Head and Neck Pathology (2023) 17:618–630
https://doi.org/10.1007/s12105-023-01562-w

RESEARCH

Loss of Caveolin-1 Expression in Tumor Cells is Associated with Increased Aggressiveness and Cell Invasion in Oral Squamous Cell Carcinoma

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Received: 19 April 2023 / Accepted: 18 May 2023 / Published online: 26 May 2023

Abstract

Background Changes in Caveolin-1 (CAV-1) expression are related to tumorigenesis. The aim of this study was to evaluate the role of CAV-1 in tumor progression in oral squamous cell carcinoma (SCC) tissue samples and the effect of CAV-1 silencing on two oral tongue SCC (OTSCC) cell lines (SCC-25, from a primary tumor, and HSC-3 from lymph node metastases).

Methods Mycroarray hybridization, mRNA expression, and immunohistochemistry were performed on OSCC tissue samples and corresponding non-tumoral margin tissues. The effects of CAV-1 silencing (siCAV-1) on cell viability, membrane fluidity, on the expression of epithelial to mesenchymal transition (EMT) markers and on cell migration and invasion capacity of OTSCC cell lines were evaluated.

Results Microarray showed a greater *CAV-1* expression (1.77-fold) in OSCC tumors than in non-tumoral tissues and 2.0-fold more in less aggressive OSCCs. However, significant differences in *CAV-1* gene expression were not seen between tumors and non-tumoral margins nor *CAV-1* with any clinicopathological parameters. CAV-1 protein was localized both in carcinoma and in spindle cells of the tumor microenvironment (TME), and CAV-1 positive TME cells were associated with smaller/more aggressive tumors, independent of the carcinoma cells' expression. Silencing of CAV-1 increased cell viability only in SCC-25 cells. It also stimulated the invasion of HSC-3 cells and increased *ECAD* and *BCAT* mRNA in these cells; however, the protein levels of the EMT markers were not affected.

Conclusion Decreased expression of CAV-1 by tumor cells in OSCC and an increase in the TME were associated with increased cell invasiveness and tumor aggressiveness.

Keywords Oral tongue squamous cell carcinoma · Caveolae · Caveolin-1 · Epithelial-mesenchymal transition · Tumor aggressiveness · Silencing of the gene

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Introduction

Oral squamous cell carcinoma (OSCC) is associated with high mortality rates worldwide and is usually diagnosed in advanced stages and with poor overall survival rates [1]. Genetic and epigenetic events occur during OSCC development, leading to the activation of oncogenes and the inactivation of tumor suppressor genes [2, 3]. Caveolin-1 (CAV-1), a protein in caveolar structures in cell membranes, acts on membrane traffic and intracellular cholesterol transport. The caveolar compartmentalization of molecules and their interaction with caveolins provide a mechanism for the regulation of signalling events, as well as a mechanism for the interaction between different signalling pathways [4].

Dysregulation of CAV-1 is associated with cell transformation and tumor progression, and its function varies according to the type and stage of the disease [4–6]. There is still controversy in OSCC research regarding the role of CAV-1 in tumorigenesis and tumor progression, with both oncogenic and tumor suppressor functions described previously [7–11]. Increased CAV-1 expression seems to favor the development of malignant characteristics, while its loss seems to be related to a decrease in migratory and invasive capacity in head and neck cancer [6–12]. However, in metastatic OSCC, CAV-1 expression was down-regulated in regard to primary tumors [7].

During carcinogenesis, cells may undergo the epithelial-mesenchymal transition (EMT) and acquire mesenchymal cell phenotype and properties. This includes negative regulation of epithelial markers, such as E-cadherin and β-catenin, and positive regulation of mesenchymal markers, including N-cadherin and Vimentin [13]. The EMT process has been associated with the ability to form buds (tumor budding), defined as fewer than five cancer cells forming a cell cluster at the invasive front of the tumor [14]. Budding has been reported as a predictive feature associated with a high risk for locoregional recurrence and shortened survival [14].

Studies have correlated the EMT process with the expression of CAV-1 [5]. Both an increase and decrease in the expression of CAV-1 were associated with the induction of EMT in different types of cancer, and different levels of CAV-1 expression seem to be associated with different phases of EMT [15, 16]. However, the pattern of CAV-1 expression in OSCC, as well as its role in EMT, is still unclear. Thus, this study aimed to evaluate the expression of CAV-1 in OSCC tumors as well as the effect of CAV-1 silencing on the EMT process, and the migratory and invasive capacity of oral tongue squamous cell carcinoma (OTSCC) cell line cells.

Material and Methods

Patient Tissue Samples

Oral squamous cell carcinoma tissue samples (tongue and floor of the mouth) and their corresponding non-tumoral tissues (patient-matched tumor-free margin specimens) were obtained by surgical resection from OSCC patients (male ≥ 40 years old, smokers) admitted for diagnosis and treatment at the Arnaldo Vieira de Carvalho Cancer Institute, Heliópolis Hospital, and Clinical Hospital (School of Medicine, University of São Paulo, Brazil). All patients have provided written informed consent to participate in this study which was approved by the Brazilian National Ethics Committee (Process #16,491) and meets the Declaration of Helsinki.

Samples were immediately snap-frozen in liquid nitrogen upon surgical removal. After histological confirmation, OSCC samples were macrodissected before processing and each sample contained at least 70% of tumor cells. Corresponding surgical margins were reported as "non-tumoral tissue", confirmed by the pathologist. GENCAPO (Head and Neck Genome Project) was responsible for sample collection and initial processing, clinical data collection, histopathological analysis, and the informed consent acquisition of each patient. The tumor aggressiveness parameter was based on the TNM system, divided into two groups T1⁄2, N + (more aggressive) and T3⁄4, N0 (less aggressive). Samples of OSCC and non-tumoral tissue (margin) were used. Tumors were divided into two groups: (1) more (T1/T2 N + , $n = 14$) and (2) less (T3/T4 N0, $n = 19$) aggressive tumors according to Xavier et al. [17].

Microarray Hybridization

Ten fresh OSCC samples and a pool of the corresponding non-tumoral tissues were used for microarray analysis. Experiments were carried out as described by Severino et al. [18].

The individual *CAV-1* expression profile in OSCC samples and their respective non-tumoral tissues were compared with each other. Results were expressed as fold variation; fold-change > 2.0-fold in mRNA levels were considered 'up-regulated' and < 0.5-fold 'down-regulated.'

The array design and raw data files are available from the Gene Expression Omnibus database (GEO) under the accession number GSE9792.

