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Stanniocalcin 2 contributes to aggressiveness and is a prognostic marker for oral squamous cell carcinoma

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Running title: Stanniocalcin 2 in oral cancer.

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ABSTRACT

Stanniocalcin 2 (STC2), a glycoprotein that regulates calcium and phosphate homeostasis during mineral metabolism, appears to display multiple roles in tumorigenesis and cancer progression. This study aimed to access the prognostic value of STC2 in oral squamous cell carcinoma (OSCC) and its implications in oral tumorigenesis. STC2 expression was examined in 2 independent cohorts of OSCC tissues by immunohistochemistry. A loss-of-function strategy using shRNA targeting STC2 was employed to investigate STC2 in vitro effects on proliferation, apoptosis, migration, invasion, epithelial-mesenchymal transition (EMT) and possible activation of signaling pathways. Moreover, STC2 effects were assessed in vivo in a xenograft mouse cancer model. High expression of STC2 was significantly associated with poor disease-specific survival (HR: 2.67, 95% CI: 1.37-5.21, p=0.001) and high rate of recurrence with a hazard ratio of 2.80 (95% CI: 1.07-5.71, p=0.03). In vitro downregulation of STC2 expression in OSCC cells attenuated proliferation, migration and invasiveness while increased apoptotic rates. In addition, the STC2 downregulation controlled EMT phenotype of OSCC cells, with regulation on E-cadherin, vimentin, Snail1, Twist and Zeb2. The reactivation of STC2 was observed in the STC2 knockdown cells in the in vivo xenograft model, and no influence on tumor growth was observed. Modulation of STC2 expression levels did not alter consistently the phosphorylation status of CREB, ERK, JNK, p38, p70 S6K, STAT3, STAT5A/B and AKT. Our findings suggest that STC2 overexpression is an independent marker of OSCC outcome and may contribute to tumor progression via regulation of proliferation, survival and invasiveness of OSCC cells.

Keywords: Stanniocalcin 2; oral cancer; prognosis; tumor progression.

1. Introduction

Oral squamous cell carcinoma (OSCC), the most common tumor in the head and neck region, displays high incidence (more than 350,000 new cases every year) and rates of mortality (approximately 177,000 deaths every year) [1]. Since OSCCs are asymptomatic at early stages, most patients are diagnosed at advanced stage of disease, resulting in a 5-year survival rate of approximately 50% [2]. Tumor invasion, lymph node metastasis and high rates of locoregional recurrence are the main factors leading to the death of patients with OSCC [3]. Therefore, biomarkers of early diagnosis, prognosis and post-therapeutic monitoring are essential to improve clinical management of OSCC. Indeed, several studies have reported potential biomarkers for predicting OSCC progression and prognosis, however, their practical usefulness remains very limited [4].

Stanniocalcins are glycoprotein hormones involved in calcium and phosphate homeostasis [5]. Two isoforms are known in mammalians, stanniocalcin 1 and stanniocalcin 2 (STC2), which are encoded by distinct genes [6]. Besides the canonical function in calcium/phosphate regulation, promoting inorganic phosphate-induced mineralization and preventing ectopic calcification, STC2 has been related to proliferation, apoptosis, invasion and epithelial-mesenchymal transition (EMT) when upregulated in tumors [7-9]. A recent systematic review and meta-analysis revealed that high STC2 levels are frequently associated with advanced disease stage, lymph node metastasis and vascular invasion, factors that suggest worse prognosis, and most important, the high expression of STC2 significantly predicted poor overall survival [10]. However, in both female and male breast cancers, STC2 expression is a favorable prognostic factor, extending disease-free and overall survival [11-13]. In head and neck squamous cell carcinomas (HNSCC), STC2 expression level was associated with aggressiveness, and in vitro studies confirmed its role regulating apoptosis, proliferation, migration and invasion of HNSCC cells [14]. Upregulation of miR-206 on HNSCC suppressed STC2 biological effects via PI3K-AKT signaling pathway [15]. Although the

emerging literature suggests the clinical importance of STC2 in human cancers as potential prognostic marker or therapeutic target, there are still very little molecular details defining the mechanisms of action of those proteins in the control of oral tumorigenesis.

In the current study we examined the expression levels of STC2 in OSCC clinical samples to determine the prognostic impact of this protein for OSCC patients. Moreover, we assessed whether STC2 knockdown influences OSCC cell proliferation, apoptosis, migration, invasion and EMT in vitro and growth of xenograft tumors in vivo. The STC2 effects on the main signaling pathways, which is known to promote oral tumorigenesis, were verified using the Luminex technology.

2.Materials and Methods

STC2 mRNA levels in previously published microarrays. To examine the expression pattern of STC2 in published microarray data, we performed a meta-analysis using data mining from the Oncomine® Research Premium Edition database (https://www.oncomine.org). The first step was to identify previously published microarray gene expression data comparing normal oral mucosa and OSCC. Filters for selection of the data were studies that included STC2 in the analysis, comparing cancer vs normal tissue, cancer type (squamous cell carcinoma) and primary tumor sites in the oral cavity. After applying those filters, a total of 9 datasets (312 samples) from published studies remained. The expression level was considered the median rank for the gene across each of the analysis, and the given p-value was based in the median-ranked analysis at a cut off 0.01 ($p<0.01$).

Tissue samples and clinicopathological data. This study included 2 independent cohorts of OSCC. The cohort 1 included 100 primary OSCCs diagnosed and treated by radical surgery at 2 reference hospitals (UOPECCAN and CEONC Cancer Hospitals) in Cascavel-Parana, between 1998 and 2008. The clinicopathological

features of these patients were described previously [16]. The cohort 2 included 119 patients with OSCC treated at the Hospital Bom Pastor in Varginha-Minas Gerais, between 1998 and 2014. In this cohort, a male prevalence was observed (n=86, 72.3%) and the average age was 60.4 ± 11.4 years (range 21-87 years). Smoking was reported by 84 (70.6%) patients and alcohol consumption by 57 (47.9%). Tumors were localized in the tongue (n=95, 79.8%) or floor of mouth (n=24, 20.2%). Regarding clinical stage, 15 patients were stage I (12.6%), 32 were stage II (26.9%), 27 were stage III (22.7%) and 45 were stage IV (37.8%). Surgery as monotherapy was performed in 17 (14.3%) patients, whereas 50 (42%) were treated by a combination of surgery and postoperative radiotherapy and 52 (43.7) by a combination of surgery and postoperative radiotherapy and chemotherapy. A total of 21 (17.6%) tumors were classified as well-differentiated, 87 (73.1%) as moderately-differentiated and 11 (9.2%) as poorly-differentiated, according to the WHO grading system. During follow up, 24 (20.2%) patients developed recurrence (17 local, 5 regional and 2 distant). The overall survival ranged between 8 to 116 months, with a mean of 84 months. Twenty patients (17.1%) died due to the tumor. The study was approved by the ethics review board of each of the hospitals affiliated with the collaborative study, and revised by the Human Research Ethics Committee of the School of Dentistry, University of Campinas (protocol number: 090/2011).

Immunohistochemistry. Three-µm tissue sections were deparaffinized and hydrated using a graded series of ethanol until water. Sections were then subjected to antigen retrieval with 0.01 M citrate buffer pH 6.0 in an electric pressure cooker and incubation with 3% aqueous hydrogen peroxide for 15 min to quench endogenous peroxidase. Two anti-STC2 antibodies were used : anti-STC2 monoclonal antibody (Abcam, USA) diluted 1:50 (for the first cohort) and anti-STC2 polyclonal antibody (Thermofisher, USA) diluted 1:100 (for the second cohort). After primary antibody, slides were incubated with EnVision reagents (EnVision® + Dual Link System-HRP kit, Dako, USA) and developed with 0.6 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (Dako,

USA) and Mayer's hematoxylin as counterstained. The control reactions were performed by the exclusion of the primary antibody.

Immunoexpression of STC2 was semiquantitatively assessed taking in consideration the number of positive cells and the intensity of staining. The number of positive cells was graded in quartiles (0, 1: 1%-25% staining, 2: 26%-50% staining, 3: 51%-75% staining, and 4: 76%-100% staining), and the intensity of staining was scored in 0: negative, 1: weak staining, 2: moderate staining and 3: strong staining. The 2 grades were added together, resulting in scores that ranged from 0 to 7, and were classified as low (0-4 score) and high (5-7 score) expression for comparative analysis.

