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ORIGINAL ARTICLE

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Overexpression of heat-shock protein 47 impacts survival of patients with oral squamous cell carcinoma

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Abstract

Background: The expression of heat-shock protein 47 (HSP47) has been linked to collagen synthesis control and implicated in fibrotic disorders, but more recent studies have demonstrated its role in solid tumors. In this study, we explored the prognostic impact of HSP47 in oral squamous cell carcinomas (OSCC) and determined the in vitro effects of its loss-of-function on viability, proliferation, migration, invasion, and resistance to cisplatin of OSCC cells.

Methods: The HSP47 expression in tumor samples was assessed by immunohistochemistry in two independent cohorts totaling 339 patients with OSCC, and protein levels were associated with clinicopathological features and survival outcomes. The OSCC cell lines HSC3 and SCC9 were transduced with lentivirus expressing short hairpin RNA to stably silence HSP47 and used in assays to measure cellular viability, proliferation, migration, and invasion.

Results: HSP47 was overexpressed in OSCC samples, and its overexpression was significantly and independently associated with poor disease-specific survival and shortened disease-free survival in both OSCC cohorts. The knockdown of HSP47 showed

no effects on cell viability or cisplatin sensitivity, but impaired significantly proliferation, migration, and invasion of OSCC cells, with stronger effects on SCC9 cells.

Conclusion: Our results show a significant prognostic impact of HSP47 overexpression in OSCC and reveal that HSP47 inhibition impairs the proliferation, migration, and invasion of OSCC cells. HSP47 may represent a potential therapeutic target for OSCC.

KEYWORDS
invasion, oral cancer, prognosis, proliferation, SERPINH1

1 | INTRODUCTION

The heat-shock protein 47 (HSP47), which is encoded by the serpin family H member 1 (*SERPINH1*) gene, was originally identified as a 47-KDa glycoprotein localized in the endoplasmic reticulum (ER) of collagen producing cells and was later characterized as a collagen-specific chaperone that participates in the translation of procollagen in the ER and in its translocation to the Golgi apparatus, then recycling back to the ER.[1] The expression of HSP47 has been involved with fibrotic disorders including scleroderma, keloids, hereditary gingival fibromatosis, and other fibrotic conditions of many organs, such as those of the kidneys, heart, liver, and lungs, by encouraging the buildup of collagen.[2,3] Aside from its canonical function as molecular chaperone for nascent chains of collagen, the studies are revealing that HSP47 influences numerous cellular processes including proliferation, apoptosis, and invasion,[4] and HSP47 expression is correlated with chemoresistance[5,6] and poor prognosis in solid tumors.[7] In the neoplastic context, the effects of HSP47, depending of the cell type, were related to activation of pathways involving transforming growth factor-beta (TGF-β),[8] Wnt/β-catenin,[9] and Akt.[10]

The expression and the prognostic significance of HSP47 in head and neck squamous cell carcinomas have been revealed in some studies[7,11–13]; however, little is known about its biological role in oral squamous cell carcinoma (OSCC), the most common malignancy in the oral cavity, which displays high prevalence and mortality worldwide. Lee et al.[14] demonstrated that HSP47 levels are significantly higher in areca quid chewing-associated OSCCs compared to normal oral tissues, and in a recent study, Fu et al.[15] demonstrated that HSP47 was one of the 18 upregulated genes in OSCC compared to normal tissue after combining two GEO microarray datasets. Although HSP47 is described as an ER-resident protein, Hebert et al.[16] demonstrated that HSP47 can be anchored in the cell membrane in a complex with CD9, where regulates motility and invasiveness of OSCC cells. In a previous screening study, which was based on laser capture microdissection coupled mass spectrometry, we have shown that HSP47 is significantly more expressed in OSCC cells compared with normal oral epithelial cells.[17] In the current study, we conducted an immunohistochemical analysis to verify the impact of HSP47 levels on OSCC prognosis, and we further explored the role of HSP47 by knocking it down in OSCC cells.