RNA Extraction, cDNA Synthesis, and qRT-PCR from Tissues

Total RNA was obtained (1 μg) from 32 fresh OTSCC tissue samples and their correspondent margins were incubated with DNase I (Invitrogen) and reverse transcribed to single-stranded cDNA using a High Capacity cDNA Reverse

Transcription Kit (Applied Biosystems™). Quantitative RT-PCR (RT-qPCR) was performed using the Applied Biosystems 7500 Real-Time PCR System with SYBR Green I Dye (Applied Biosystems). Primer sequences were designed from a specified exon–exon junction (*HPRT1*—F: 5′-CCACCACCCTGTTGCTGTA-3′; R: 5′-TCCCCGTTGACTGGTCAT-3′; *CAV-1*—F: 5′-CCCTAAACACCTCAACGATG-3′; R: 5′-GCCTTCCAAATGCCGTCAAA-3′) using GeneTool 2.0 software (Biotools). *HPRT1* was used as a housekeeping gene for data normalization and relative quantification was performed using the Pfaffl mathematical model [19]. For comparisons between tumor and non-tumoral tissues, one sample from normal mucosa was used as the calibrator sample, while non-tumoral tissue from each case was the calibrator sample for comparisons between less aggressive and more aggressive tumors.

Immunohistochemistry

Twenty formalin-fixed paraffin-embedded tissue Sects. (5-µm thickness) and 10 corresponding margins were used. An immunohistochemical assay was performed according to the protocol by Prosdocimi et al. [20]. Briefly, the slices were incubated with CAV-1 rabbit polyclonal primary antibody (3238, Cell Signaling – 1:200) in a humid chamber at 4 °C overnight. Human colon tumor biopsies were used as a positive control.

Immunohistochemical staining of tumor and stroma cells were evaluated by light microscopy at × 200 magnification throughout each entire section. Distribution of CAV-1 positivity was scored semi-quantitatively, according to Koo et al. [21], as negative (−, no staining), weak (+, staining of less than 30% of the carcinoma or stromal cells), moderate (++, staining of 30–60% of carcinoma or stromal cells), and strong (+++, staining of more than 60% of carcinoma or stromal cells). The expression of CAV-1 was considered positive when any value greater than weak staining was identified. Expression in stromal cells was considered only in stromal spindle cells, excluding endothelial cells (internal control). For statistical analysis, both negative and weak staining were grouped as "CAV-1 negative expression" and moderate and strong staining were considered "CAV-1 positive expression", all analyzed and confirmed by 2 experienced pathologists.

Cell Culture

The human OTSCC primary tumor cell line SCC-25 (ATCC, Wesel, Germany, CRL-1628, obtained in August 2014) and metastatic cell line HSC-3 (JCRB, Osaka, Japan, JCRB0623, obtained in June 2013) were tested and authenticated using microsatellite markers in September 2020. For this test, the GenePrint 24 system allows co-amplification and detection of 24 human loci (22 autosomal STR loci and Amelogenin and DYS391 for gender identification). These loci collectively provide a genetic profile with a random match probability of < 1 in 2.92×10 9 (calculated based on the 10 markers shared with the GenePrint 10 kit). Identified genetic profile is compared to publicly available data to count allelic identity.

Cells were cultured in 1:1 DMEM)/ F-12 (Gibco; Thermo Fisher Scientific) supplemented with 10% heat-inactivated FBS (Gibco; Thermo Fisher Scientific), 100 U/mL penicillin, 100 µg/mL streptomycin, 50 µg/mL ascorbic acid, 250 ng/mL amphotericin B and 0.4 ng/mL hydrocortisone (all from Sigma Aldrich). Cells were maintained at 37 °C with 5% CO_2. Cells were regularly mycoplasma tested with an EZ-PCR Mycoplasma test kit (Biological Industries, Beit-Haemek, Israel).

Cav-1 siRNA Transfection

Silencing of the Caveolin-1 gene was performed in SCC-25 and HSC-3 cells with interference RNA (siRNA) using Lipofectamine (Invitrogen). Three validated, commercially available human CAV-1 silencers were used (CAV-1 Silencer select Validated siRNA ID: s2446 (1), s2447 (2) and s2448 (3), Ref. 4,427,038, Ambion – ThermoFisher Scientific). siRNA was incubated for 48 h in final concentrations of 10 nM and 50 nM. QPCR and Western Blotting evaluated the silencing capacity.

Cell Viability Analysis

After 24 h, 48 h, and 72 h of gene silencing, Alamar Blue (Resazurin sodium salt, R7017, Sigma-Aldrich) was used to evaluate cell viability according to the manufacturer's protocol. Fluorescence levels after 3 h were analysed using 544/15 and 595/60 nm filters in the Victor3V 1420 Multilabel Counter equipment (Perkin Elmer Life & Life Technologies). Results represent the average of three independent experiments, performed in triplicate.

Membrane Fluidity

A Membrane Fluidity kit (Abcam), was used according to the manufacturer's protocol, to measure membrane fluidity. Briefly, 1.5×10^4 cells were cultured in a 96-well plate and silenced with siCAV-1 and then kept for 48 h. Then, the membrane fluidity was evaluated immediately (0 h) and 24 h after silencing. Fluorescence was recorded using 405/10 nm (excitation) and 460/30 nm (emission) filters on the VICTOR® Nivo ™ system equipment (PerkinElmer).

RNA Extraction, cDNA Synthesis, and qRT-PCR from Cells

Total RNA was extracted from control and siCAV-1 OTSCC cells using PureLink RNA mini kit (Ambion, Life Technologies) according to the manufacturer's instructions. cDNA was synthesized from 1 µg of DNAse I (ThermoFisher) treated RNA using the RevertAid First Strand cDNA Synthesis Kit (K1622, ThermoFisher), according to the manufacturer's protocol. qPCR analysis was performed with FastStart MasterMix with ROX (Roche Diagnostics), according to the manufacturer's instructions, on a Rotor-Gene 3000 (Corbett Research) machine. The primers (final concentration 0.3 µM) used for gene expression are described in SF 1. The housekeeping gene used was *GAPDH* (F: 5′-CAC CAACTGCTTAGCACCC; R:5′-GCAGGGATGTTC TGGA). Relative gene expression analysis was performed according to the 2-ΔΔCT method based on 3 to 5 different experiments.

Western Blotting

Control and siCAV-1 OTSCC cells were lysed with elution buffer [50 mM Tris–HCl pH 7.5, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% (v/v) Brij-35 (Sigma Aldrich) including Complete EDTA-free protease inhibitor cocktail (Roche). Thirty micrograms of soluble protein were separated under reducing conditions on a 10% or 12% SDS-PAGE gel and then the proteins were transferred to an Immobilon-P membrane (Millipore). The membrane was blocked with 5% milk powder (Bio-Rad) in Tris-buffered saline/0.1% Tween 20 and incubated overnight at 4 °C with the following antibodies CAV-1 (1:1,000, ab32577 3238, Abcam), E-cadherin (1:1,000, 24E10, Cell Signalling), N-cadherin (1:1,000, 18–0224, Invitrogen), β-catenin (1:2,500, ab32572, Abcam), vimentin (1:750, M0725, Dako) or anti-β-actin (1:2,000, ab8226, Abcam), followed by a biotinylated anti-rabbit IgG (1:5,000, E035301-2, Dako) or anti-mouse IgG (dilution 1:5,000, E035401-2, Dako) secondary antibodies and Vectastain ABC kit (Vector Laboratories). Immunocomplexes were visualized using a Pierce ECL Western blotting substrate (Thermo Scientific) and the Luminescent image analyzer LAS-3000 (Fujifilm). Quantification of protein levels was performed with Fiji software 1.51w [24] and β-actin was as an endogenous control protein for data normalization. The results represent the average of three to five independent experiments.

