cell growth (47).
The current study investigated, in vitro, the differential expression of genes involved in stem cell signalling pathways in OSCC CSC-like cells (CSCLCs), as well as the effects of GLI3 knockdown on cellular proliferation, invasion and stemness. Furthermore, GLI3 protein expression was also evaluated in OSCC and non-tumour tissues and its association with the patient clinicopathological parameters and overall survival was examined. To the best of our knowledge, this is the first study to investigate the role of GLI3 in OSCC.

Materials and methods

Cell lines. SCC4 (CRL-1624™) and SCC9 (CRL-1629™) human tongue squamous cell carcinoma cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultivated in DMEM/F12 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and supplemented with 10% fetal bovine serum (FBS), 400 ng/ml hydrocortisone (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 100 µg/ml penicillin and 100 µg/ml streptomycin with 5% CO₂ at 37°C.

Flow cytometry. Fluorescent-activated cell sorting (FACS) was performed using a FACSAria II flow cytometer (BD Biosciences, San Jose, CA, USA) and analysed using FACSDiva software (Divac version 6.1.1). Initially, the SCC4 and SCC9 cells were detached from the cultures using Accutase™ cell detachment solution (SCR005; MilliporeSigma, Burlington, MA, USA) at 37°C and stained with anti-CD44 antibody (anti-CD44-FITC, clone G4-26, 1:100, 555478; BD Biosciences) for 30 min in the dark. DAPI nuclear dye (Sigma-Aldrich; Merck KGaA) was used at 200 ng/ml to exclude dead cells and IgG2ak-FITC was used as an isotype control. The whole population was fractionated into CD44^{high} (CSCLC), representing the top 5% of CD44-expressing cells, and CD44^{low}, corresponding to the bottom 5% of differentiating cells. FACS-sorted CD44^{high} and CD44^{low} subpopulations were then submitted to colony and sphere formation, as well as gene expression assays for stemness-related signalling pathways (PCR array) and markers (qPCR).

Subsequently, the cells were co-stained with anti-CD44 and anti-ESA antibodies (anti-ESA-PE, clone EBA-1, 1:100, 347198; BD Biosciences), for 30 min at room temperature, washed with PBS, incubated with DAPI and analysed using a FACSCanto II Flow cytometer (Divac software version 6.1.1; BD Biosciences) to evaluate the CSCLC fractions representing both EMT-CSCLC (CD44^{high}/ESA^{low}) and non-EMT-CSCLC (CD44^{high}/ESA^{high}).

Colony and sphere formation assays. To examine the clonogenic abilities of the SCC4 and SCC9 subfractions (CD44^{high} and CD44^{low}), the cells were plated at clonal density (100 cells/ml) in each well of 6-well plates and cultured for 14 days. Following fixation in 4% paraformaldehyde, the cells were stained for 5 min at room temperature with crystal violet (0.04% in 1% ethanol) and colonies measuring at least 2 mm in diameter were counted visually.

To assess the capacity of the SCC4 and SCC9 subfractions for growth as tumour spheres in suspension, 1x10^5 cells/ml were seeded in 24-well ultra-low attachment plates per well (#3473; Corning, New York, NY, USA). After 2 weeks, the number of tumour spheres >5 µm was counted under a microscope at x200 magnification (Carl Zeiss Micro-Imaging GmbH, Jena, Germany).

GLI3 knockdown. A total of 3x10^5 SCC9 cells were transfected with 4 shRNA-GLI3 sequences (Origene TG301348) or shRNA-scrambled (Origene, Rockville, MD, USA) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer’s instructions. This cell line was selected as we have previously observed that the SCC9 cell line, as well as the CD44^{high} and CD44^{low} fractions are able to form tumours in vivo (49). Transfected cells were selected in a medium with 0.5 µg/ml of puromycin (Invitrogen; Thermo Fisher Scientific, Inc.) for 21 days. The transfection efficiency of the cells was examined. To the best of our knowledge, this is the first study to investigate the role of GLI3 in OSCC.