2 | MATERIALS AND METHODS

2.1 | Samples and clinicopathological data

The clinical samples explored in the present study were previously described in detail.[18] The Cohort 1 included 254 whole tumor sections derived from patients with OSCC diagnosed and treated in hospitals in Brazil, Chile, and Finland, and Cohort 2 included primary OSCCs and matched nonmalignant oral epithelial tissues, and 17 lymph node metastases, included in a tissue microarray (TMA), from patients treated at the Jewish General Hospital, Montreal (Canada). The clinicopathological features are shown in Table S1. The study was carried out with approval of the Human Research Ethics Committee (CAAE: 55927322.0.0000.5418).

2.2 | Immunohistochemistry

Immunohistochemistry was performed with standard method,[19] using an anti-HSP47 antibody (HPA029198, Sigma-Aldrich, USA). The semi-quantitative evaluation of HSP47 expression was performed by two qualified pathologists, who scored HSP47 intensity of staining and percentage of positive tumor cells as described elsewhere.[19]

2.3 | Cell culture

HGK, an immortalized normal human gingival keratinocyte cell line, was cultured in serum-free, low calcium media (Gibco's keratinocyte-SFM: Invitrogen, USA), and OSCC cell lines HSC3, SCC4, SCC9, SCC15, SCC25, and CALF27 were cultured in 1:1 Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12; Invitrogen) containing 10% fetal bovine serum, 400 ng/mL hydrocortisone (Sigma-Aldrich), and antibiotics in an incubator at 37°C with 5% CO_2.

2.4 | Stable cells with HSP47 downregulation

Lentiviral vectors containing short hairpin RNA (shRNA) targeting human HSP47 (sequence HSP47#1: 5′GCCTTTGAGTTGGACACAGAT3′, and sequence HSP47#2: 5′CCTCTACAACTACTACGACGA3′) or control shRNA (MISSION® pLKO.1-puro non-Mammalian shRNA Control) were produced by Sigma-Aldrich. In growth medium containing 8 mg/mL

of polybrene (Sigma-Aldrich), HSC3 and SCC9 cells were incubated with control or HSP47 shRNA lentiviral particles at a multiplicity of infection of 2.0 for 8 h. The cells were then washed with phosphate-buffered saline and cultivated for a further 48 h in fresh medium. To sort out resistant cells, the cells were cultivated for 15 days with puromycin dihydrochloride (Sigma-Aldrich) at a concentration of 1 mg/mL for SCC9 cells and 2 mg/mL for HSC3 cells.

2.5 | Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) and western blot analysis

The quantification of HSP47 mRNA was performed using specific primers (HSP47 forward 5′GTGAGACCAAATTGAGCTAGGG3′ and reverse 5′TAGTTGGGAGAGGTTGGGATAG3′, and cyclophilin A-PPIA forward 5′GCTTTGGGTCCAGGAATGG3′ and reverse 5′GTTGTCCACAGTCA GCAATGGT3′) and Sybr green chemistry (Applied Biosystems, USA). The $2^{-\Delta\Delta Ct}$ quantification method was used, with the housekeeping PPIA (Cyclophilin A) as the reference gene for data normalization.

Western blot analysis was conducted according to Ervolino De Oliveria et al.,[20] using antibodies against HSP47 diluted 1:500 (HPA029198, Sigma-Aldrich) or β-actin diluted 1:50000 (clone AC-15; Sigma-Aldrich).

2.6 | Viability, apoptosis, and proliferation assays

Cells were seeded into 96-well or 6-well plates and cultured for 24 h, followed by viability assay (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay, Promega, USA) or by apoptosis analysis with a flow cytometric apoptosis assay (Annexin V Apoptosis Detection Kit, ThermoFisher Scientific, USA), both in accordance with manufacturer's instructions.

The proliferation was measured by bromodeoxyuridine (BrdU) incorporation into DNA using a BrdU cell proliferation ELISA kit (Roche Applied Science, USA), or by growth curves. For the growth curves, cells were seeded in 96-well plate at 1000 cells/well, and proliferation was determined every 24 h, up to 96 h, with the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega).

2.7 | Transwell migration and invasion assays

The 24-well plates with transwell inserts of polycarbonate with 6.5 mm diameter and 8 μm pore were used to evaluate the migration and invasion of the cells, as previously reported by us.[21] In the invasion assays, the membrane of inserts was covered with myogel.