Horizontal Cell Migration and Invasion Assays

96-well ImageLock™ plates (Essen BioScience) were coated with 50 µl of 0.3 mg/ml Myogel [22] and incubated overnight. Then, 2.5×10^4 OTSCC cells were seeded, and 24 h later, they were silenced with siCAV-1. Cell layers were scratched with the WoundMaker™ tool (Essen BioScience). The cell migration was followed in IncuCyte® S3 (Essen BioScience) supplied with the Scratch Wound assay module and figures were taken every 2 h. The figures were analyzed with Fiji software 1.51w where the wound areas were measured and calculated as a percentage, considering zero time as 100%. The results reflect the average of 3 independent experiments in triplicate.

For invasion assay, after scratching, Myogel/fibrin (2.4 mg/mL Myogel, 0.5 mg/mL fibrinogen (Merck, Darmstadt, Germany), 0.3 U/mL thrombin (Sigma-Aldrich), and 33.3 µg/ml aprotinin (Sigma-Aldrich) were added; these reagents were diluted in DMEM/F12 media with 10% FBS. Pictures were obtained every 2 h on Incucyte® S3 Live-Cell Analysis System microscope and analysed with Image J software, where the wound areas were measured and calculated as a percentage, considering zero time as 100%. The results reflect the average of 3 independent experiments in triplicate.

Statistical Analysis

Wilcoxon nonparametric test was used to assess the differences in CAV-1 gene expression levels between "tumor vs non-tumoral tissues", while the Mann–Whitney test was performed to analyze CAV-1 gene expression levels between "less aggressive vs more aggressive tumors" in qRT-PCR analysis. The cut-off was set up at the values < 2.0 (negative) and ≥ 2.0 (positive) for gene expression analysis by qRT-PCR.

The Fisher's exact test was used to estimate the statistical difference between *CAV-1* gene/protein expression levels and clinicopathological parameters such as mean age, tumor location, tumor size-pT, nodal metastasis-pN, pathological grade, lymphatic and/or perineural invasion and recurrence. For this analysis, only OSCC samples paired with their respective non-tumoral tissue in which *CAV-1* exhibited detectable expression by qRT-PCR and immunohistochemistry were included. Spearman Correlation was used to compare tumoral and stromal protein expression. Kaplan–Meier product-limit estimation with log-rank ($p < 0.05$) was used for survival analysis from lifetime data according to gene expression levels and immunoexpression of CAV-1 in tumor and stroma cells. Overall survival was defined as the time from surgery to the day of death or last follow-up.

One-way analysis of variance (ANOVA) with post hoc comparisons based on Tukey's multiple comparisons test were applied to cell viability and invasion capacity analysis. Student t-tests or Mann–Whitney U test were applied to membrane fluidity and protein expression analysis. The gene expression of each gene compared to the control was analyzed by t student test, while the association between genes

was analyzed through the Kruskall-Wallis test. To evaluate migration capacity, two-way ANOVA with Bonferroni correction, used for post hoc comparison, was applied.

Data were analyzed using GraphPad Prism 5 software (GraphPad Software, Inc., CA, USA). The level of significance considered was 5% ($p \leq 0.05$). Results with a p-value ≤ 0.05 were considered significant (*), and those with a p-value < 0.01 (**), or < 0.001 (***), very significant.

Results

Microarray Data and CAV-1 mRNA Expression Analysis

General analysis of the microarray data revealed that CAV-1 transcripts presented a differential expression pattern which varied greatly between OTSCC tissue samples. The mean fold-change for CAV-1 in OSCC samples, in relation to their matched pool of non-tumoral tissue, was 1.77. Five OSCC samples (5/10) showed CAV-1 up-regulation (≥ 2.0-fold) and in the remaining five OSCC samples, CAV-1 was considered not differentially expressed (values between 0.5 and 2.0) (Fig. 1A and SF 1). Considering tumor aggressiveness, *CAV-1* mean fold change was 1.48 and 2.95 in less aggressive and more aggressive tumors, respectively. mRNA of *CAV-1* was highly expressed in three (70%) of the more aggressive tumor samples, while in less aggressive tumors only one sample showed *CAV-1* up-regulation (Fig. 1B and SF 2).

To validate data from microarray analysis, *CAV-1* gene expression was also evaluated by using RT-qPCR from a 3x-bigger cohort of 32 OSCC samples. Comparing "tumors vs non-tumoral margin", no statistically significant difference was found in *CAV-1* expression (median value = 0.42 and 0.68, respectively, $p = 0.29$, Fig. 1B). Additionally, there was no significant difference in *CAV-1* expression between more and less aggressive tumors (median value = 0.65 and 0.57, respectively, $p = 0.19$, Fig. 1C).

Sample characterization and the association of CAV-1 expression levels with clinicopathological features and disease outcomes were examined and are shown in SF 3 and Fig. 1D. In general, there was no significant association between gene expression levels and age group, tumor location, pTNM classification, pathological grade, lymphatic and/or perineural invasion, and survival.

CAV-1 Immunoexpression

CAV-1 reactivity was detected in the membrane and/or cytoplasm in OSCC samples. In non-tumoral oral tissue, CAV-1 was detected in basal epithelial cells and blood vessels (internal control) (Fig. 2A).

In OSCC, CAV-1 was detected in both carcinoma cells (Fig. 2B, C), and in the spindle cells of the tumor microenvironment (TME) (Fig. 2D). CAV-1 expression in carcinoma cells was positive in 12 cases (60%), and CAV-1 in TME cells was positive in 13 cases (65%) (Table 1). Interestingly, different patterns were found for CAV-1 protein expression: positive carcinoma vs negative TME ($n = 4$) (Fig. 2B), positivity within both carcinoma and TME cells ($n = 8$) (Fig. 2C), and negative carcinoma and TME ($n = 5$) (Fig. 2D). Only three samples were negative in both locations.

The immunoexpression levels of CAV-1 in carcinoma cells and TME cells were associated with clinicopathological features and disease outcomes (Fig. 2E and Table 1). CAV-1 expression in TME cells was associated with larger tumor size and with OSCC tissue compared to margin ($p = 0.05$). Additionally, CAV-1 positive carcinoma cells tended to be associated with low or negative CAV-1 in TME cells ($p = 0.064$; Fisher exact test). A negative correlation was identified between CAV-1 immunoexpression in carcinoma and TME spindle cells ($r = -0.09$, $p = 0.69$; Spearman correlation) but with no statistical significance.