Cell culture. The immortalized normal human gingival keratinocyte cell line (HGK) was cultured in serum-free, low calcium media (Gibco's Keratinocyte-SFM; Invitrogen, USA) containing bovine pituitary extract, epidermal growth factor and antibiotics. Human OSCC cells SCC-4, SCC-9, SCC-15 and SCC-25 were purchased from the American Type Culture Collection (ATCC, USA) and cultured as recommended in a 1:1 mixture of Dulbecco's modified Eagle's media and Ham's F12 media (DMEM/F12; Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS), 400 ng/ml hydrocortisone (Sigma-Aldrich, USA) and antibiotics. HSC-3, a highly invasive OSCC cell line, was obtained from Japan Health Sciences Foundation (Osaka, Japan) and cultured in DMEM/F-12 media (Invitrogen, USA) supplemented with 10% FBS, 50 μg/ml ascorbic acid (Sigma-Aldrich, USA), 400 ng/ml hydrocortisone (Sigma-Aldrich, USA) and antibiotics. All cells were growth at 37°C in a humidified atmosphere of 5% CO_2. Cells were regularly tested for mycoplasma infection.

Quantitative PCR (qPCR). Total RNA from cell lines was isolated with TRIzol® reagent (Invitrogen, USA) according to the manufacturer's protocol. Following DNase I treatment in order to eliminate genomic DNA contamination, 1 μg of total RNA per sample was used to generate cDNA using Oligo-dT (Invitrogen, USA) and reverse

transcriptase (Superscript II RT enzyme, Invitrogen, USA). Resulting cDNAs were subjected to qPCR using specific primers and PowerUp™ SYBR® Green Master Mix (Applied Biosystems) in the StepOnePlus Real Time PCR (Applied Biosystems). Gene expression was determined by the $^{\Delta\Delta}$CT method and the housekeeping gene PPIA (cyclophilin A) was used as a reference gene for data normalization. All primers used in this study are described in Supplementary Table 1.

Stable short hairpin RNA (shRNA) knockdown of STC2. Stable knockdown of STC2 expression in OSCC cell lines was achieved by the construction of lentiviral vectors containing shRNA targeting human STC2 (MISSION® shRNA Lentiviral Transduction Particles, Sigma-Aldrich, USA) or scrambled control shRNA (MISSION® pLKO.1-puro Non-Mammalian shRNA Control, Sigma-Aldrich, USA) carrying a puromycin antibiotic resistance gene. Oligonucleotide sequence for STC2 shRNA (NM_003714.1) was 5'CCGGGAACAGTCTGAGTATTCTGATCTCGAGATCAGAATACTCAGACTGTTCTTTTTTG3'. In essence, SCC-15 and HSC-3 cells grown in a 12-well plate at confluence of 70% were incubated with control or STC2 shRNA lentiviral particles at a multiplicity of infection (MOI) of 1.5 in culture media containing 8 mg/ml of polybrene (Sigma-Aldrich, USA) for 4 h. After washing with PBS, cells were cultured in fresh media for an additional period of 48 h. Cells were then cultured for 10 days in the presence of 1 µg/ml of puromycin dihydrochloride (Sigma-Aldrich, USA) to select resistant cells. Efficacy of STC2 knockdown was determined by qPCR and western blot.

Western blot analysis. Proteins extracted from silenced cells were separated by 10% or 12% SDS-PAGE under reducing conditions and transferred onto nitrocellulose membranes. The membranes were blocked with 10% non-fat dry milk in PBS containing 0.1% Tween-20, rinsed in the same buffer, and incubated for 2 h with the primary antibodies: anti-STC2 (1:100, Abcam, USA), anti-AKT (1:1000, Cell Signaling, USA),

anti-pAKT (pS473, 1:1000, Cell Signaling, USA), anti-mTOR (1:1000, Cell Signaling, USA), anti-pmTOR (pS2448, 1:1000, Cell Signaling, USA), anti-PI3K (1:1000, Cell Signaling, USA), anti-E-cadherin (1:1000, clone 24E10, Cell Signaling, USA), anti-vimentin (1:1000, clone D21H3, Cell Signaling, USA), anti-Snail1 (1:1000, clone C15D3, Cell Signaling, USA), anti-Snail2 (1:1000, clone C19G7, Cell Signaling, USA), anti-β-actin (1:30000, Sigma-Aldrich, USA) and anti-GAPDH (1:3000, Santa Cruz, USA). After washing, the protein bands were detected using enhanced chemiluminescence western blotting system (GE Healthcare, USA) and signals captured with an Alliance 9.7 instrument (UVITEC, UK).

Bromodeoxyuridine (BrdU) incorporation assay. Proliferation rates were determined by measuring BrdU incorporation into DNA using the Cell Proliferation ELISA BrdU (Colorimetric) kit (Roche Applied Science, Germany). Briefly, BrdU antigen was added to the cultures in 1:10 dilution and kept for 2 h at 37°C in 5% CO_2. After incubation, the medium was removed and manufacturer's protocol was followed. Absorbance was measured at 450 nm with correction at 655 nm.

Apoptosis analysis. Apoptosis index was determined by annexin V labeling. Briefly, cells were harvested, washed and resuspended in the binding buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$) containing annexin V-PE and 7-AAD (BD Biosciences, USA). Apoptosis was analyzed on a FACScalibur flow cytometer equipped with an argon laser (BD Biosciences, USA) and quantified as the number of annexin V-PE positive and 7-AAD negative cells divided by the total number of cells.

Invasion and migration assays. Transwell migration and invasion assays were performed in 6.5 mm inserts with 8 μm pore size (Corning, USA). For invasion assay, membranes were coated with 50 μl of myogel [17]. Starved cells were plated into the

upper chamber in 200 µl of serum-free DMEM/F12. As chemoattractant, 500 µl of complete medium was used in the lower chamber. Experiment times varied between 24 h for migration and 48-72 h for invasion assays. Assessment of migration or invasion was performed by gently removing cell in the interior part of the insert with a cotton swab. Cells on the bottom of the membrane were fixed in 10% formalin for 30 min and stained with 1% toluidine blue in 1% borax solution. The excess dye was washed out and cells were then eluted in 1% SDS solution for 5 min. Absorbance was measured at 655 nm.

Gelatin zymography for MMP-2 and MMP-9. Zymography was performed with conditioned media of the cells. Briefly, samples were submitted to 1-D electrophoresis on 10% SDS-PAGE gels containing 1 mg/ml gelatin under nonreducing conditions. Gels were stained with Coomassie blue, and gelatinolytic activity was identified as clear bands against a blue background.

Tumorigenicity assay. To assess the growth of xenograft tumors in nude mice, HSC3 cells (HSC-3 shRNA Control and HSC-3 shRNA STC2) were injected s.c. in the flank of 12-week-old nude mice (10 animals per cell line). At 4-weeks, all mice were euthanized, and tumors and multiple organs were examined grossly, collected, fixed in 10% formalin and then embedded in paraffin. At this moment, tumor size was measured with a caliper, and volumes are reported as mm^3, calculated by using the formula volume=0.5 x length x $width^2$. Five-micrometer sections were stained with hematoxylin and eosin for histopathologic analysis and subjected to STC2 immunohistochemistry. The animal experiment was reviewed and approved by the Ethical Committee on Animal Research at the University of Campinas (protocol number 5279-1/2019).

Analysis of phosphorylated proteins by **Luminex technology.** Total proteins from shRNA Control and shRNA STC2 cells were extracted and subjected to analysis of the phosphorylated proteins CREB (pS133), ERK (pT185/pY187), JNK

(pT183/pY185), p38 (pT180/pY182), p70 S6K (pT412), STAT3 (pS727), STAT5A/B (pY694/699), AKT (pS473) using a multiplex kit (Milliplex MAP Multi-Pathway #48-680MAG, Millipore, USA), according to the manufacturer's instructions. Positive and negative controls (cellular lysates provided by the kit) were included in all analyses.

Statistical analysis. Associations between immunoexpression of STC2 and clinicopathological parameters of the tumors were performed using cross-tabulation and chi-square test. Survival curves were constructed based on the Kaplan-Meier method and compared with the Log-rank test. For multivariate survival analysis, the Cox proportional hazard model with a stepwise method was applied. All functional assays were performed at least three times. Mann-Whitney U test or one-way analysis of variance (ANOVA) with post-hoc comparisons based on the Tukey's multiple comparisons test were applied. P values ≤0.05 were considered as significant.

3. Results

STC2 expression is associated with worse outcome of patients with OSCC

At first, we performed a meta-analysis with published microarray data compiled from Oncomine® Research Premium Edition database. Comparison across microarray gene expression studies showed that STC2 mRNA is significantly overexpressed in OSCC samples compared with normal oral mucosa (p=0.01). The association between STC2 expression and clinicopathological characteristics was verified in the cohort containing 100 cases of OSCC (cohort 1). All cases showed cytoplasmatic staining of STC2 in the tumor cells, with variable distribution and intensity. A high expression of STC2 was detected in 64% of the tissue samples (Fig. 1A), while low expression was found in 36% (Fig. 1B). As depicted in Table 1, the high expression of STC2 in OSCC tissues was significantly associated with advanced clinical stage (p=0.02), and a tendency towards association was observed with recurrence (p=0.07) and development of a second primary tumor (p=0.06).