2.8 | Treatments with hydrogen peroxidase and cisplatin

For hydrogen peroxidase treatment, cells (5000 cells/well in 96 well-plate) were incubated with 125 μM of hydrogen peroxidase diluted directly in complete medium for 1 h. For detection of the cytotoxicity and IC_{50} of cisplatin, cells were incubated with different concentrations (0, 2.5, 5, 10, 20, and 40 μM) of cisplatin (Sigma-Aldrich) for 24 or 48 h. After incubation, cellular viability assays were performed as described above.

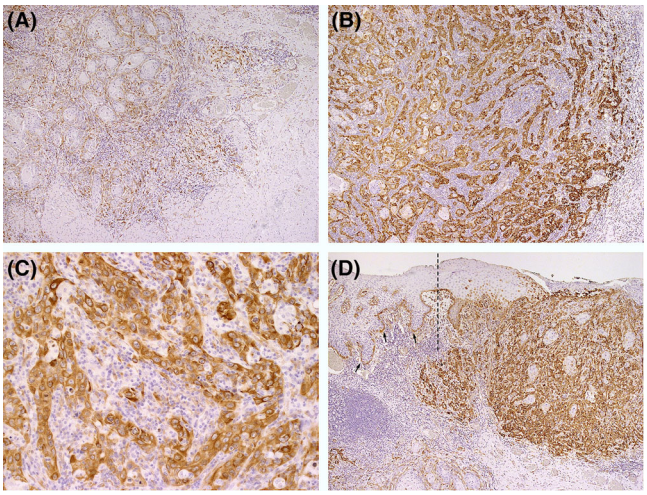

FIGURE 1 Immunohistochemical detection of heat-shock protein 47 (HSP47) in oral squamous cell carcinoma (OSCC) samples. Tumor cells demonstrated a clear cytoplasmic HSP47 expression, with variable intensity. The central regions of the tumor islands, occupied by differentiating cells with accumulation of cytokeratin, were frequently negative. (A) Representative invasive front of a tumor classified with low expression of HSP47, whereas (B) displays representative samples classified with high HSP47 expression. (C) High power view of the sample represented in (B), showing the positive immunostaining for HSP47 in the cytoplasm of the tumor cells. (D) One of the samples of study with a transition area from dysplastic oral epithelia to invasive carcinoma (dashed line). The expression of HSP47 increases in intensity and number when it goes from dysplastic cells (arrows highlighting positivity restricted to the basal cells) to invasive tumor cells (×100, original magnification for A, B, and D; ×200, original magnification for C).

2.9 | Statistical analysis

The immunohistochemical expression of HSP47 among normal, primary, and lymph node metastasis was compared with the Kruskal–Wallis test. In the table with clinicopathological characteristics, the differences were assessed with chi-square test. The survival methods included Kaplan–Meier curves with comparison based in log rank test, and both univariate and multivariate Cox regression analyses.

All in vitro experiments were repeated at least three times, and the final experimental data are expressed as mean ± standard deviation. For analysis of the in vitro assays, analysis of variance with post hoc comparisons using the Tukey's multiple comparisons test was applied. $p < 0.05$ was considered statistically significant.

TABLE 1 Cancer-specific survival and disease-free survival of 254 patients with oral squamous cell carcinoma (Cohort 1) based on univariate and multivariate analysis.