Silencing of Caveolin-1 Increases Cell Viability of Non-Metastatic Cells but Not of Metastatic Cells

To understand the role of *CAV-1* in OSCC tumorigenesis, OTSCC cell lines were silenced for *CAV-1*. In order to select the best RNA silencer, silencing was performed using three different interference RNA (siRNA) for *CAV-1* and the knockdown was confirmed by the evaluation of mRNA and protein expression. Silencer 1 (s2446) was selected as the best interference RNA. (SF 4).

We analysed the effect of CAV-1 silencing on OTSCC cell viability and membrane fluidity. The silencing of CAV-1 in non-metastatic SCC-25 cells increased its cell viability ($p = 0.0025$, t student test, Fig. 3A). Membrane fluidity was not affected immediately after silencing of CAV-1 ($p = 0$, 17, student t-test) but a tendency towards increasing the membrane fluidity was observed after 24 h ($p = 0.0650$, Mann–Whitney test, Fig. 3B). In metastatic HSC-3 cells, the silencing of *CAV-1* did not change cell viability or membrane fluidity (Fig. 3C, D).

Silencing of CAV-1 Did Not Affect Any EMT Marker at the Protein Level

Gene expression of the EMT markers (*ECAD, NCAD, βCAT*, and *VIM*) was not affected by siCAV-1 in SCC-25 cells (Fig. 4A). On the other hand, in HSC-3 cells, *NCAD* gene expression increased at 6 h ($p = 0.0260$, Mann–Whitney) and *βCAT* gene expression increased 24 h after siCAV-1 ($p = 0.0436$, teste t student, Fig. 4B). There was no difference in the evaluated markers' gene expression

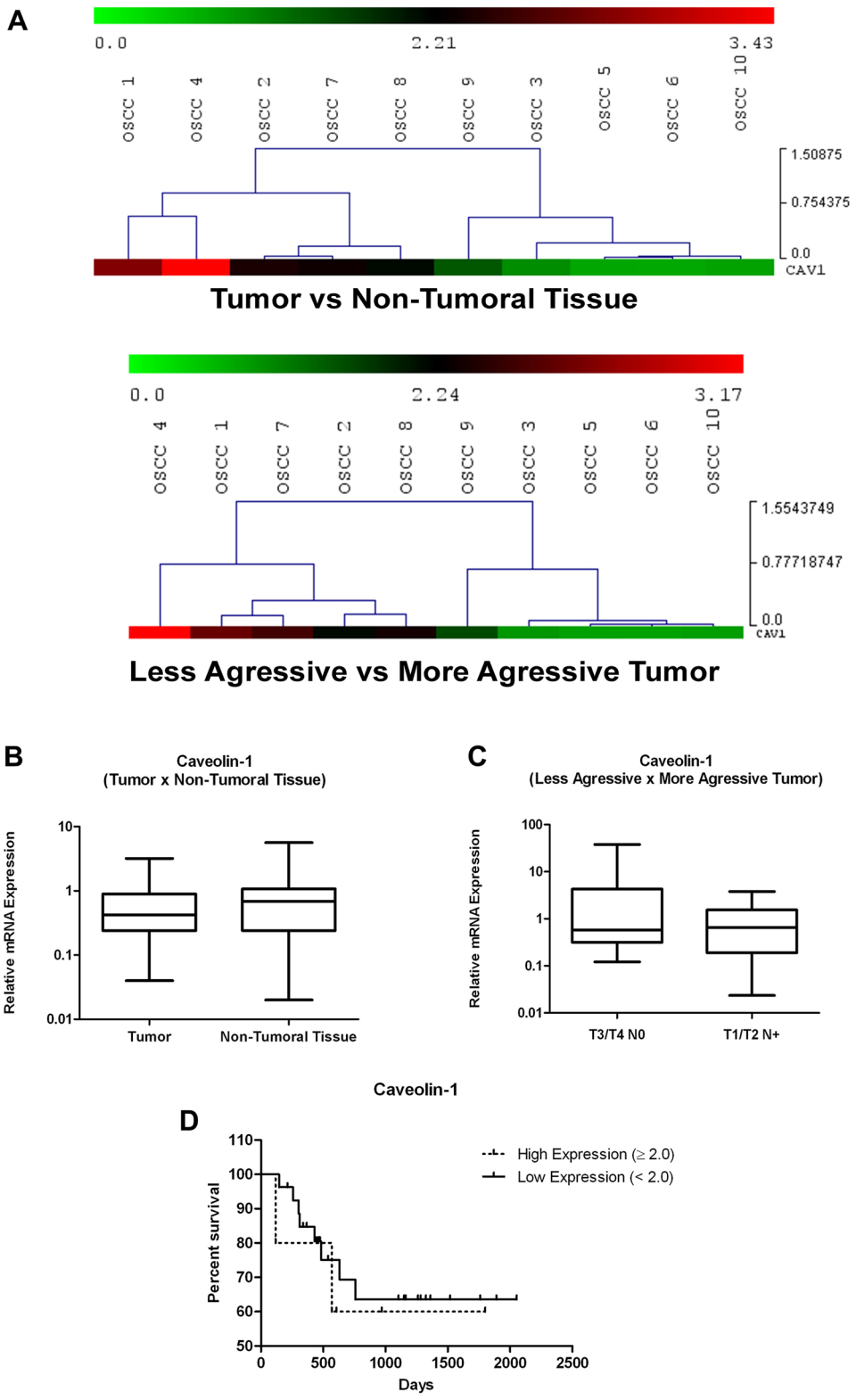

Fig. 1 **A** Hierarchical cluster diagram of *CAV-1* expression in OSCC samples. Gene expression levels in non-tumoral tissues were used as the baseline. Data are visualized colorimetrically with heat plots, "red" representing elevated gene expression and "green" decreased gene expression in tumor vs non-tumoral tissue and less aggressive vs more aggressive tumors. Relative expression ratio (log10) of CAV-1 mRNA expression analysis by qRT-PCR in OSCC samples. Housekeeping gene: HPRT1. **B** and **C** Differential gene expression in tumor vs non-tumoral tissue, and less vs more aggressive tumor. **D** Overall survival of OSCC patients according to positive and negative CAV-1 gene expression

between either cell line cells. (Kruskal–Wallis, Fig. 4A, B). However, even though we detected some induction in the EMT marker genes (*NCAD and βCAT*) in HSC-3 cells, we could not see any changes in the protein level of any