We next assessed the association between STC2 expression and clinical prognosis of OSCC patients. Outcomes were categorized as disease-specific survival, time from treatment initiation until death due to cancer or last known date alive, and disease-free survival, time from treatment initiation until diagnosis of the first recurrence (local, regional or distant) or last follow-up information for those without recurrence. Only survivors with follow-up information of at least 5 years were included. In comparison with low expression, patients with high STC2 expression had a significantly shorten disease-specific survival (HR: 2.68, 95% CI: 1.56-4.62, p=0.002) and a high risk of recurrence (disease-free survival), yielding a HR of 2.37 (95% CI: 1.20-4.69, p=0.02) (Table 2). On univariate analysis, significant associations between age (p=0.02) and advanced clinical stage (p=0.02) with shorten disease-specific survival were detected (Table 2). On multivariate survival analysis, age (HR: 1,95, 95% CI: 1.12-3.38, p=0.01) and STC2 expression (HR: 2.67, 95% CI: 1.37-5.21, p=0.001) were independently associated with cancer-specific survival, whereas for disease-free survival, only STC2 (HR: 2.80, 95% CI: 1.07-5.71, p=0.03) was significantly associated (Table 3).

Validation of association of STC2 with poor survival in an independent cohort

To confirm our findings, the expression levels of STC2 were investigated in an independent set of OSCCs (cohort 2; details provided in materials and methods section), applying a different anti-STC2 polyclonal antibody. Patients with high STC2 expression showed a 5-year disease-specific survival of 46.0% compared with 84.5% for patients with low STC2 immunoreactivity, yielding a HR of 2.68 (95% CI: 1.14-6.32, p=0.02, Fig. 2A). After 5 years, 88.5% of patients classified with low STC2 expression remained without recurrence compared with 48.7% of those with high STC2 expression (HR: 2.66, 95% CI: 1.16-6.14, p=0.01, Fig. 2B).

Efficiency of STC2 knockdown in OSCC cells

We investigated the expression of STC2 in one normal oral keratinocyte and five OSCC cell lines. The STC2 mRNA levels were significantly higher in the OSCC cell lines SCC-4 ($p<0.01$), SCC-9 ($p<0.01$), SCC-15 ($p<0.0001$) and HSC-3 ($p<0.001$) compared with the spontaneously immortalized, but not transformed oral keratinocyte cell line HGK (SFig. 1). Although high, the expression of STC2 in SCC-25 did not reach a significant level. Considering SCC-15 and HSC-3 presented the highest STC2 expression among OSCC cell lines, they were chosen for testing the effects of STC2 knockdown. We then transduced SCC-15 and HSC-3 with a retroviral vector encoding a STC2 shRNA. Compared to control cells (transduced with a universal shRNA control), STC2 mRNA and protein levels were significant lower in STC2-silenced cells (Fig. 3).

STC2 controls proliferation, apoptosis, migration and invasion of OSCC cells

shRNA Control and shRNA STC2 cells were analyzed for cell proliferation, apoptosis, migration and invasiveness. As shown in Fig. 4A, the proliferation of SCC-15 (~73% reduction, $p<0.0001$) and HSC-3 (~64% reduction, $p<0.001$) was significantly reduced following STC2 knockdown. The annexin V/7-AAD labeling assay was employed to measure apoptosis of the shRNA. Flow cytometry revealed that the number of apoptotic cells in the cells expressing the specific shRNA against STC2 was significantly higher compared to respective controls (Fig. 4B). For SCC-15 shRNA STC2 cells, the increase in apoptosis rate was of approximately 40% ($p<0.01$) and for HSC-3 shRNA STC2 cells was approximately 19% ($p<0.01$). Both migration and invasion were attenuated by STC2 knockdown. Compared to shRNA Control cells, the migration of SCC-15 shRNA STC2 cells was reduced in 55% ($p<0.001$), whereas the reduction of HSC-3 shRNA STC2 cell migration was approximately of 46% ($p<0.01$) (Fig. 4C). Fewer SCC-15 (~50% reduction, $p<0.001$) and HSC-3 (~54% reduction, $p<0.001$) cells with STC2 knockdown invaded the extracellular matrix compared to their corresponding controls (Fig. 4D).

STC2 is associated with EMT

To investigate whether STC2 is associated with acquisition of EMT properties, we assessed the expression of EMT markers, including epithelial marker E-cadherin and mesenchymal marker vimentin, as well as EMT transcription factors, after knockdown STC2 expression. As indicated in Fig. 5A, cells expressing STC2-targeting shRNA had significantly higher mRNA levels of E-cadherin ($p<0.01$ for SCC-15 cells and $p<0.001$ for HSC-3 cells) and significantly lower expression of vimentin ($p<0.05$ for both SCC-15 and HSC-3 cells) compared to control cells. Analysis of EMT transcription factors showed a consistent decrease in the expression of Snail1 ($p<0.0001$ for both SCC-15 and HSC-3), Twist ($p<0.05$ for SCC-15 and $p<0.01$ for HSC-3) and Zeb2 ($p<0.001$ for both SCC-15 and HSC-3) in shRNA cells compared with controls (Fig. 5B). Zeb1 expression was only significantly downregulated in HSC-3 cells ($p<0.001$), whereas no effects on Snail2 were detected in both cell lines (Fig. 5B). In the western blot analysis, levels of E-cadherin were increased and levels of vimentin, Snail1 and Snail2 were decreased in SCC-15 shRNA STC2 cells compared to SCC-15 shRNA Control cells (Fig. 5C). In addition, we investigated MMP-2 and MMP-9 by qPCR and zymography. Levels of MMP-2 and MMP-9 mRNA were significantly lower in STC2-silenced cells compared to their corresponding control cells (Fig. 5D). In consonance, the gelatinolytic activities of MMP-2 (band with 72 kDa) and MMP-9 (band with 92 kDa) were lower in conditioned medium from shRNA STC2 cells compared to shRNA Control cells (Fig. 5E).

Expression of STC2 is reactivated in xenograft tumors

To further investigate the effects of STC2 on tumor growth in vivo, HSC-3 shRNA control and HSC-3 shRNA STC2 cells were injected subcutaneously into nude mice. The tumor nodules were visible in all mice, but no significant differences in volume were found (SFig. 2). The histological features of tumors were quite similar, and immunohistochemical analysis showed a clear cytoplasmic STC2 staining in HSC-3 shRNA STC2 cells, mainly in the peripheral cells of the tumor islands. Although some

cells were completely negative, a reactivation of STC2 in vivo was observed in the tumors formed by shRNA STC2 cells (SFig. 3).

Cell signaling in STC2 knockdown cells

A previous study demonstrated that the pro-tumorigenic effects of STC2 are mediated by PI3K-AKT-Snail signaling [14]. We therefore examined the PI3K-AKT-mTOR signaling pathway and their phosphorylation status through western blot. No differences were observed in both SCC-15 and HSC-3 control and silencing cells (Fig. 6A). In order to further investigate the effects of STC2, we analyzed key proteins associated with different pathways using a multiplex assay containing phosphorylated CREB (pS133), ERK (pT185/pY187), JNK (pT183/pY185), p38 (pT180/pY182), p70 S6K (pT412), STAT3 (pS727), STAT5A/B (pY694/699), AKT (pS473). None of the investigated proteins was consistently and statistically different between shRNA Control and shRNA STC2 cells, including AKT (Fig. 6B).

4. Discussion

Although STC2 was originally associated with calcium/phosphate regulation during mineralization, it has been speculated that it is actually an oncoprotein since its overexpression in many cancer types can predict outcome. Nonetheless, studies on STC2 roles in OSCC are still limited. In this study, we found that STC2 is overexpressed in OSCCs in comparison with normal oral mucosa, and its expression is associated with advanced clinical stage, shortened disease-free survival and poor disease-specific survival. These results are in line with previous ones regarding many solid cancers [10] and with the findings of Yang and collaborators [14] on 298 patients with HNSCC (tumors from oral cavity, oropharynx, pharynx and larynx). Interestingly, the expression levels of STC2 were significantly related to the clinical stage of tumor but there were no large alterations in the hazard ratios and its significance on multivariate compared to univariate analysis in our survival analysis, and our findings were validated with a different antibody

in an independent cohort, supporting that STC2 is a promising and independent prognostic biomarker for OSCCs.