PCR array. To assess the expression of genes related to different stem cell signalling pathways in the CD44^{high} and CD44^{low} cells from the SCC4 and SCC9 cells, we used the commercially available Human Stem Cell Signalling RT² Profiler PCR Array (30231; Qiagen, Germantown, MD, USA). Immediately after FACS, sorted cells were submitted to RNA extraction using the RNeasy Mini kit following the manufacturer’s instructions (74104; Qiagen). cDNA was synthesized using 500 ng of RNA with the aid of the RT² First Strand kit (330401; Qiagen). A list of genes on this PCR array is available at https://www.qiagen.com/us/shop/primer-sets/rT2-profile-rPCR-arrays/#catno=PAHS-047Z#orderinginformation. The relative expression level of the target gene in the CD44^{high} cells to that in the CD44^{low} cells was evaluated using the 2⁻ΔΔCt method (48) using the software RT² Profiler PCR Array Data Analysis version 3.5 (Qiagen). This software classifies the average threshold cycle of the gene as follows: ‘A’ [relatively high (>30) in either the control or the test sample, and is reasonably low in the other sample (<30)]; ‘B’ [relatively high (>30), meaning that its relative expression level is low, in both the control and test samples, and the P-value for the fold change is either unavailable or relatively high (P>0.05)]; ‘C’ [either not determined or greater than the defined cut-off value (default 35), in both samples meaning that its expression was undetected, making this fold change result erroneous and uninterpretable] and ‘OKAY’. Only genes classified as ‘A’ or ‘OKAY’ by the software and with a fold change >2 were considered as overexpressed.

GLI3 expression analysis. The differential and uninterpretable] and ‘OKAY’. Only genes classified as ‘A’ or ‘OKAY’ by the software and with a fold change >2 were considered as overexpressed.

RNA extraction, cDNA synthesis and RT-qPCR. RNA was extracted using the RNeasy® micro kit (Qiagen) followed by...
the reverse transcription of 500 ng of total RNA with the High Capacity cDNA Archive kit, according to the manufacturer's instructions. qPCR was performed on an ABI 7500 real-time PCR system using SYBR®-Green mix (all from Applied Biosystems, Foster City, CA, USA) and primers for CD44, BMI1, POUSF1 (OCT4), CD133, NANOG, Involucrin (IVL), S100A9 (Calgranulin B), SNAI2 (SLUG) and GAPDH gene amplification, which was used for normalization of the target gene. The qPCR cycling conditions were as follows: 95°C for 10 min, (95°C for 15 sec, 60-62°C for 60 sec) (40 cycles), followed by dissociation curve analysis. Positive samples used to generate a standard curve included SCC9 (for CD44, BMI1 and POUSF1 genes) and embryonic stem cells (for CD133 and NANOG genes). Primer sequences were designed using the GeneTool software (BioTools Inc., Edmonton, AB, Canada) and are described in detail in Table I. Gene expression was calculated by the $\Delta \Delta C_q$ method (48) and the fold change values were calculated by the ratio of house-keeping gene (CD44) to the CD44 was assessed using ImageJ software (BioTools Inc., Edmonton, AB, Canada).

**Cell proliferation assays.** The SCC9 control cells and SCC9 cells in which GLI3 was knocked down (1x10^5) were initially plated in 24 well-plates and counted daily in a Neubauer chamber (Celeromics, Cambridge, UK) for 5 days (24, 48, 72, 96 and 120 h). Independent experiments were performed in triplicate and the mean value, standard deviation and statistical analyses were calculated. The bromodeoxyuridine (BrdU) labelling index was performed as previously described by Rodrigues et al (50). In summary, the SCC9 control cells and SCC9 in which GLI3 was knocked down were plated on chamber slides and serum-starved for 48 h. After this period, cell culture medium with 10% FBS was added and the cells were cultivated for an additional 24 h. The cells were then incubated with BrdU (1:100) for 2 h at 37°C and the reactions were revealed using the BrdU Staining kit (both from Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The BrdU-positive cells were determined by counting 1,000 cells in 3 independent reactions using the Kontron 400 image analysis system (Zeiss Axio Imager A1; Zeiss, Dublin, CA, USA).

**Cell invasion assay.** To analyse cell invasion, cell culture inserts with an 8-µm PET membrane coated with Matrigel (#354480, Corning® BioCoat® Matrigel® Invasion Chambers; Corning) were used. The cells (1x10^5) were suspended in serum-free DMEM/F12 and seeded onto the upper compartment of the Transwell chamber. DMEM/F12 containing 5% FBS was used in the lower chamber as a chemoattractant. Following 24 h of incubation, cells in the upper chamber were removed and the inserts were fixed in 4% paraformaldehyde followed by 20 min in methanol 100%. The cells on the lower surface were stained for 5 min at room temperature with crystal violet (0.04% in 1% ethanol; Sigma-Aldrich; Merck KGaA). A cotton swab was used to mechanically removed cells that did not migrate through the pores. Five random areas from each membrane were photographed under x400 magnification using a light microscope (Axioplan 2; Zeiss, Oberkochen, Germany) and the mean number of cells was calculated.