	Cancer-specific survival		Disease-free survival	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
Univariate analysis				
Age (years)				
≤63 years	1		1	
>63 years	1.67 (1.16–2.41)	0.006	1.29 (0.85–1.94)	0.23
Gender				
Male	1		1	
Female	0.92 (0.62–1.35)	0.66	0.92 (0.59–1.43)	0.70
Clinical stage (7th ed.)				
Early (I + II)	1		1	
Advanced (III + IV)	1.73 (1.20–2.48)	0.003	1.52 (1.01–2.27)	0.04
Tumor site				
Tongue	1		1	
Floor of mouth	0.97 (0.57–1.66)	0.94	0.83 (0.46–1.53)	0.58
Other	1.28 (0.76–2.15)	0.31	1.35 (0.75–2.41)	0.23
Histopathological grading				
Well differentiated	1		1	
Moderately differentiated	1.05 (0.70–1.57)	0.82	0.74 (0.46–1.17)	0.17
Poorly differentiated	1.46 (0.77–2.73)	0.19	0.92 (0.44–1.90)	0.82
Treatment				
Surgery	1		1	
Surgery + radiotherapy	1.30 (0.85–1.98)	0.18	0.85 (0.54–1.35)	0.50
Surgery + radiotherapy + chemotherapy	0.90 (0.56–2.44)	0.91	1.29 (0.76–2.19)	0.28
Margin status				
≥5 mm	1		1	
<5 mm	0.98 (0.63–1.53)	0.93	1.25 (0.76–2.04)	0.37
HSP47				
Low expression	1		1	
High expression	2.26 (1.52–3.35)	0.0001	1.97 (1.25–3.10)	0.0035
Multivariate analysis (stepwise method)				
Age (>63 vs. ≤63 years)	1.72 (1.15–2.56)	0.008	-	-
Clinical stage (advanced stage vs. early stage)	1.59 (1.07–2.36)	0.02	1.59 (1.03–2.46)	0.03
HSP47 (high expression vs. low expression)	2.34 (1.58–3.47)	0.0001	1.87 (1.21–2.88)	0.005

3 | RESULTS

3.1 | HSP47 levels are associated with OSCC prognosis

The cytoplasmic immunoreactivity for HSP47 was observed in all OSCC samples, with variable distribution and intensity in the tumor cells (Figure 1A–C). The positivity was localized mainly along the advancing front of the tumor and limited within the central differentiated regions of the tumor islands. A progressive increase in the intensity and extension of HSP47 immunoreactivity was observed from dysplastic adjacent areas towards areas with tumor invasion (Figure 1D). In the normal mucosa samples, immunoreactivity for HSP47 was weaker and restricted to the basal layer (Figure S1A). The expression of HSP47 was significantly higher in primary tumors ($p < 0.0001$) and lymph node metastases ($p < 0.0001$) than in normal tissues (Figure S1B–D).

The association of the clinicopathological features of the tumors with HSP47 expression is depicted in Tables S2 and S3. High expression of HSP47 in tumor cells in Cohort 1 was significantly associated with recurrence ($p = 0.026$), and in Cohort 2, HSP47 was significantly associated with advanced clinical stage ($p = 0.02$) and tumor location ($p = 0.03$), with tumors of the oral tongue more frequently classified with high HSP47 expression than tumors in other locations (Table S3). Kaplan–Meier curves showed that tumors that overexpressed HSP47

TABLE 2 Univariate and multivariate analysis of cancer-specific survival and disease-free survival of the validation cohort composed of 85 cases of oral squamous cell carcinoma.

	Cancer-specific survival		Disease-free survival	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
Univariate analysis				
Age (years)				
≤63 years	1		1	
>63 years	1.12 (0.57–2.21)	0.73	1.43 (0.76–2.68)	0.26
Gender				
Male	1		1	
Female	0.92 (0.46–1.81)	0.80	1.33 (0.70–2.49)	0.36
Clinical stage (7th ed.)				
Early (I + II)	1		1	
Advanced (III + IV)	2.46 (1.23–4.92)	0.01	1.50 (0.78–2.89)	0.22
Tumor site				
Tongue	1		1	
Floor of mouth	0.50 (0.16–1.58)	0.29	0.95 (0.31–2.94)	
Other	0.69 (0.2–1.48)	0.28	0.62 (031–1.23)	0.44
Histopathological grading				
Well differentiated	1		1	
Moderately differentiated	1.94 (0.79–4.71)	0.27	1.42 (1.12–5.24)	
Poorly differentiated	3.67 (1.25–10.7)	0.02	3.21 (1.26–8.20)	0.10
Treatment				
Surgery	1		1	
Surgery + radiotherapy	0.53 (0.25–1.09)	0.29	0.84 (0.23–5.27)	
Surgery + radiotherapy + chemotherapy	0.82 (0.18–2.83)	0.69	1.62 (0.55–4.75)	0.17
Margin status				
≥5 mm	1		1	
<5 mm	1.14 (0.50–2.58)	0.75	1.36 (0.66–2.78)	0.39
Perineural invasion				
No	1		1	
Yes	1.39 (0.62–3.11)	0.41	1.68 (0.76–3.71)	0.20
Lymphovascular invasion				
No	1		1	
Yes	2.56 (1.18–5.56)	0.02	1.69 (0.80–3.56)	0.17
HSP47				
Low expression	1		1	
High expression	2.00 (1.08–4.04)	0.04	2.01 (1.04–3.91)	0.03
Multivariate analysis (stepwise method)				
Clinical stage (advanced stage vs. early stage)	3.04 (1.34–6.87)	0.008	3.35 (1.48–7.56)	0.004
Lymphovascular invasion (yes vs. no)	2.20 (1.03–4.61)	0.04	—	—
HSP47 (high expression vs. low expression)	2.70 (1.31–5.56)	0.007	2.10 (1.05–4.19)	0.04