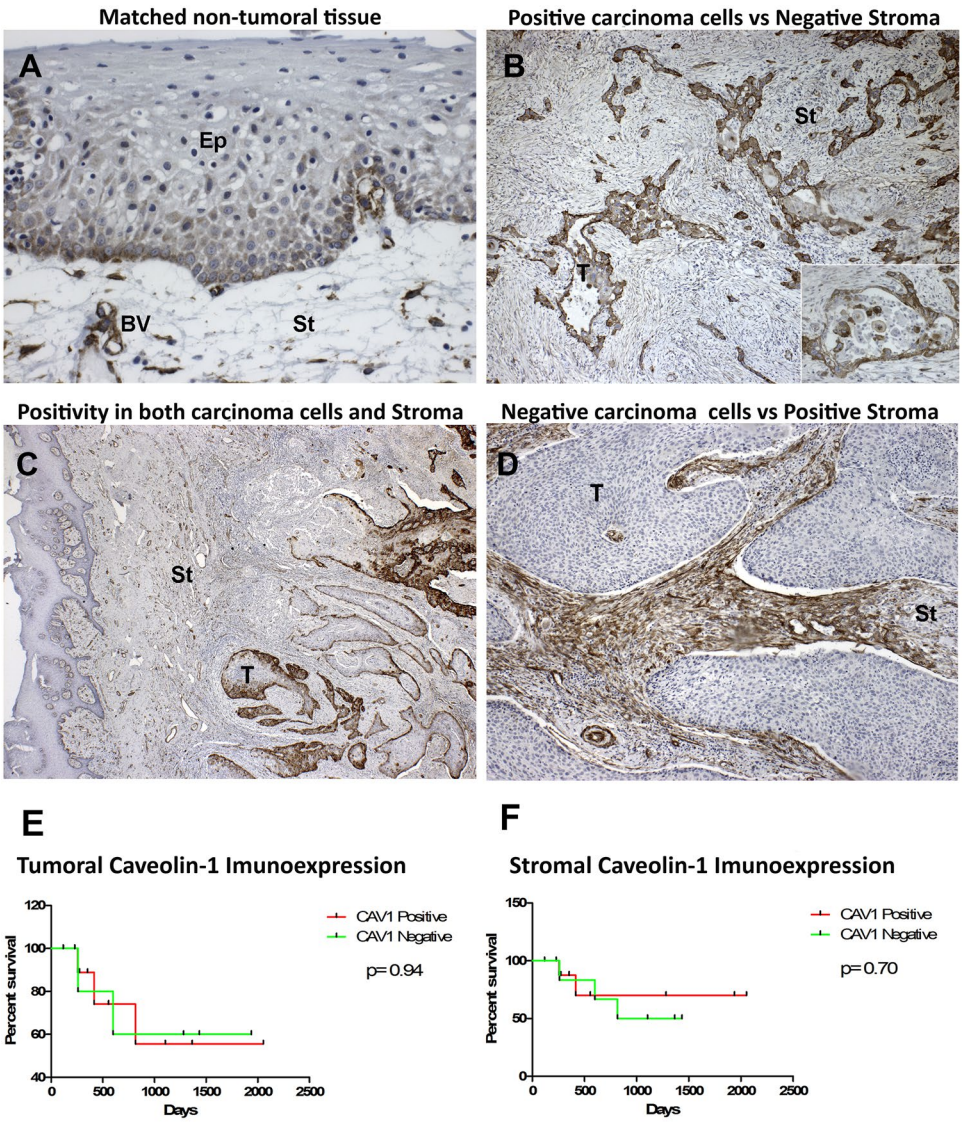

Fig. 2 Immunoexpression of CAV-1 in OSCC and non-tumoral tissue. CAV-1 shows cytoplasmic immunoexpression in matched non-tumoral tissue in basal /suprabasal layers (**A**), intense membranous/cytoplasmic immunoexpression in carcinoma (**B** and **C**), and TME (**C** and **D**). Overall survival of OSCC patients according to positive and negative CAV-1 in carcinoma (**E**) and TME (**F**) immunoexpression (*Ep* epithelium; *St*: stroma; *T*: tumor; *BV*: blood vessel)

EMT marker after silencing *CAV-1*, in either of the cell lines (Fig. 4C, D).

CAV-1 Knockdown Induces the Invasion of Metastatic HSC-3 Cells

There was no difference in the migration ability of SCC-25 and HSC-3 siCAV-1 cells in relation to their corresponding controls in Myogel coated wound healing assay (Fig. 5A–D). Invasion capacity in Myogel was also not affected by siCAV-1 in SCC-25 cells (Fig. 5E, F). However, the invasion capacity of HSC-3 siCAV-1 cells was significantly increased compared to control cells ($p = 0.0458$, Mann–Whitney) (Fig. 5G, H).

Discussion

In the present study, the microarray analysis revealed *CAV-1* overexpression in OSCC tissues compared to the corresponding non-tumoral samples, and, particularly, it was overexpressed in more aggressive oral cancers. CAV-1 immunopositivity in stromal spindle cells was significantly associated with smaller/more aggressive tumors. Silencing of CAV-1 increased the invasive capacity of metastatic OTSCC cell line cells in vitro.

In several cancers, CAV-1 plays a dual role, as a tumor suppressor early on, but as a tumor promoter in more advanced and metastatic stages [4, 9, 11]. Here, *CAV-1* expression assessed by microarray was also higher in

Table 1 Association between CAV-1 protein localization in tumor cells and TME compartments and clinicopathological findings and disease outcome

Clinicopathological features	N° of cases	CAV-1 expression			CAV-1 expression		
		Tumor cells			TME cells		
		Negative (−/+)	Positive (++/+++)	*p* value	Negative (−/+)	Positive (++/+++)	*p* value
Tumor location							
Tongue	8	4	4	0.64$^{\phi}$	4	4	0.35$^{\phi}$
Floor of mouth	12	4	8		3	9	
T classification							
T1/2	14	7	7	0.32$^{\phi}$	7	7	**0.05**$^{\phi}$
T3/4	6	1	5		0	6	
N classification							
N0	7	2	5	0.64$^{\phi}$	2	5	1.00$^{\phi}$
N+	13	6	7		7	13	
Pathological grade							
Well-differentiated	7	3	4	1.00$^{\phi}$	3	5	1.00$^{\phi}$
Moderately differentiated	13	5	8		4	8	
Lymphatic invasion (LI)*							
LI-	3	2	1	0.50$^{\phi}$	1	2	1.00$^{\phi}$
LI+	11	3	8		5	6	
Blood invasion (BI)*							
BI-	2	1	1	1.00$^{\phi}$	0	2	0.51$^{\phi}$
BI+	15	6	9		6	9	
Perineural invasion (PI)							
PI-	7	1	6	0.15$^{\phi}$	2	5	0.63$^{\phi}$
PI+	11	6	5		5	6	
Survival							
Alive	11	5	6	0.94†	3	8	0.70†
Dead of index cancer	5	2	3		3	2	
Disease vs Margin							
Tumor	20	8	12	0.24	7	13	**0.05**$^{\phi}$
Non-tumoral	10	7	3		8	2	

*Missing data, $^{\phi}$Fisher's exact test,† Log-rank test

more aggressive tumors (T1/T2, N+) compared to less aggressive tumors (T3/T4, N0). However, these findings were not confirmed by RT-qPCR in our series of OSCC. An association between increased *CAV-1* expression in the step-wise OSCC carcinogenesis has been shown in the Jaafari-Ashkavandi and Aslani [23] study, where they found a lower expression of *CAV-1* in dysplastic compared to OSCC tissue samples which suggests that *CAV-1* expression increases during carcinoma formation. Similarly, Hung et al. [7] reported a step-wise increase in *CAV-1* expression from the normal oral mucosa, non-cancerous matched tissue, and oral potentially malignant lesions up to primary OSCC. Here, the immunohistological analysis showed more positivity in the stromal cells of the tumors, especially more aggressive tumors, when compared to non-tumoral margins. Our immunohistological finding of the association of CAV-1 expression in TME with the more-aggressive tumor may suggest that in OSCC, CAV-1 is expressed differently during the progression of this cancer.