**Cell apoptosis assay.** The SCC9 control and SCC9 shRNA-GLI3 were triple-stained for 30 min at room temperature with Annexin V-FITC (Invitrogen; Thermo Fisher Scientific, Inc.), DAPI, or anti-CD44-APC (BD Pharmingen). Samples were examined on a FACS Canto II Flow cytometer and analysed with the FACS Diva version 6.1.1 (both from BD Biosciences) software.

**Immunohistochemistry.** Forty-five tongue OSCC samples were obtained by surgical resection from patients (male, ≥40 years of age) admitted for diagnosis and treatment at the Hospital of the University of São Paulo, School of Medicine (Brazil) from January, 2011 to November, 2014. Samples and clinical data collection, and histopathological analysis were performed with the informed consent of each patient by the GENCAPO (Head and Neck Genome Project) Consortium. OSCC diagnosis was performed following the WHO Classification of Tumors. Clinicopathological TNM staging was established according to the classification determined by the Union for International Cancer Control (UICC) (51). This study was approved by the Brazilian National Ethics Committee (Process #16491) and meets the requirements of the Declaration of Helsinki.

Immunohistochemical analysis of GLI3 was performed in 45 OSCC samples and in 10 non-tumour margins. Tissue sections (4-µm-thick) were cut and, following dewaxing and hydration in graded alcohol solutions, the sections were incubated with 3% H₂O₂, for 20 min. After washing in PBS, antigen recovery was performed by treatment with a 100 mM citrate buffer target retrieval solution, pH 6.0 at 95°C, in a water bath for 20 min. The sections were incubated with protein block (X0909; Dako, Carpenteria, CA, USA) for 10 min followed by overnight incubation with monoclonal anti-GLI3 antibody (ab6050; Abcam) diluted at 1:300 in Antibody Diluent with Background Reducing components (S3022; Dako). The sections were then incubated with the Envision+Dual Link visualization system (K4061) and 3,3-diaminobenzidine tetrahydrochloride (DAB, K3468) (both from Dako) was used
as a chromogen. The negative control consisted of the omission of the primary antibody. A human kidney was used as the positive control. Immunostaining evaluations were performed by two independent pathologists who had no knowledge of the clinicopathological parameters. GLI3 immunoexpression was scored as 0 (<5%), 1 (5-30%), 2 (30-60%) and 3 (60-100%), as previously described by Schneider et al (52). For the final analyses, scores were dichotomised according to protein expression as low expression (scores 0 and 1) and high expression (scores of 2 and 3). The immunoexpression pattern was determined independently by two investigators (M.F.S.D.R. and L.M.) and its association with clinicopathological parameters such as mean age, tumour location, tumour size-pT, nodal metastasis-pN, lymphatic, vascular or perineural invasion, as well as overall survival was examined.

Statistical analysis. All assays were independently repeated at least 3 times and the significance of the differences was calculated using unpaired t-tests with the Welch correction. A value of P<0.05 was considered to indicate a statistically significant difference. Error bars represent the standard error of the mean (SEM). Fisher’s exact test was used to estimate the statistically significant differences between protein expression and the clinicopathological parameters. The Kaplan-Meier product-limit estimation with the log-rank test (P<0.05) was used for survival analysis from lifetime data according to GLI3 protein expression (high vs. low). Overall survival was defined as the time from surgery to the day of death or the last follow-up. Statistical package GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis and P-value <0.05 were considered to indicate statistically significant differences.

Results

Characterization of the SCC4 and SCC9 CD44 high and CD44 low cell fractions. The FACS-sorted CD44 high and CD44 low fractions from SCC4 and SCC9 (Fig. 1A) were immediately plated for clonogenic (Fig. 1B-D) and sphere formation assays (Fig. 1E) in order to investigate the CSC properties. As expected, the CD44 high cells exhibited a significantly higher number of colonies with holoclone morphology (P<0.001, Fig. 1C and D) and spheres (Fig. 1E) in relation to the CD44 low cells from both SCC4 and SCC9 cell lines. Additionally, gene expression evaluation revealed increased mRNA expression levels of CD44, POU5F1 (OCT4) and BMI1 (P<0.05) in the CD44 high cells in relation to the CD44 low cells (Fig. 1F).

Expression of genes related to stem cell signalling pathways in SCC4 and SCC9 CD44 high and CD44 low cell fractions. The results of the PCR array assay revealed that the vast majority of the genes were overexpressed in the CD44 high cells when compared to the CD44 low cells, including genes related to the Notch, transforming growth factor (TGF)β, fibroblast growth factor (FGF), Hedgehog, Wnt and pluripotency maintenance pathways. The top 10 upregulated genes with the highest fold change (>2.0) in CD44 high in relation to the CD44 low fractions, in both the SCC4 and SCC9 cell lines, are presented in Table II and Figs. 2 and 3.