Modulation of STC2 expression has been investigated in different contexts and cell lines. In this study, we found that STC2 knockdown inhibits proliferation, migration and invasion, and promotes apoptosis of OSCC cells. In consonance with our results, previous studies have demonstrated that STC2 regulates proliferation and colony formation of lung and cervical cancer cells [8,18], and STC2-silencing reduces the migration and invasion of colorectal cancer cells [19]. Moreover, loss of STC2 increased radiation sensitivity of nasopharyngeal carcinoma cells, resulting in accumulation of the cells at G_1 and G_2/M phases of the cell cycle [20]. Through inhibition of STC2, miR-485-5p, miR-184, miR-206 and miR-1-3p inhibited proliferation, migration and invasion of hepatocellular carcinoma, glioblastoma, HNSCC and gastric cancer cells, respectively [15,21-23]. One recent study showed that STC2 overexpression in colon adenocarcinoma, which allows its pro-tumorigenic roles, is dependent on Sp1 promoter activation [24]. In contrast, overexpression of STC2 resulted in a significant impairment of growth, migration and invasion in breast cancer cells [25,26]. In mouse xenograft models of colorectal cancer [9] and HNSCC [14] and in neuroblastoma experimental formation on the chick chorioallantoic membrane [27], STC2 accelerated tumor growth and facilitated early metastasis. However, in our xenograft model, the expression of STC2 was reactivated in most of the peripheral cells of tumor islands, and the cells transduced with STC2 shRNA had quite similar tumor growth pattern than control cells. It is possible that the complex in vivo tumor microenvironment set in this xenograft model produces regulatory factors that reactivated STC2, such as hypoxia inducible factor 1 (HIF-1), a STC2 target gene that promotes proliferation and invasion in hypoxia, a feature of this xenograft model [28,29]. Our findings support that overexpression of STC2 promotes oral tumorigenesis by regulation of proliferation, survival, migration and invasion of OSCC cells, but STC2 might play different regulatory functions in the context of cell tumor origin.

EMT comprises the biological process by which cells transit between epithelial and mesenchymal states and may acquire potent pro-tumorigenic features, including motility, invasiveness, stemness, drug-resistance and the ability to form metastases [30]. This transition can be regulated at multiple levels, but transcriptional control of gene expression by EMT-inducing transcription factors, including Snails, Zebs and Twist, is generally considered the master step in this process [31]. The main hallmarks of EMT are the loss of the epithelial cell-cell adhesion molecule E-cadherin and acquisition of mesenchymal cell marker vimentin, inducing phenotypical plasticity of cells [32]. Downregulation of STC2 was associated with gain of E-cadherin expression and loss of vimentin, suggesting EMT regulation. In addition, STC2-knockdown cells showed consistent inhibition of MMP-2 and MMP-9 and of EMT-inducing transcription factors Snail1, Twist and Zeb2. Consistent with our findings, previous studies have revealed that overexpression of STC2 may trigger EMT and MMP expression in ovarian [33], colorectal [19,34] and HNSCC [14]. In this context, STC2 was closely related with chemotherapeutic drug resistance in cervical, liver and colorectal cancer [18,34,35], and cells overexpressing STC2 showed elevated expression of the pluripotency and self-renewal markers Oct4 and Nanog [7]. Yang and collaborators [14] reported that STC2 could promote EMT in vitro through activating PI3K-AKT signaling. Unexpectedly, we were unable to detect any alteration in expression or phosphorylation of PI3K, AKT and mTOR concomitant with STC2 downregulation. Other signaling pathways frequently altered in OSCCs, including CREB, ERK, JNK, p38, p70 S6K, STAT3 and STAT5A/B, were not affected by silencing of STC2. Based on our results, we concluded that overexpression of STC2 in OSCC cells plays an important role on acquisition of EMT properties, but this effect is independent of PI3K-AKT-mTOR pathway.

In closing, our findings show that STC2 is upregulated in patients with OSCC and its overexpression is significantly and independently associated with relapse and poor outcome. In vitro downregulation of STC2 drove proliferation, survival and EMT properties associated with migration and invasiveness of OSCC cells. Therefore, the

potential clinical applications of STC2 as a prognostic marker and as a therapeutic target should be considered in OSCC.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Authors contributions

AFC, MRD, CEO, DCB, CBD, GAB, ENSG and RDC carried out data acquisition and analysis. IS-C and LMRP have contributed with the samples and their clinical information. EG, TAS, RAF and HCG have participated in the design of the study. RDC has designed and implemented the research protocol, and drafted the manuscript. All authors have critically revised the manuscript.

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Figure legends

Fig. 1. Immunoexpression of STC2 in OSCCs. Representative immunohistochemical analysis of STC2 in OSCC specimens. OSCC tumor cells showed variable distribution and intensity of STC2. (A) OSCC specimen classified as low STC2 expression and (B) sample with high expression of STC2.

Fig. 2. Kaplan-Meier curves. Curves for cancer-specific survival (A) and disease-free survival (B) of patients with OSCC of cohort 2 based in STC2 expression levels.

Fig. 3. STC2 knockdown efficiency in SCC-15 and HSC-3 cells. Cells were transduced with lentivirus expressing shRNA sequences against STC2 (shRNA STC2 cells) and control (shRNA Control cells) as outlined in the methods. shRNA STC2 cells showed a marked reduction in both mRNA (A) and protein levels (B) when compared with shRNA Control cells. *p<0.0001

Fig. 4. Knockdown of STC2 modulates proliferation, apoptosis, migration and invasion of OSCC cells. (A) Downregulation of STC2 significantly inhibited the proliferative potential of SCC-15 and HSC-3, as revealed decreased BrdU incorporation. (B) The proportion of apoptotic cells was increased in shRNA STC2 cells compared to controls, as assessed by annexin V/7AAD binding assay. (C) Migration of SCC-15 and HSC-3 cells was significantly decreased by STC2-specific shRNA, as revealed by transwell migration assay. (D) Invasion of SCC-15 and HSC-3 cells was significantly inhibited after STC2 knockdown. *p<0.01, **p<0.001, ***p<0.0001

Fig. 5. Modulation of STC2 mRNA levels affects epithelial-mesenchymal transition-related markers. (A) Downregulation of STC2 significantly increased the expression of E-cadherin while reduced vimentin in both SCC-15 and HSC-3 cells. (B) shRNA STC2 cells showed consistent decrease in the expression of Snail1, Twist and Zeb2 compared

with control cells. Zeb1 expression was only significantly downregulated in HSC-3 cells. (C) Western blot analysis confirmed STC2 downregulation in SCC-15 cells is accompanied by increased levels of E-cadherin and decreased levels of vimentin, Snail1 and Snail2. (D) Downregulation of STC2 significantly decreased the expression of MMP-2 and MMP-9 in SCC-15 and HSC-3 cells as determined by qPCR. (E) Zymography analysis of MMPs secreted by OSCC cells showed the inhibition of gelatinolytic activity of MMP-2 and MMP-9 in shRNA STC2 cells. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Fig. 6. (A) Effects of STC2 knockdown on expression and phosphorylation of PI3K-AKT-mTOR signaling proteins. Western blotting of PI3K and both phosphorylated and total AKT and mTOR in shRNA Control and shRNA STC2 cells. (B) Activation of key-proteins related to CREB, ERK, JNK, p38, p70 S6K, STAT3 and STAT5A/B pathway. Values represent mean ± standard derivation of 3 independent experiments for each cell line.