In a study evaluating head and neck cancer, lymph node metastases presented lower levels of CAV-1 than primary tumors, and the restoration of CAV-1 expression substantially reduced tumor growth and inhibited lung metastasis in a highly metastatic xenograft mouse model as well as inhibited invasion in vitro [9]. In addition, CAV-1 is not frequently expressed in the metastatic OSCC sample, which suggests that during invasion and metastasis, CAV-1 expression decreases [7].

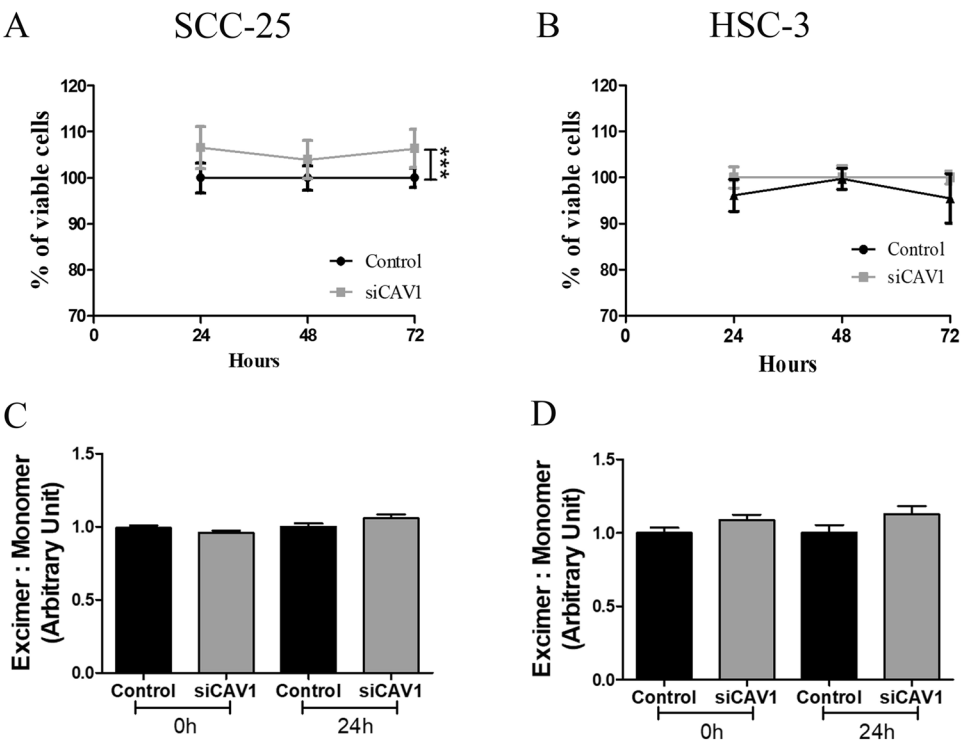

Fig. 3 Effect of siCAV-1 in cell viability and membrane fluidity. Analysis of % of viable cells by Alamar Blue assay after siCAV-1 in SCC-25 cells (**A**) and HSC-3 cells (**B**). Evaluation of membrane fluidity by fluorescence assay using a Membrane Fluidity kit in SCC-25 (**C**) and HSC-3 (**D**) cells after siCAV-1. (***$p < 0.001$; Error bars represent the standard deviation of 3 or more experiments)

In general, loss of CAV-1 expression in tumor stromal cells has been associated with aggressive disease and poor outcomes in various tumors, including oral tongue cancer [4, 7, 11]. Here, CAV-1 positivity in stromal cells was associated with the presence of cancer and with smaller tumor size, suggesting that its expression in the TME may promote tumor progression. In our OSCC samples, we could not verify a similar significant association between the TME CAV-1 expression and overall survival, although there was a similar trend as observed by Vered et al. [10]. In the stroma, the expression of CAV-1 in spindle cells refers most likely to carcinoma-associated fibroblasts (CAFs), which contribute to tumor invasion [10]. Using 3D in vitro human myoma disc assay, Vered et al. [10] also showed a higher CAV-1 expression in the TME mimicking myoma discs compared to the invading HSC-3 cancer cells. They demonstrated that CAF-like cells surrounding the invading HSC-3 cells had a triple positive profile of CAV-1/alpha-smooth muscle actin/Twist, and the CAV-1-TME positivity correlated with the CAF density [10]. These findings suggest the possible role of CAV-1 either in EMT or in fibroblasts undergoing transdifferentiation towards CAFs. Jung et al. [24] observed that the decrease in *CAV-1* expression in head and neck cancer samples was characterised by a high propensity for rapid distant metastasis when compared to tumor samples with less invasive capacity and that *CAV-1* negative expression was associated with poor prognosis. They also reported that *CAV-1* negative expression enables cells to undergo the EMT process, observed by the reduction of E-cadherin and β-catenin protein expression and increased migration and invasion capacity in SCC-9 OTSCC cells. However, in our experiments, using different OTSCC cell lines, we could not confirm any changes in any EMT markers after silencing CAV-1 in the protein level, although in HSC-3 cells, silencing CAV-1 induced *NCAD* and *βCAT* genetic expressions.

To further investigate the functional role of *CAV-1* in cell viability, migration, and invasion, we compared normal and si*CAV-1* cell lines. In the primary OTSCC tissue-derived cell line, SCC-25, the silencing of *CAV-1* improved cell viability but did not affect migration or invasion. On the other hand, in the metastatic HSC-3 cells, *siCAV-1* improved their invasion significantly but did not change their viability. These results suggest a possible association between *CAV-1* negative expression in aggressive cancer cells leading to more effective invasion and progression of cancer. Here, migration capacity in HSC-3 cells was not affected, which could be associated with the significant late increase in *βCAT* expression and the slight increase, but not significant, in *ECAD* expression. Figure 6 summarises the results obtained in this work. (Fig. 6)**.** In contrast, Nohata et al. [12] showed that *CAV-1* silencing decreased the migratory and invasive capacity of HSC-3 cells [12]. However, there are some differences regarding the in vitro cell culture methodology, which should be taken into account. First, Nohata et al. [12] used a vertical transwell assay with Matrigel® membrane filter inserts, while our horizontal invasion assay was performed with Myogel (derived from human