The Frizzled-1 (FZD1; Wnt pathway), signal transducer and activator of transcription 3 (STAT3; pluripotency pathways) and GLI3 (Shh pathway) genes were >6-fold upregulated in the SCC4 CD44 high and SCC9 CD44 high cells in relation to the CD44 low cells. Thus, we then selected to knockout the GLI3 gene in order to investigate the role of this transcription

| Gene     | GenBank     | Primers 5'→3' | Temperature (˚C) | Product size (bp) |
|----------|-------------|---------------|------------------|------------------|
| POU5F1   | NM_001173531.1 | F: acttcactgcactgctacctc  R: aggttctttctctcctctctc | 62 | 159 |
| NANOG    | NM_024865.2  | F: catctggaacctcagctgtaaca  R: ttgctttttcgccaggggt | 62 | 99  |
| CD133    | NM_001145848 | F: ggcggagtagctacaaaactc  R: egcctctctacgtgtaatttt | 62 | 71  |
| CD44     | NM_000610.3  | F: caacgctgggagaaatacctc | 60 | 226 |
| BMI1     | NM_005180.8  | F: gctgccgaatgcccaatcct | 60 | 189 |
| SNAI1    | NM_003068.4  | F: caaggaatacctcagctcgg  R: cactgtgggtgccctggg | 60 | 218 |
| S100A9   | NM_002965.3  | F: aaagagctgctgagagaaaga  R: gtgtcaggctccatctca | 60 | 92  |
| IVL      | NM_005547.3  | F: caagacttcaaccacsctct  R: tagcggacccgaaaataagtt | 60 | 185 |
| GAPDH    | NM_001256799 | F: gcagctggtgcatacactgta  R: ccacaccctctgtgctgta | 60 | 162 |
factor in stemness, as well as in cell proliferation, invasion and apoptosis.

**GLI3 knockdown decreases clonogenicity, as well as the epithelial and mesenchymal CSCLC fractions in the SCC9 cell line.** The SCC9 cell line transfected with the shRNA (sequence 2) for GLI3 gene silencing exhibited a decrease in the expression of GLI3 of approximately 80% at both the mRNA (Fig. 4A) and protein (Fig. 4B) level. To assess the effects of GLI3 gene silencing on CSCLC fractions previously identified in OSCC (14), the cells transfected with shRNA-GLI3 and the control cells were stained for CD44 and ESA and analysed by flow cytometry in 3 different subpopulations: The CD44$^{\text{high}}$/ESA$^{\text{low}}$ (EMT-CSCLC), CD44$^{\text{high}}$/ESA$^{\text{high}}$ (EPI-CSCLC) and CD44$^{\text{low}}$ (NON-CSCLC) populations. GLI3 knockdown significantly decreased the percentage of CD44$^{\text{high}}$/ESA$^{\text{low}}$ (P=0.02) and CD44$^{\text{high}}$/ESA$^{\text{high}}$ (P<0.0001) cells and consistently increased the percentage of CD44$^{\text{low}}$ cells (P<0.0001) (Fig. 4C and D).

To determine whether the decrease in the CD44$^{\text{high}}$/ESA$^{\text{low}}$ (EMT-CSCLC) and CD44$^{\text{high}}$/ESA$^{\text{high}}$ (EPI-CSCLC) cell populations was associated with a functional decrease in stemness, the cells transfected with shRNA-GLI3 and the control cells were plated to determine their clonogenicity and sphere formation ability. As shown in Fig. 4E and F, GLI3 knockdown was associated with significantly lower numbers of colonies and spheres formed (P=0.03 and P=0.004, respectively).

We also examined whether the decrease in stemness in the shRNA-GLI3-transfected cells was associated with the decreased expression of stem cell- and EMT-related genes. As expected, GLI3 gene silencing resulted in a significant decrease in CD44 ($P<0.0001$, $BMI1$ ($P<0.0001$, $POU5F1$ ($OCT4$) ($P<0.0001$)
and SNAI2 (SLUG) \((P<0.001)\) gene expression in relation to the control cells. Additionally, the shRNA-GLI3-transfected cells exhibited an increased gene expression of the epithelial differentiation markers, IVL and S100A9 (Calgranulin B) in relation to the control cells, indicating a shift towards differentiation (Fig. 4G).