Supplementary figure legends

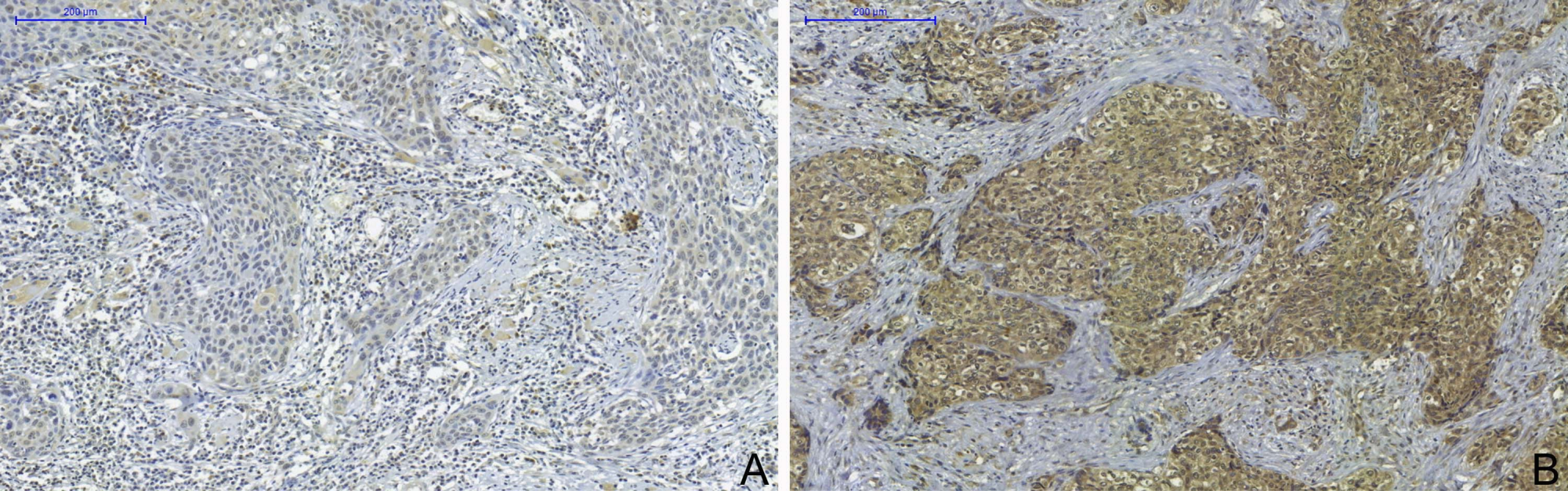
SFig. 1. STC2 is overexpressed in OSCC-derived cell lines. Total RNA from the normal human gingival keratinocyte cell line (HGK) and from 5 human OSCC cell lines (SCC-4, SCC-9, SCC-15, SCC-25 and HSC-3) were converted in cDNA and subjected to qPCR. The STC2 mRNA levels were significantly higher in OSCC cells lines than in HGK, with exception of SCC-25. 8p<0.01, **p<0.0001.

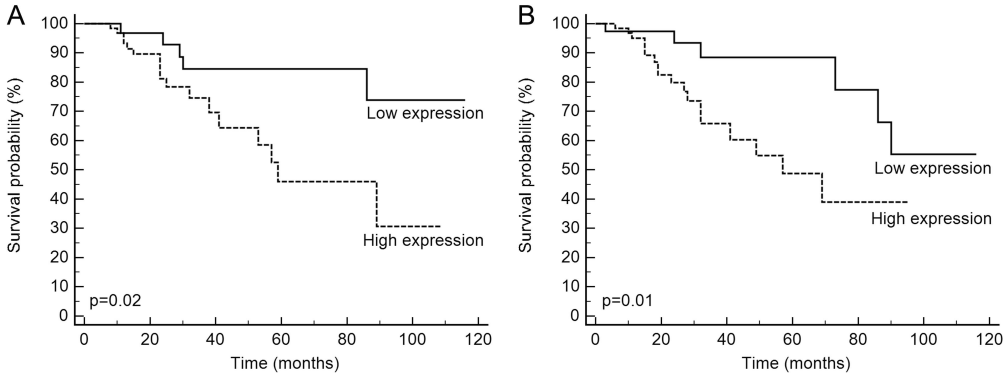
SFig. 2. The volume of xenograft tumors formed by HSC-3 shRNA Control and HSC-3 shRNA STC2 cells. No differences in volume of tumors formed by HSC-3 shRNA Control and HSC-3 shRNA STC2 cells were observed.

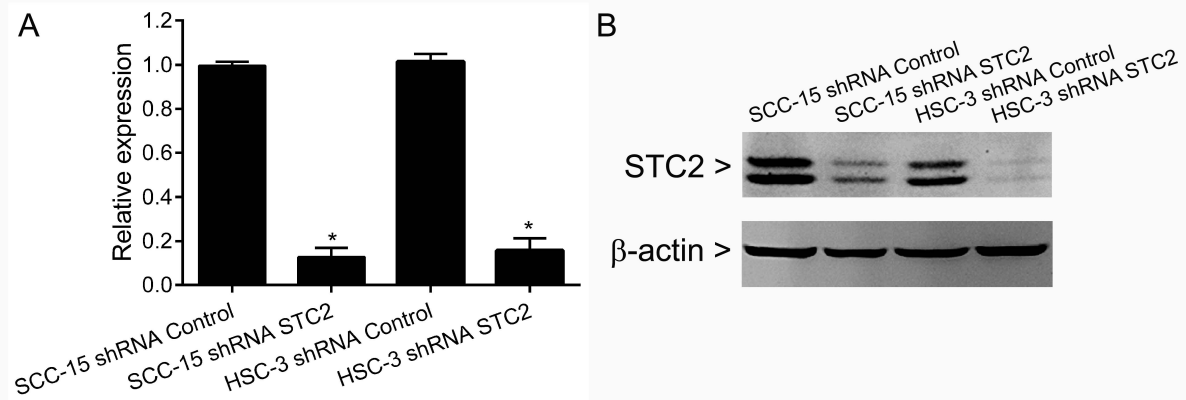
SFig. 3. Representative images of the xenograft tumors formed by HSC-3 shRNA Control cells (A and C) and HSC-3 shRNA STC2 cells (B and D). Tumors showed similar histological features, and unexpectedly, the levels of STC2 were reactivated in the peripheral HSC-3 shRNA STC2 cells of the tumor islands