showed statistically poorer cancer-specific survival ($p = 0.0001$ for Cohort 1, Figure S2A and $p = 0.042$ for Cohort 2, Figure S2C) and shortened disease-free survival ($p = 0.005$ for Cohort 1, Figure S2B and $p = 0.031$ for Cohort 2, Figure S2D) compared with low expressing tumors.

Univariate survival analysis in Cohort 1 showed a significant association of age ($p = 0.006$), clinical stage ($p = 0.003$) and HSP47 expression ($p = 0.0001$) with cancer-specific survival, and clinical stage ($p = 0.04$) and HSP47 expression ($p = 0.0035$) with disease-free survival (Table 1). To determine whether HSP47 is an independent

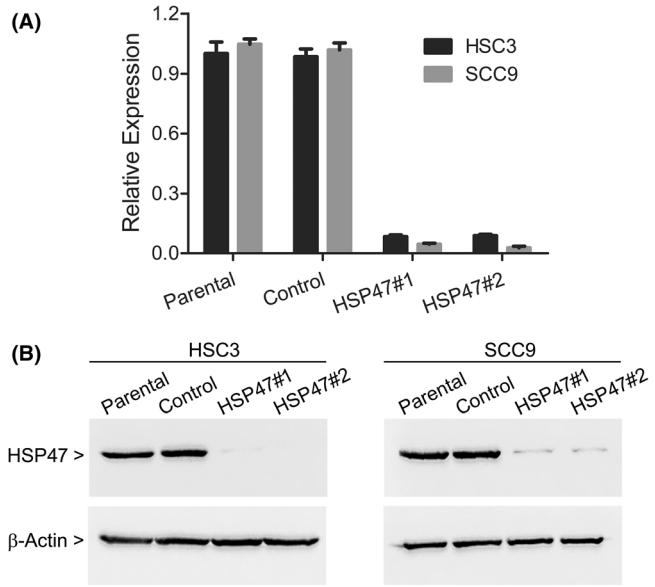

FIGURE 2 Knockdown of heat-shock protein 47 (HSP47) in HSC3 and SCC9 cells. After transduction and selection with puromycin for 15 consecutive days, cells were subjected to RT-qPCR and western blot. Parental cells were also included in the analyses. Results from both RT-qPCR (A) and western blot (B) demonstrated an effective downregulation of HSP47 levels in the cells transduced with lentivirus expressing specific short hairpin RNAs against HSP47 mRNA.

marker of survival, multivariate survival analysis based on the stepwise model of the Cox regression test was employed. Expression of HSP47 remained as independent prognostic factor for both cancer-specific survival (HR: 2.34, 95% CI: 1.58–3.47, $p = 0.0001$) and disease-free survival (HR: 1.87, 95% CI: 1.21–2.88, $p = 0.005$). In Cohort 1, age >63 years withstood as an independent parameter for death due to cancer, and advanced clinical stage (Stages III and IV) was independently associated with both poor cancer-specific survival and shortened disease-free. The results of the impact of HSP47 expression on survival were replicated on Cohort 2. The univariate survival analyses confirmed a significant association of HSP47 expression with cancer-specific survival (HR: 2.00, 95% CI: 1.08–4.04, $p = 0.04$) and disease-free survival (HR: 2.01, 95% CI: 1.04–3.91, $p = 0.03$), which remained as independent prognostic factor in the multivariate analyses (Table 2).