**Downregulation of GLI3 reduces cell proliferation and invasion.** Subsequently, we performed BrdU and proliferation curve assays to address the effects of GLI3 knockdown on the proliferation rates of the SCC9 cells. As demonstrated in Fig. 5A and B, the SCC9-shRNA-GLI3-transfected cells exhibited a significant decrease in the number of BrdU-positive cells \((P=0.01)\) in relation to the control cells. Moreover, there was a significant decrease in the number of shRNA-GLI3-transfected cells after 48 \((P=0.001)\), 72 \((P=0.0079)\), 96 \((P=0.0068)\) and 120 h \((P=0.001)\) when compared to the control cells (Fig. 5C). No differences in the number of Annexin V-positive cells were observed between the control and shRNA-GLI3-transfected cells (Fig. 5D). Invasion assay revealed significantly lower numbers of invasive shRNA-GLI3-transfected cells in relation to the control cells \((P=0.03)\) (Fig. 5E and F).

**GLI3 protein expression in OSCC samples and its association with patient clinicopathological characteristics.** GLI3 protein expression was detected in the cytoplasm and nucleus of normal and malignant epithelial cells. In non-tumour margins, GLI3 expression was observed mainly in the basal and suprabasal cells (Fig. 6A). In the OSCC samples, GLI3 was highly expressed in the tumour islands, as well as in isolated neoplastic cells. GLI3 was considered to have a low expression \((48,88\%)\) in 22/45 OSCC samples and a high expression \((51,12\%)\) in 23/45 samples. Invasive OSCC areas were highly positive for GLI3.

The association between GLI3 expression and the patient clinicopathological characteristics, as well as survival was evaluated and is described in Table III and Fig. 6D. There were no significant associations between GLI3 protein expression and metastasis \((pN)\), or blood, lymphatic and/or perineural invasion. However, a GLI3 \(^{high}\) expression was associated with tumour size T3/T4 \((P=0.03)\). To determine the prognostic significance of GLI3 in patients with OSCC, Kaplan-Meier survival analysis was performed. There were no significant differences observed in the 5-year survival rate of patients with a GLI3 \(^{high}\) expression when compared to those with a GLI3 \(^{low}\) expression \((P=0.15)\) (Fig. 6D).

**Discussion**

The primary aim of this study was to evaluate the differential expression of genes related to stem cell signalling pathways in CD44\(^{high}\) in relation to CD44\(^{low}\) cells. The upregulation of receptors and transcription factors belonging to the Wnt, TGFβ,
Notch, SHH and pluripotency maintenance of the stem cells was observed in the CD44\textsuperscript{high} cells. This observation demonstrates that in OSCC, the aberrant and constitutive activation of developmental signalling pathways in CSCLC may favor the acquisition and maintenance of malignant behavior. Secondly, we explored the role of \textit{GLI3} in OSCC stemness, proliferation and invasion, speculating that this gene, among others that were overexpressed in both cell lines, could be important to oral CSCLC. The clonogenic ability, sphere formation and number of CSCLCs with epithelial and mesenchymal phenotypes, identified by the surface markers, CD44\textsuperscript{high}/ESA\textsuperscript{high} and CD44\textsuperscript{high}/ESA\textsuperscript{low}, respectively, were significantly reduced in the cells in which \textit{GLI3} was knocked down. Of note, the downregulation of the stemness genes, \textit{BMI1}, \textit{POU5F1} (OCT4) and \textit{EMT} gene \textit{SNAI2} (SLUG), was observed, as well as an increase in the expression of the epithelial differentiation markers, \textit{S100A9} (Calgranulin B) and \textit{IVL}. Taken together, these results indicate that \textit{GLI3} is a transcription factor that may play an important role to play in the maintenance of CSCLCs in OSCC and may thus be considered a potential target for the eradication of CSC fractions in OSCC.

CSCs play an important role in OSCC development and progression; different cell surface markers have been used to identify and characterise these cells (5,13,14). In this study, we used the CD44 marker to isolate the CD44\textsuperscript{high} (CSCLC) and the CD44\textsuperscript{low} (NON-CSCLC) populations, the first being associated with an increased ability to form spheres and colonies with holoclone morphology and the upregulation of the stem cell-related genes, \textit{POU5F1} (OCT4) and \textit{BMI1}. CD44 is the most frequently observed CSC marker. It was used by Prince \textit{et al} to identify the CSC population in OSCC, which exhibited a high \textit{BMI1} gene expression, as well as an exclusive ability to form tumours in immunodeficient mice and to recreate tumour heterogeneity (5). We have previously demonstrated that SCC9 CD44\textsuperscript{high} cells are able to produce a tumour in immunodeficient mice, even at lower concentrations, and that the derived tumours exhibit a high expression of CD44, cytokeratin19, β-catenin and E-cadherin (51), demonstrating that this cell line and CD44 are suitable for the investigation of CSCLCs in OSCC.