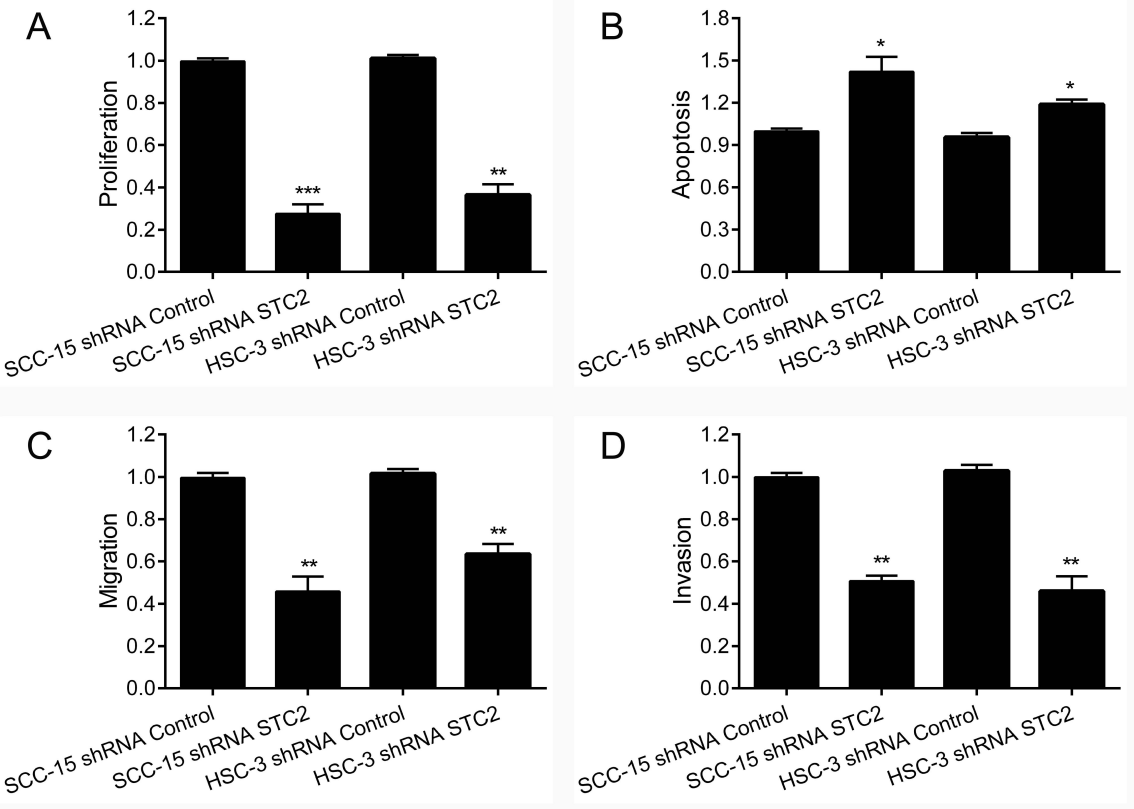
Highlights

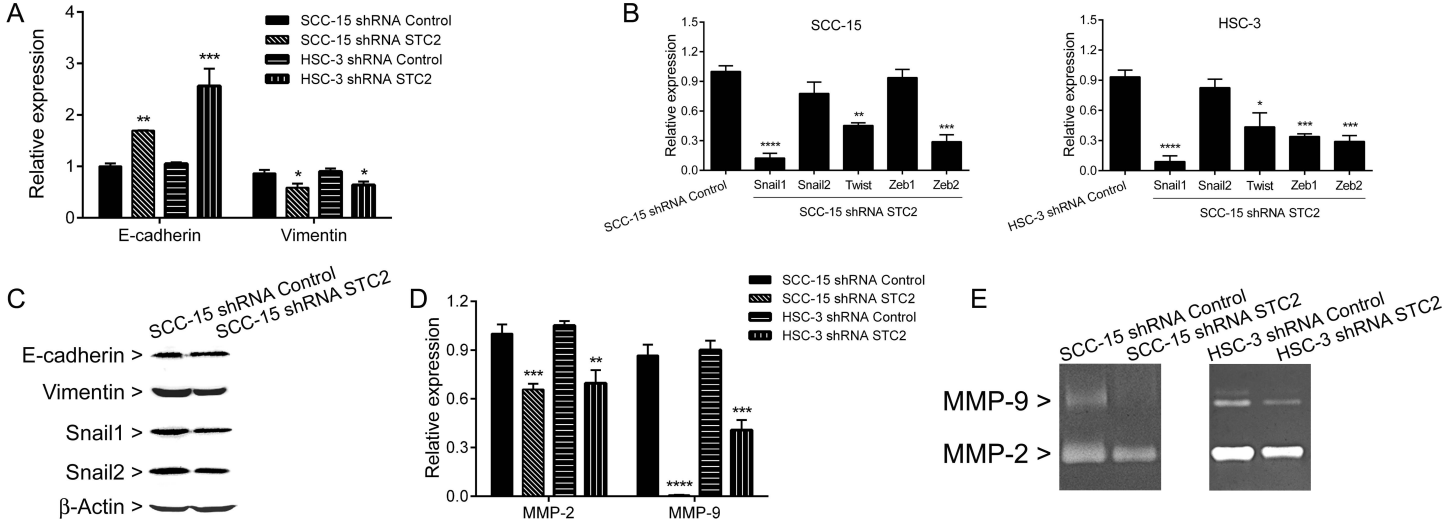
- Expression STC2 is a determinant of OSCC prognosis.
- STC2 A promotes proliferation, migration and invasion, and inhibits apoptosis.
- STC2 controls EMT phenotype of OSCC cells, with regulation on E-cadherin, vimentin, Snail1, Twist and Zeb2.


200 µm
A
200 µm
B


A
Survival probability (%)
100
90
80
70
60
50
40
30
20
10
0
Low expression
High expression
p=0.02
0
20
40
60
80
100
120
Time (months)
B
Survival probability (%)
Low expression
High expression
p=0.01
Time (months)


A
Relative expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
SCC-15 shRNA Control
SCC-15 shRNA STC2
HSC-3 shRNA Control
HSC-3 shRNA STC2
B
SCC-15 shRNA Control
SCC-15 shRNA STC2
HSC-3 shRNA Control
HSC-3 shRNA STC2
STC2 >
β-actin >


A
Proliferation
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
**
SCC-15 shRNA Control
SCC-15 shRNA STC2
HSC-3 shRNA Control
HSC-3 shRNA STC2
B
Apoptosis
1.8
1.5
1.2
0.9
0.6
0.3
0.0
*
*
SCC-15 shRNA Control
SCC-15 shRNA STC2
HSC-3 shRNA Control
HSC-3 shRNA STC2
C
Migration
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
**
SCC-15 shRNA Control
SCC-15 shRNA STC2
HSC-3 shRNA Control
HSC-3 shRNA STC2
D
Invasion
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
**
SCC-15 shRNA Control
SCC-15 shRNA STC2
HSC-3 shRNA Control
HSC-3 shRNA STC2


A
Relative expression
SCC-15 shRNA Control
SCC-15 shRNA STC2
HSC-3 shRNA Control
HSC-3 shRNA STC2
E-cadherin
Vimentin
B
SCC-15
HSC-3
Relative expression
SCC-15 shRNA Control
Snail1
Snail2
Twist
Zeb1
Zeb2
SCC-15 shRNA STC2
HSC-3 shRNA Control
C
SCC-15 shRNA Control
SCC-15 shRNA STC2
E-cadherin >
Vimentin >
Snail1 >
Snail2 >
β-Actin >
D
Relative expression
MMP-2
MMP-9
E
SCC-15 shRNA Control
SCC-15 shRNA STC2
HSC-3 shRNA Control
HSC-3 shRNA STC2
MMP-9 >
MMP-2 >

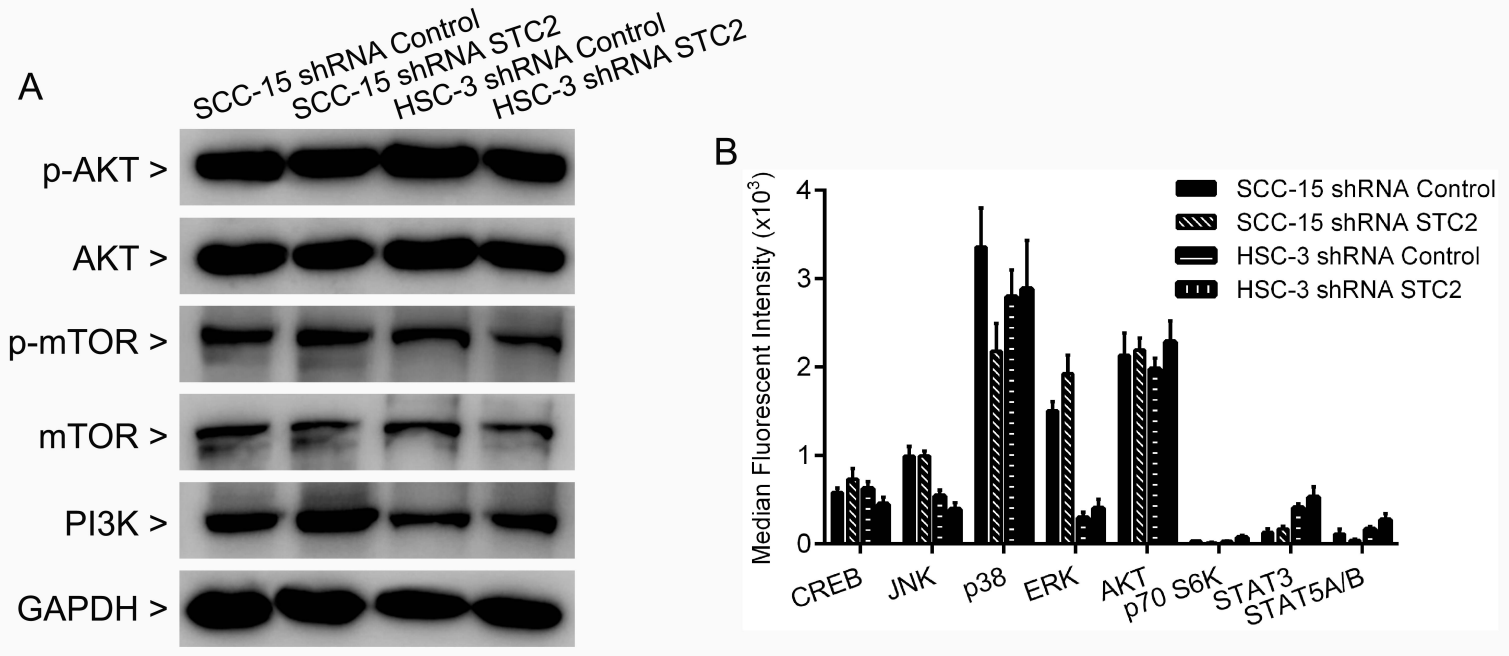

A
SCC-15 shRNA Control
SCC-15 shRNA STC2
HSC-3 shRNA Control
HSC-3 shRNA STC2
p-AKT >
AKT >
p-mTOR >
mTOR >
PI3K >
GAPDH >
B
Median Fluorescent Intensity (x10^3)
SCC-15 shRNA Control
SCC-15 shRNA STC2
HSC-3 shRNA Control
HSC-3 shRNA STC2
CREB
JNK
p38
ERK
AKT
p70 S6K
STAT3
STAT5A/B

Table 1. Association of the clinicopathological parameters of the OSCC with the immunohistochemical expression of stanniocalcin 2 (STC2).