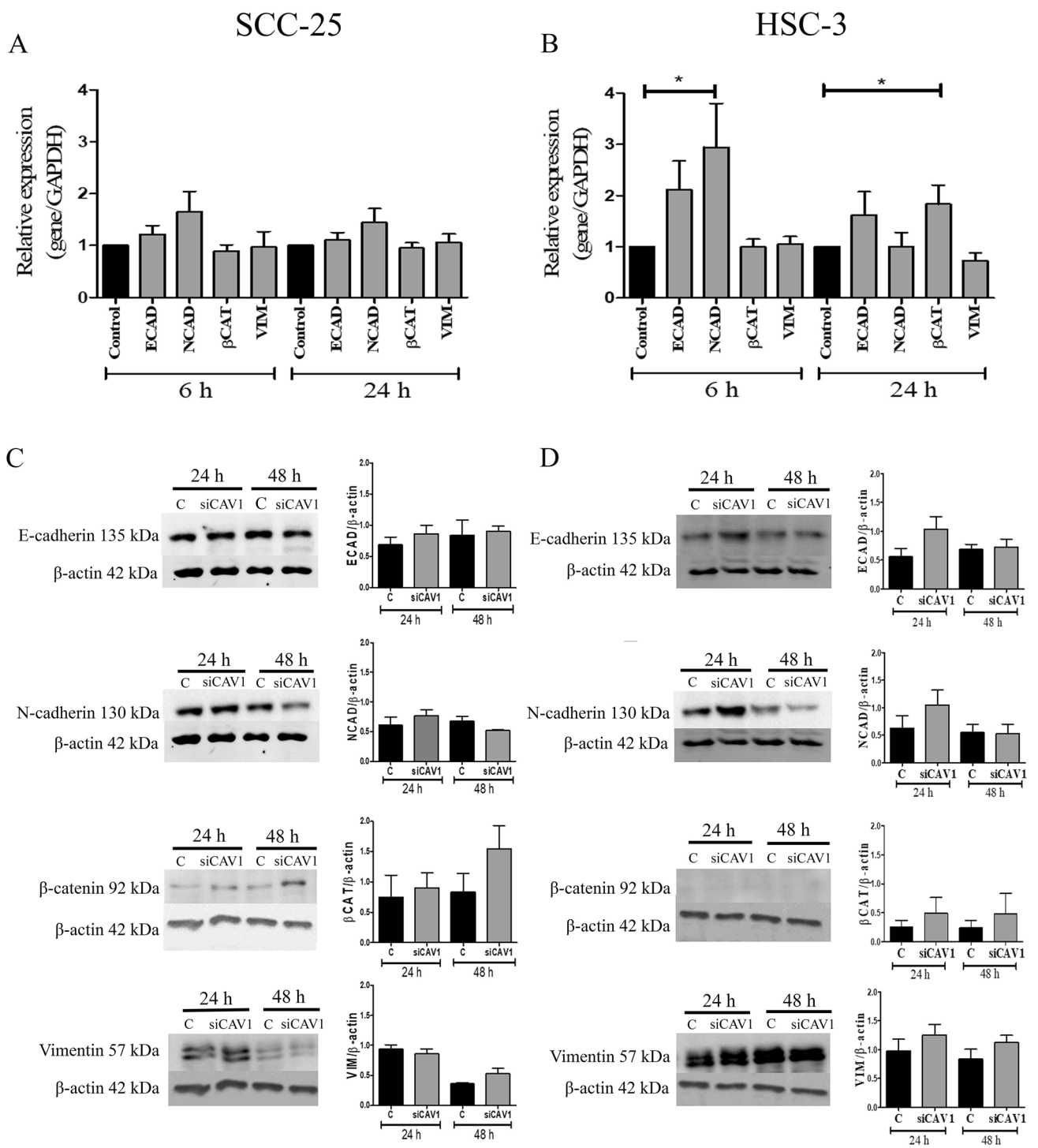

Fig. 4 Effect of siCAV-1 on gene and protein expression. Analysis of gene expression of EMT markers by qRT-PCR in SCC-25 cells (**A**) and HSC-3 cells (**B**). Western Blotting experiments for evaluation of protein expression of EMT markers in SCC-25 cells (**C**) and HSC-3 cells (**D**). (*$p < 0.05$; Error bars represent the standard deviation of 3 or more different experiments)

uterus leiomyoma tissue) through which the cells invade after scratching the wounds. In a recent study, the invasion of different OSCC cells in distinct matrices indicates that invasion speed is different depending on the type of matrix used and the characteristics of the cancer cells [25]. The most commonly used Matrigel®, derived from mouse tumors, confers disadvantages for human-derived cell culture studies. Myogel, derived from a human tumor,