CD44 is a glycoprotein receptor that binds to hyaluronan (HA) and can vary in size due to post-translational modifications and alternative splicing (11). This receptor actively interacts with the extracellular matrix (HA, collagen and fibronectin) and activates intracellular events that promote resistance to apoptosis, EMT, migration and metastatic...
CD44 also plays an important role in the crosstalk between CSCs and their niche, including the activation and recruitment of resident cells to form CSC niches and pre-metastatic niches (54).

In cancer, to sustain their stem cell properties, CSCs upregulate several signalling pathways that control normal stem cells, including the Notch, Wnt, Hedgehog and the pluripotency pathways mediated by interleukin (IL)6/STAT3 (17).

In this study, we observed that the vast majority of receptors, co-receptors and transcriptional factors belonging to these pathways were upregulated in the CD44<sup>high</sup> cells in relation to the CD44<sup>low</sup> cells in OSCC and may thus contribute to the maintenance of stemness in this malignancy.

Notch signalling, a key regulator of stem cell fate, is frequently overexpressed in various types of cancer and contributes to angiogenesis and drug resistance via interaction...
with oncogenic pathways (17,55). In this study, we observed an upregulation in the expression of NOTCH2, PSEN1, PSEN2 and PSENEM in the CD44high cells. Zhao et al (2016) observed that pharmacological Notch1 inhibition was associated with a
Table III. Association of GLI3 protein expression with the clinicopathological characteristics of patients with oral squamous cell carcinoma.

| Clinicopathological characteristics | Number of cases | GLI3 expression | P-value |
|-------------------------------------|-----------------|-----------------|---------|
| Age (years)                         |                 |                 |         |
| >60                                 | 17              | 9               | 8       | 0.76   |
| <60                                 | 28              | 13              | 15      |        |
| pT classification                   |                 |                 |         |
| T1/T2                               | 27              | 17              | 10      | 0.03   |
| T3/T4                               | 18              | 5               | 13      |        |
| pN classification                   |                 |                 |         |
| N+                                  | 29              | 13              | 16      | 0.54   |
| N-                                  | 16              | 9               | 7       |        |
| Blood invasion (BI)*                |                 |                 |         |
| BI+                                 | 7               | 2               | 5       | 0.40   |
| BI-                                 | 35              | 19              | 16      |        |
| Lymphatic invasion (LI)*            |                 |                 |         |
| LI+                                 | 18              | 6               | 12      | 0.12   |
| LI-                                 | 25              | 15              | 10      |        |
| Perineural invasion (PI)            |                 |                 |         |
| PI+                                 | 19              | 9               | 10      | 1.00   |
| PI-                                 | 26              | 13              | 13      |        |

*Missing patient data. Values in bold indicate statistical significance (P<0.05; P-values were calculated using Fisher’s exact test).

reduction in OSCC CSC fractions and self-renewal capacity, as well as with an increase in the response to conventional therapy (56), bringing with it the promise of an OSCC therapy. In addition, Notch-mediated signalling is associated with the induction of EMT under hypoxic conditions in OSCC (57). It is also associated with high mortality and T classification in OSCC, as well as with an increase in cell migration and invasion (58,59).

Wnt signalling plays an important role during embryogenesis and in the determination of cell fate during development (60). The activation of the canonical Wnt pathway begins when the ligand binds with the Frizzled receptors and co-receptors (low-density lipoprotein receptor-related protein (LRP)) which then leads to β-catenin stabilization and translocation to the nucleus. This results in the transcriptional activation of tumour-promoting genes, including CCND1, MYC, MMP7, and CD44 (61). In this study, a large number of genes related to the Wnt pathway, such as FZD receptors (FZD1 and FZD2), co-receptors (LRP6), and transcription factors (BCL9L, CTNNB1, NFAT5, NFATC3, and TCF7L1), were overexpressed in the CSCLC fraction. This result infers that this pathway may play an important role in the maintenance of these cells in OSCC. In fact, it was previously demonstrated that CSCs in head and neck squamous cell carcinoma isolated based on Hoechst dye exclusion exhibited an aberrant activation of the Wnt/β-catenin pathway (62). The strength of their findings supports the concept that these cells subpopulations are lost through cell differentiation. This was further confirmed by the upregulation of the epithelial differentiation markers, S100A9 (Calgranulin B) and IVL in the shRNA-GLI3-transfected cells. Prince et al (5) observed that in tumours derived from CD44high cells, well differentiated areas negative for CD44 exhibited a strong expression of IVL. The loss of clonogenicity, increase in CD44low fraction used to identify CSCs are targets of this pathway, including CD44, CD24 and ESA, which can contribute to the enrichment of this population (63). Additionally, this pathway plays an important role in the induction of the EMT, in which cells necessarily acquire CSCs traits and exhibit an upregulation of SNAI1, ZEB1 and ZEB2 (61,63).