Parameter	Low STC2 n (%)	High STC2 n (%)	p value
Age			
<57 years	18 (50)	31 (48.4)	
≥57 years	18 (50)	33 (51.6)	0.88
Gender			
Male	30 (83.3)	53 (82.8)	
Female	6 (16.7)	11 (17.2)	0.94
Smoking habit			
No	5 (16.1)	8 (15.7)	
Yes	26 (83.9)	43 (84.3)	0.96
Drinking habit			
No	8 (27.6)	13 (29.5)	
Yes	21 (72.4)	31 (70.5)	0.85
Clinical stage			
Early (I + II)	22 (62.9)	24 (38.1)	
Advanced (III + IV)	13 (37.1)	39 (61.9)	0.02
Tumor site			
Tongue	19 (52.8)	28 (43.8)	
Others	17 (47.2)	36 (56.2)	0.38
Treatment			
Surgery	14 (38.9)	26 (40.6)	
Surgery + Radiotherapy	19 (52.8)	32 (50)	
Surgery + Radiotherapy + Chemotherapy	3 (8.3)	6 (9.4)	0.96
Histological grade			
Well-differentiated + Moderately-differentiated	34 (94.4)	54 (84.4)	
Poorly-differentiated	2 (5.6)	10 (15.6)	0.13
Margin status			
≥ 5 mm	35 (100)	60 (95.2)	
< 5 mm	0	3 (4.8)	0.19
Recurrence			
No	27 (77.11)	38 (59.4)	
Yes	8 (22.9)	26 (40.6)	0.07
Second Primary Tumor			
No	24 (68.6)	54 (84.4)	
Yes	11 (31.4)	10 (15.6)	0.06

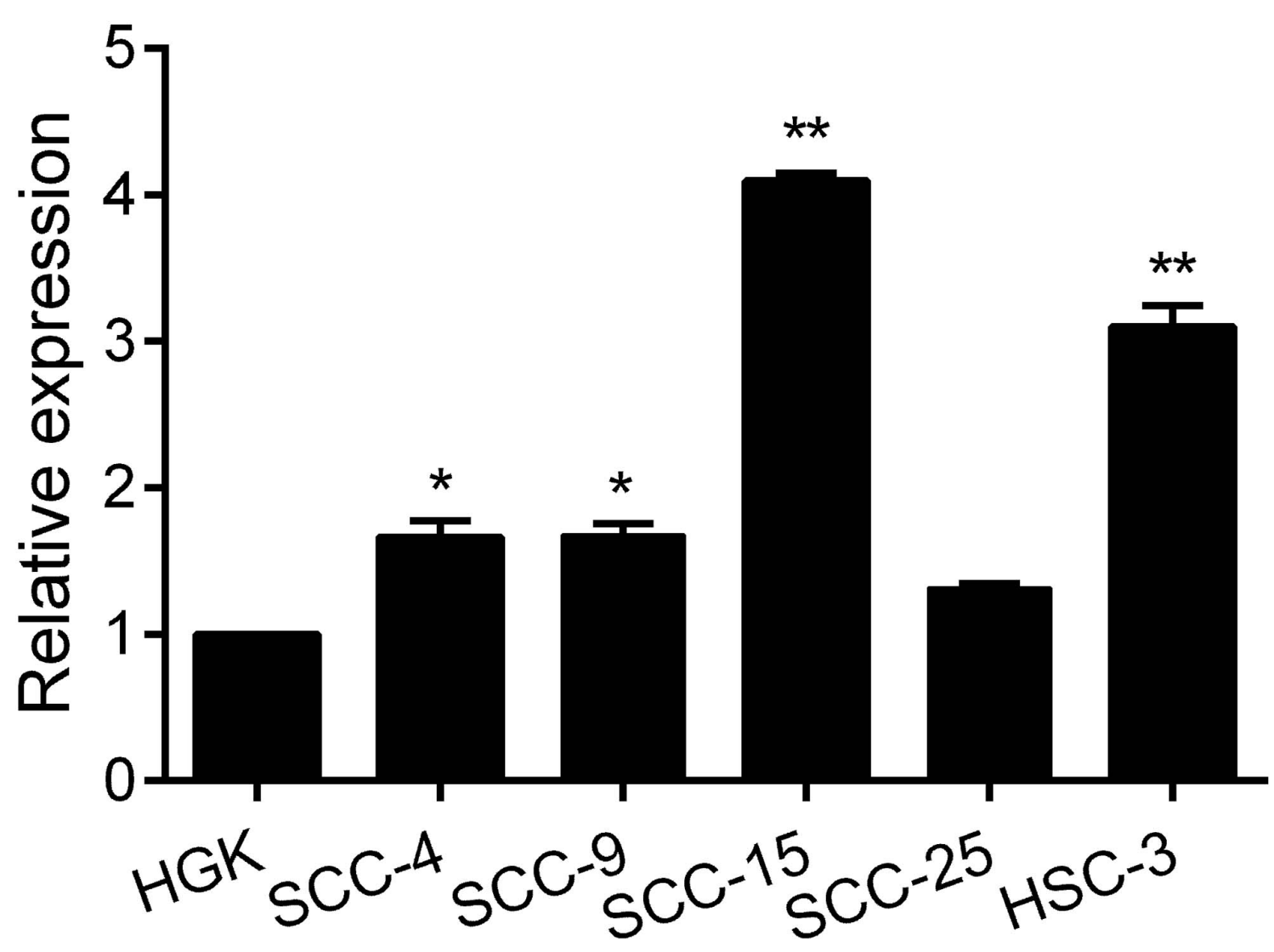
Table 2. Univariate analysis for disease-specific survival and disease-free survival of the OSCC patients.

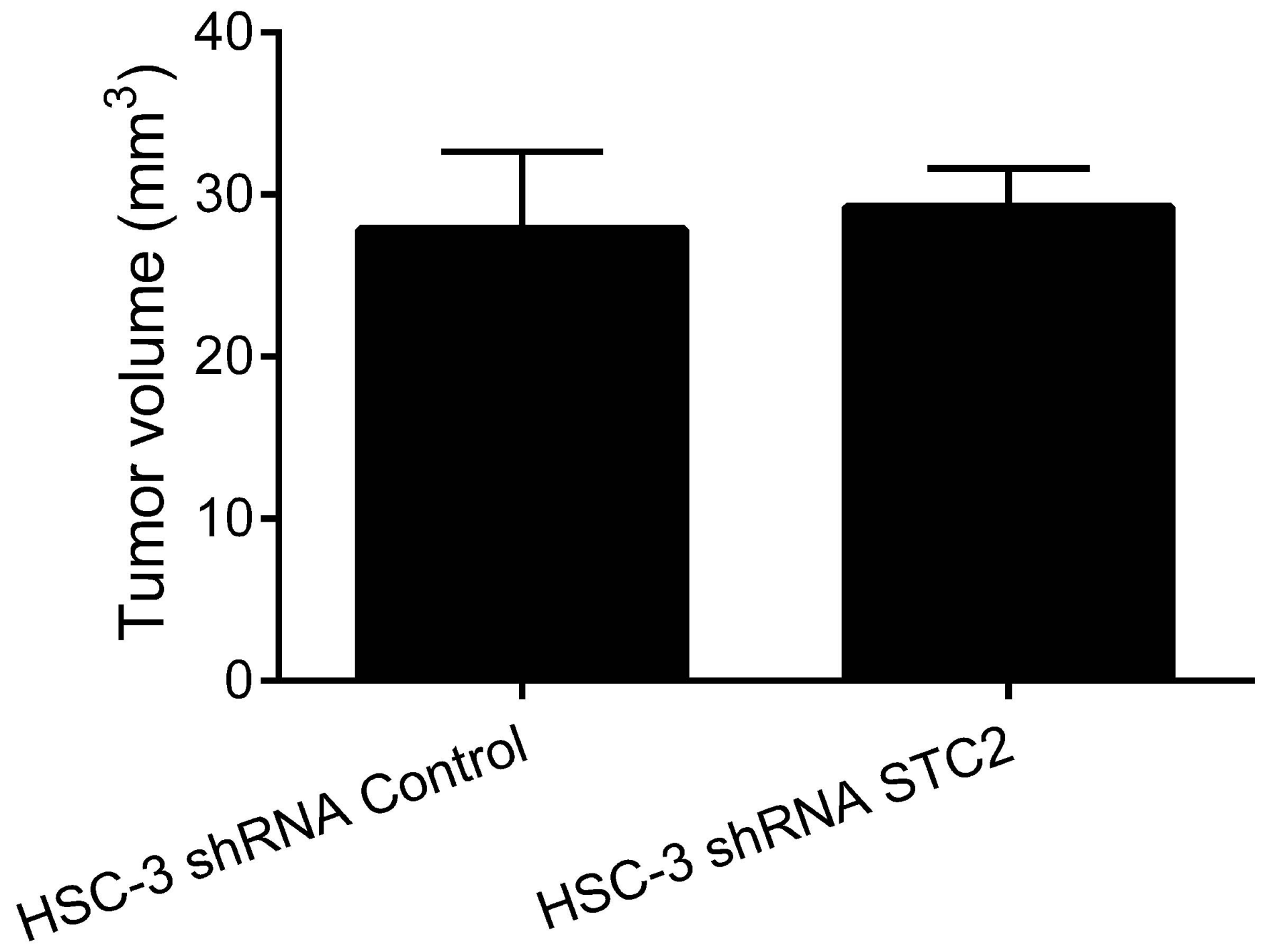
Parameter	Disease-specific survival		Disease-free survival	
	% in 5 years	HR (95% CI)/p value	% in 5 years	HR (95% CI)/p value
Age				
<57 years	57.1	Reference	62.4	Reference
≥57 years	34.0	1.84 (1.07-3.16)/0.02	57.8	1.22 (0.62-2.39)/0.55
Gender				
Male	45.3	Reference	63.5	Reference
Female	42.9	1.16 (0.55-2.48)/0.66	46.2	1.59 (0.64-3.97)/0.23
Smoking habit				
No	51.8	Reference	68.3	Reference
Yes	46.9	1.42 (0.60-3.38)/0.35	47.1	1.99 (0.63-6.23)/0.12
Drinking habit				
No	51.8	Reference	82.8	Reference
Yes	46.9	1.60 (0.82-3.12)/0.20	59.8	1.67 (0.68-4.06)/0.29
Clinical stage				
Early (I + II)	54.2	Reference	62.2	Reference
Advanced (III + IV)	34.7	1.92 (1.11-3.31)/0.02	56.3	1.13 (0.57-2.24)/0.72
Tumor site				
Tongue	54.0	Reference	63.2	Reference
Others	34.7	0.87 (0.51–1.50)/0.63	58.4	1.05 (0.53-2.07)/0.87
Treatment				
Surgery	53.3	Reference	60.1	Reference
Surgery + Radiotherapy	50.0	1.22 (0.69-2.15)/0.51	59.2	1.13 (0.55-2.31)/0.72
Surgery + Radiotherapy + Chemotherapy	35.4	1.34 (0.55-3.27)/0.43	64.3	0.87 (0.26-2.89)/0.83
Histological grade				
Well-differentiated + Moderately-differentiated	45.3	Reference	74.1	Reference
Poorly-differentiated	34.1	1.80 (0.66-4.93)/0.13	59.5	1.48 (0.44-4.95)/0.58
Margin status				
≥ 5 mm	44.0	Reference	62.1	Reference
< 5 mm	50.0	0.92 (0.12-7.21)/0.94	100	NA
STC2				
Low	63.4	Reference	74.5	Reference