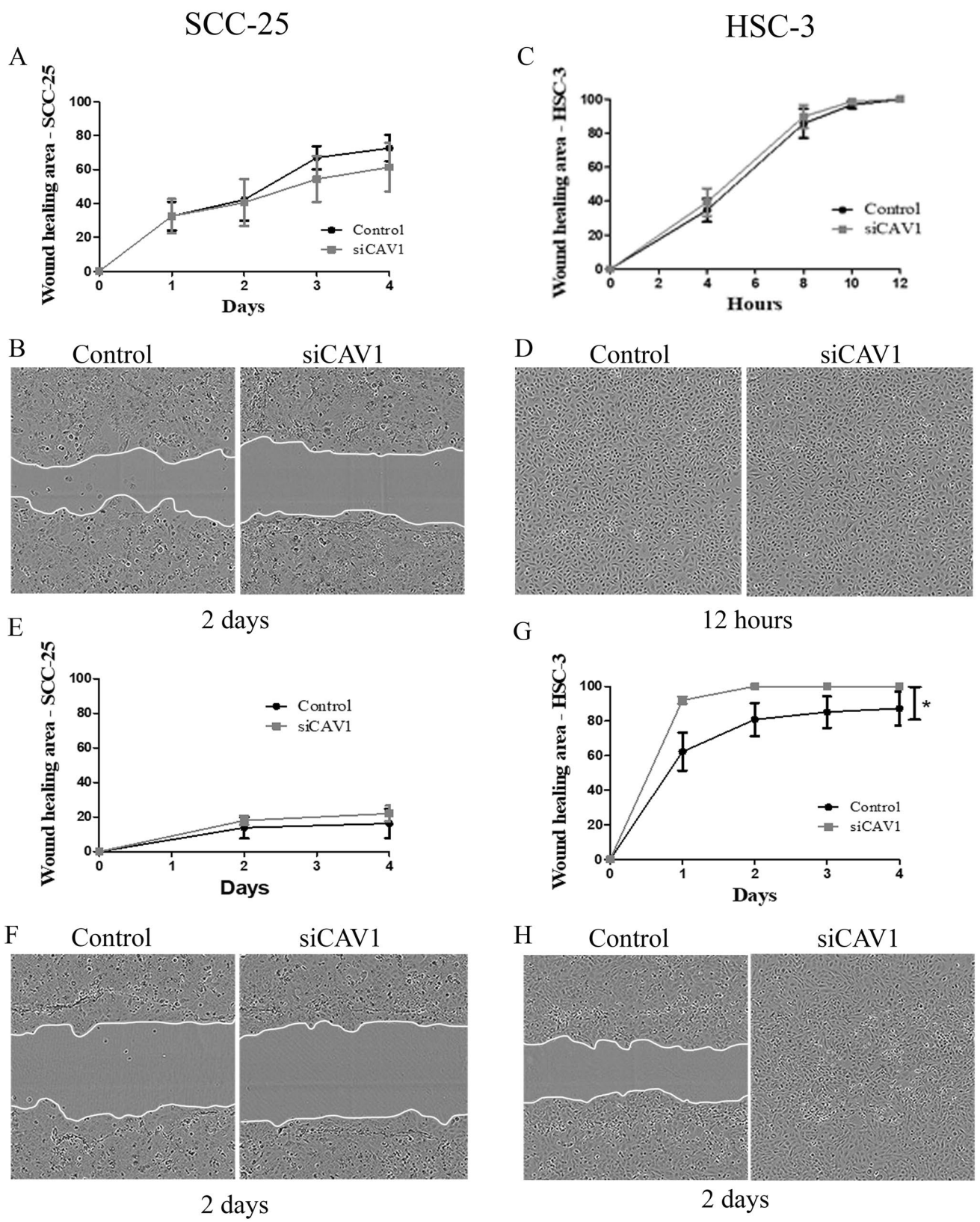

Fig. 5 Effect of siCAV-1 on cell migration and invasion. Migration assay was evaluated by scratch wound healing assay on Myogel coating in both SCC-25 (**A** and **B**) and HSC-3 (**C** and **D**) cells after 48 h siCAV-1. A wound healing assay was performed in Myogel-fibrin for evaluating the effect of silencing of CAV-1 on the invasion capacity of SCC-25 (E and F) and HSC-3 (**G** and **H**) cells (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Error bars represent the standard deviation of 3 or more different experiments)

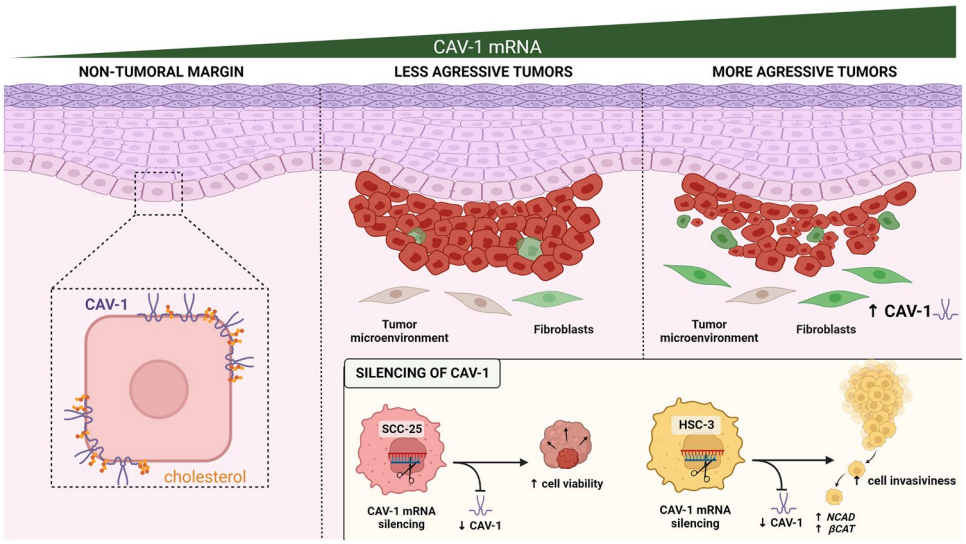

Fig. 6 Summary of results. Normal cells present cholesterol-rich membranes with the presence of caveolar structures containing Caveolin-1 (CAV-1) protein. An increase in CAV-1 expression was observed in tumors when compared to non-tumor margin tissues and in more aggressive tumors when compared to less aggressive tumors. This increase in CAV-1 expression was greater in tumor microenvironment cells, such as cancer-associated fibroblasts. In addition, the silencing of CAV-1 in OSCC cells induced an increase in the cell viability of non-metastatic SCC-25 cells but increased the invasive capacity of metastatic HSC-3 cells, showing that the effect of CAV-1 expression varies in different cellular profiles of the same tumor type

provides a soluble human TME-mimicking matrix. Compared with the Matrigel®, Myogel contains e.g. latent and active MMP-2, tenascin-C, and collagen types XII and XIV, which are not in Matrigel®. Myogel also has neutral and stable pH, and it has been shown to efficiently induce the invasion of cancer cells [22].

Although some studies have shown that changes in the cholesterol content of the plasma membrane affect the CAV-1 and the membrane fluidity [26, 27], in our study, the silencing of *CAV-1* did not affect membrane fluidity, suggesting that just the change in CAV-1 expression may not be enough to impact membrane fluidity, nor be sufficient to affect the cholesterol content in the cell membrane.

Conclusion

Increased CAV1 mRNA was observed in tumors compared to non-tumors, with a greater expression seen in more aggressive tumors. According to immunohistochemistry, an increased expression of CAV-1 by stromal cells was noted. In addition, siCAV1 in OSCC cells that present a metastatic profile potentiated the invasive capacity of these cells, which suggests that CAV1 expression varies at different stages of the disease. Given these results, there seems to be an association between the loss of CAV1 expression in tumor cells and the gain of expression by stromal cells, which induces a greater metastatic and invasive capacity.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12105-023-01562-w.

Acknowledgements GENCAPO (Head and Neck Genome Project) Consortium for the sample collection and initial processing, clinical data collection, providing histopathological analysis of tissue samples, and the acquisition of informed consent from each patient. We declare the paper was reviewed for grammar and syntax by an English expert.

Author Contributions All authors have contributed to this article as follow: RBN performed all the in vitro experiments, analysis, data interpretation, and wrote the initial draft of the manuscript. MJ and TAS designed the in vitro experiments and data interpretation. LHSS performed IHC experiments. KBSP, MFSDR, COR, FCAX performed the microarray and qRT-PCR and their analysis of tumor samples. FCAX and FDN analysed IHC images and statistical analysis. RDC, RVML and FDN are members of GENCAPO and collected the tissue samples and clinical data. FCAX, KBSP and TAS were the advisors of RBN PhD thesis. All authors have reviewed and approved the final version.

Funding This work was supported by the National Council for Scientific and Technological Development (CNPq) [Grant No. 421249/2018–8] and Higher Education Personnel Improvement Coordination (RB Nascimento fellowship—CAPES).

Declarations

Conflict of interest The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval "All procedures performed in studies involving human participants were in accordance with the ethical standards of the Brazilian National Ethics Committee (Process #16491) and with

the 1964 *Helsinki declaration* and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

Consent for Publication For this type of study consent for publication is not required.

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