3.2 | Downregulation of HSP47 impairs proliferation, migration, and invasion of OSCC cells

We next tested HSP47 expression in six OSCC cell lines and in the normal keratinocyte cell line HGK and found the highest HSP47 levels of mRNA and protein in HSC3 and SCC9 cells (Figure S3). These cell lines were then selected to examine HSP47 effects on viability, proliferation, migration, invasion, and resistance to cisplatin after stably knockdown HSP47 through lentivirus-mediated expression of two different sequences of shRNA against its mRNA. The cells expressing the specific HSP47 sequences demonstrated a significant reduction in both mRNA and protein levels in comparison with parental cells or cells transduced with the control, nontargeting sequence (Figure 2). For the functional assays, only the cells transduced with the nontargeting sequence (HSC3 Control or SCC9 Control) were used as control.

The stable HSP47 knockdown had no effects on viability and apoptotic rates of the cells (Figure S4), even when the cells were damaged/stressed with hydrogen peroxide (Figure S5). To assess the effect of HSP47 on proliferation, BrdU assay was initially carried out. Down regulated expression of HSP47 decreased significantly BrdU incorporation into DNA synthesis of HSC3 and SCC9 cells (Figure 3A). To confirm that HSP47 knockdown alters proliferation, growth curves ranging from 24 to 96 h were constructed (Figure 3B,C). The HSP47 knockdown resulted in a significant decrease in the cell number at 72 and 96 h. The silencing of HSP47 impaired the migration and invasion, but the effects were more pronounced (and significant in the case of migration) in SCC9 than in HSC3 (Figure 4). Dose–response curves with cisplatin for 24 and 48 h were constructed to explore the cytotoxicity of cisplatin in the cells with HSP47 silencing (Figure S6). No significant differences on IC_{50} values were observed.

4 | DISCUSSION

Although HSP47 has been originally described as a unique collagen-specific binding protein expressed by collagen-producing cells, later studies have demonstrated that HSP47 is expressed in many organs and cell types, in both normal and pathological conditions (The Human Protein Atlas, https://www.proteinatlas.org/ENSG00000149257-SERPINH1). Dysregulated expression of HSP47 has been reported in several cancers, but its impact in OSCC is still uncertain. Using proteomic analysis, we identified several proteins differentially expressed between OSCC and adjacent normal tissues, including HSP47.[17] Interestingly, HSP47 upregulation was consistently observed in all OSCC samples compared to the normal counterparts, suggesting that this protein is possibly involved in OSCC pathogenesis. The present study initially performed immunohistochemical analysis to determine the prognostic role of HSP47 expression in two independent cohorts of OSCC. As the samples in the cohorts were available as whole tumor sections (Cohort 1) or as 1-mm core, composing a TMA (Cohort 2), we decided to perform the analyses separately, as there is no complete consensus regarding agreement on immunohistochemical analysis between whole sections and TMAs. Of note, the results showed a substantial agreement, revealing that HSP47 displays a significant independent prognostic value for OSCC survival.

In line with previous studies,[14,17] we demonstrated that the expression of HSP47 was significantly higher in primary OSCCs and OSCC metastatic lymph nodes when compared with healthy oral mucosa. This same HSP47 upregulated profile was detected in gastric cancers,[9] esophageal squamous cell carcinomas,[22] clear cell renal cell