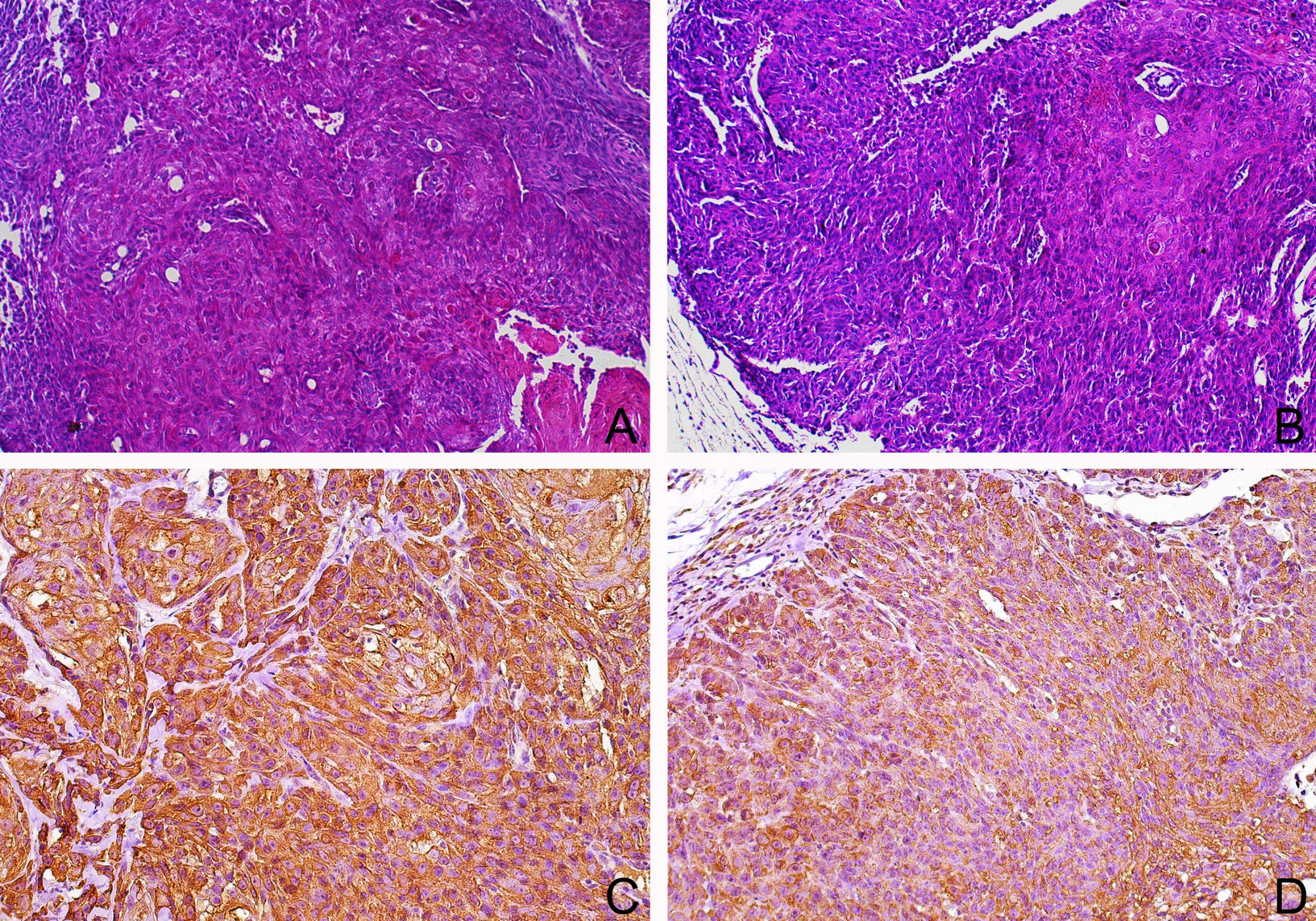
High	34.4	2.68 (1.56-4.62)/0.002	52.0	2.37 (1.20-4.69)/0.02

Table 3. Cox multivariate analysis for the risk of death and recurrence.

Parameter	Disease-specific survival		Disease-free survival	
	HR (95% CI)	p value	HR (95% CI)	p value
Age				
<57 years	Reference			
≥57 years	1,95 (1.12-3.38)	0.01		
STC2				
Low	Reference		Reference	
High	2.67 (1.37-5.21)	0.001	2.80 (1.07-5.71)	0.03


Relative expression
5
4
3
2
1
0
*
*
**
**
HGK
SCC-4
SCC-9
SCC-15
SCC-25
HSC-3


40
30
20
10
0
Tumor volume (mm3)
HSC-3 shRNA Control
HSC-3 shRNA STC2


A
B
C
D

Supplementary Table 1. Description of primers used in the qPCR.

Primers	Sequence (5’ → 3’)
STC2	Forward: GCATGTCACACGCAGGATTCT
	Reverse: TGTCCGTTCCGCGAACA3
E-cadherin	Forward: ACAGCCCCGCCTTATGATT3
	Reverse: TCGGAACCGCTTCCTTCA
Vimentin	Forward: GGCTCGTCACCTTCGTGAAT
	Reverse: TCAATGTCAAGGGCCATCTTAA
Snail1	Forward: GCGTGTGCTCGGACCTTCT
	Reverse: ATCCTGAGCAGCCGGACTCT
Snail2	Forward: GGAGCATACAGCCCCATCA
	Reverse: TGGTAGCTGGGCGTGGAA
Zeb1	Forward: GCTTTCCCATTCTGGCTCCTA
	Reverse: TCTTGGTCGCCCATTCACA
Zeb2	Forward: AAGATAGGTGGCGCGTGTTT
	Reverse: ACTGACGTGTTACGCCTCTTCTAA
Twist	Forward: GCGCTGCGGAAGATCATC3
	Reverse: GCTTGAGGGTCTGAATCTTGCT
MMP-2	Forward: GTTCATTTGGCGGACTGT
	Reverse: AGGGTGCTGGCTGAGTAG
MMP-9	Forward: CTTTGGACACGCACGAC
	Reverse: CCACCTGGTTCAACTCACT
PPIA	Forward: GCTTTGGGTCCAGGAATGG
	Reverse: GTTGTCCACAGTCAGCAATGGT