One of the mechanisms used by the SHH pathway, which can contribute to cancer development, is the upregulation of the transcription factors, GLI1, GLI2 and GLI3 (19). The majority of the studies in the literature have investigated the role of GLI1 in cancer, as it acts as a transcription activator in cancer. However, GLI proteins are highly dynamic and function in a contextual and combinatorial manner (64). For example, GLI3 can function as either a transcription activator or inhibitor, depending on the presence or lack of SHH ligands (19,28).

In the absence of ligands, the GLI protein function is turned off, GLI1 is not transcribed and GLI2/GLI3 are processed in a truncated repressor protein form that binds to the GLI sites in the SHH promoters, leading to the inhibition of target genes (19,28). On the other hand, with the presence of SHH ligands (canonical activation), GLI2/GLI3 in its activated form (full-length) is produced and positively regulates genes which control cell proliferation (CCND1, MYC), cell death (BCL2), EMT (e.g. SNAI), angiogenesis (e.g. ARGL1/2) and stemness (SOX2, NANOG) (65,66). In addition, GLI1 is a direct target of GLI2/GLI3 and favours malignant behavior (63).

Some studies have implicated the SHH pathway in CSC regulation and maintenance in addition to participating in metastatic progression and the acquisition of the EMT phenotype (38,67). Its inhibition results in a decrease in stem cell propagation and cell renewal (68). For these reasons, natural and synthetic antagonists of SMO and GLI proteins are being tested to evaluate their efficacy alone or in combination to target the CSC in a wide range of malignant tumours (28). For example, the combined target therapy of PI3K/Akt/mTOR and SMO inhibitor was associated with a decrease in CSC self-renewal by inhibiting the NANOG, Pou5f1 (Oct4), Sox2, Myc and GLI1 transcription factors in pancreatic cancer (69). In non-small cell lung cancer (NSCLC), GLI1 inhibition abrogated the CSC population and cooperated with EGFR inhibitors to impair the viability of malignant cells (70).

Biddle et al (2011) have demonstrated that CSC in OSCC can switch between two different phenotypes. One of the said phenotypes is preferentially epithelial and proliferative, characterised by the phenotype CD44high/ESAhigh (EPI-CSC); the other is preferentially migratory, with the CD44low/ESAlow (EMT-CSC) phenotype (14). In this study, we observed that GLI3 gene silencing was able to decrease the percentage of CD44high/ESAlow and the CD44low/ESAlow cells, which was then followed by an increase in CD44low cells. Moreover, the clonogenic ability, primarily a property of the CD44high/ESAlow cells, was strongly inhibited in the cells transfected with shRNA-GLI3. This supports the concept that these cell subpopulations are lost through cell differentiation. This was further confirmed by the upregulation of the epithelial differentiation markers, S100A9 (Calgranulin B) and IVL in the shRNA-GLI3-transfected cells. Prince et al (5) observed that in tumours derived from CD44high cells, well differentiated areas negative for CD44 exhibited a strong expression of IVL. The loss of clonogenicity, increase in CD44low fraction
and increase in the mRNA expression of the differentiation markers described above indicates that the SHH signalling pathway may function as a form of ‘differentiation therapy’ in OSCC (71,72) and may potentiate the effects of therapeutic agents such as cisplatin. However, additional studies are warranted to support this concept.

We also observed that the levels of stemness-related genes, BMI1 and POU5F1 (OCT4), were downregulated following GLI3 knockdown. BMI1 is a member of the polycomb repressive complex 1 and plays an important role in CSC self-renewal, tumour initiation, undifferentiated state maintenance and therapeutic resistance in OSCC (73). Nør et al (74) demonstrated that OSCC treatment with cisplatin promoted enrichment of the CSC fraction. It was also shown that the BMI1 gene actively participated in the acquisition of resistance, which was influenced by IL6 (74). POU5F1 (OCT4) also plays an important role in CSC and its overexpression, increases the capacity for tumour initiation, tumour sphere formation, invasion and the acquisition of the EMT phenotype in OSCC cell lines (75,76).

Of note, sphere formation assay, normally used to investigate the ‘functional’ state of CD44high/ESAlow fractions, has also been shown to be reduced in cells transfected with shRNA-GLI3. In addition, in this study, we observed a significant decrease in the mRNA expression of SNAI2 (SLUG), a transcription factor that participates in the EMT process. The EMT-CSC fraction in OSCC exhibits significant resistance to radiation and various chemotherapeutic agents (6,10). As it is responsible for tumour invasion and metastasis, therapies that can reduce or eradicate this cell fraction are greatly needed (77). Considering our results as a whole, they highlight the important role that GLI3 plays in controlling CSC self-renewal, its distinct phenotypes in OSCC and its involvement with genes involved in the maintenance of these subpopulations.

Also of note, a significant decrease in both cellular proliferation and invasion was observed following GLI3 gene silencing. To the best of our knowledge, this is the first study to demonstrate that GLI3 participates in the regulation of both processes in OSCC. Multiple oncogenic pathways can also increase GLI function (non-canonical activation), including K-ras, PI3K/Akt and TGFβ (78-80), contributing to tumour development and formation. It should be noted that the increase in cyclin-D2 observed after SHH activation depends on GLI3 activity (18). Thus, it can be speculated that the inhibition in cell proliferation observed in GLI3 knockdown cells may be the result of the modulation of different target genes, including cyclins and the GLI1 protein, which in turn activate pro-proliferative genes. Shih et al recently demonstrated that GLI1 expression was induced by the oncogene, ROS1, leading to an increase in cellular proliferation but not in cell migration and invasion (81). Nevertheless, additional studies are required in order to elucidate the molecular targets of GLI3 which control cell proliferation.

In this study, GLI3 inhibition decreased cellular invasion in OSCC. We can speculate that the reduction in the CSCCLC-EMT fraction may have contributed, possibly via SLUG downregulation. The EMT process is associated with an enhancement in motility and cellular invasion (82), contributing actively to tumour progression. Yan et al (2011) have observed that the pharmacological inhibition of Shh/Gli pathway leads not only to the inhibition of cellular proliferation by the downregulation of GLI1, GLI2, CCND1 and BCL2 and upregulation of caspase-3, but also to a significant decrease in cell migration (83).

In this study, non-tumoural margins exhibited a weak GLI3 expression, mainly located in the basal cell layer, where the normal epithelial stem cells reside. GLI3 expression was correlated with tumour size, although no association with lymph node metastasis or overall survival was found. There seems to be only one study in the literature evaluating GLI3 expression in OSCC; the authors found that GLI3 is weakly/weakly expressed in OSCC. They did not find any correlation to survival in a cohort of 60 patients (52). We believe that additional studies are required to investigate the prognostic value of GLI3 in OSCC.

Taken together, the findings of this study demonstrate that the signalling pathways which control normal stem cells are aberrantly upregulated in CSCLC, isolated based on CD44 expression in OSCC. Moreover, GLI3 inhibition leads to a significant decrease in the number and function of the CD44high/ESAhigh (EPI-CSCLC) and CD44high/ESAlow (EMT-CSCLC) cell fractions and an increase in the CD44low subpopulation. These results indicate the relevant role this gene plays in CSC self-renewal, maintenance and differentiation. In addition to its effects on stemness, GLI3 also inhibits cell proliferation and invasion, both processes which are necessary for tumour progression. This study highlights that GLI3 is a possible target to be investigated in the future, either isolated or in combination with other drugs, for therapies based on CSCs in OSCC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

MFSDR was the major contributor to the conceptualization and design of the project, acquisition, interpretation and analysis of all data and writing, revising and formatting of the manuscript. LM contributed with acquisition, interpretation and analysis of the majority of the data with cell culture and immunohistochemistry, also collaborated with writing and formatting the manuscript. NPaA provided and analysed flow cytometry data. DH contributed to the acquisition of the cell culture data. COR contributed to the FACS analysis, and to the writing, drafting and critical revision of the manuscript. RAM, TNT, RRG and EET provided the clinical data and samples.
for immunohistochemical analysis. FDN oversaw the study conceptualization and design, writing, drafting and critical revision of the manuscript. All authors have read and approved the publication of this version.

Ethics approval and consent to participate

The study was performed with approval from the Brazilian National Ethics Committee (Process #16491) and in accordance with the Declaration of Helsinki. The collection of samples, clinical data, and histopathological analysis was performed after acquisition of a signed informed consent form from each patient.

Patient consent for publication

Not applicable.

Competing interests

All authors declare that there are no competing interests.

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