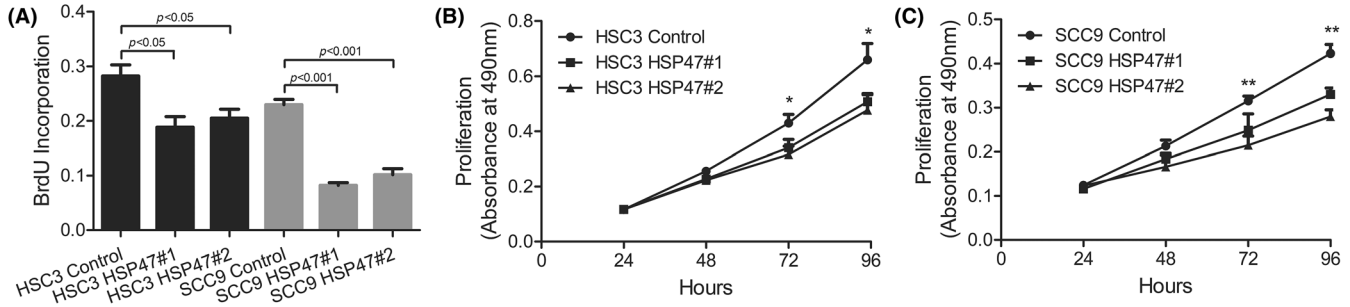

FIGURE 3 Heat-shock protein 47 (HSP47) regulates the proliferation of oral squamous cell carcinoma (OSCC) cells. (A) The proliferation of the cells with HSP47 knockout was significantly decreased, as revealed by bromodeoxyuridine (BrdU) incorporation assay. (B) The proliferative rate of the cells lines was also determined with a growth curve over a time-course from 24 to 96 h. At 72 and 96 h, differences statistically significant were detected, indicating a reduced proliferative potential of the HSP47 silenced cells. Data are presented as the mean ± standard deviation of three independent assays, each performed in quadruplicate. $^{*}p < 0.05$; $^{**}p < 0.01$.

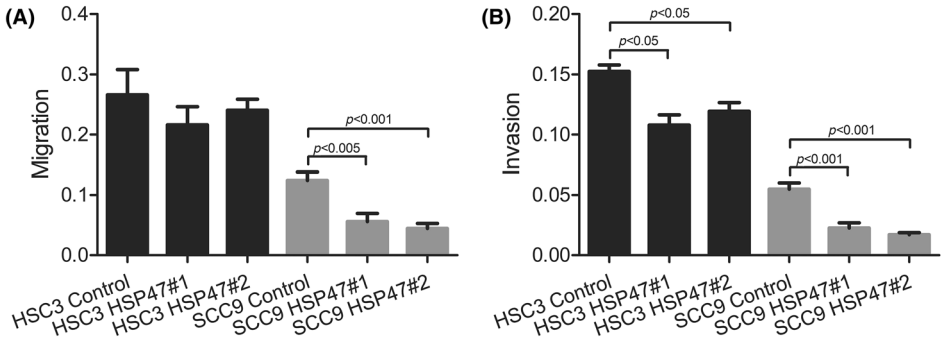

FIGURE 4 Knockdown of heat-shock protein 47 (HSP47) modulates migration and invasion of oral squamous cell carcinoma (OSCC) cells. Vertical migration and invasion assays were performed with transwells, with pores of the membrane covered by myogel in the invasion experiments, as outline in material and methods. Transwell experiments demonstrated that the migration (A) and invasion (B) abilities of the HSP47 silenced cells were inhibited in comparison with controls. Invasion effects reached significant levels for both cell lines, whereas for migration, only SCC9 clones demonstrated levels statistically significant. Data are presented as the mean ± standard deviation of three independent assays, each performed in quadruplicate.

carcinomas,[23] and colorectal cancers.[24] In Cohort 1, higher levels of HSP47 were significantly associated with recurrence, whereas high HSP47 levels in samples from Cohort 2 demonstrated significant association with clinically advanced cases and tumors located in the oral tongue. In colorectal cancer, the expression of HSP47 was significantly associated with high T stage, presence of lymph node metastasis, and advanced TNM clinical stage.[24] Comparing primary OSCCs with and without lymph node metastasis, Nikitakis et al.[25] did not find differences in the immunohistochemical intensity of HSP47 between groups, which is in agreement with our findings in Cohort 2. More important, our findings showed a significant association between high HSP47 expression and worse clinical outcomes, where patients with tumors displaying high expression levels of HSP47 had substantially poor cancer-specific survival and increased chance of relapse after treatment (shorter disease-free survival) than did patients with tumors expressing low HSP47 levels. In both cohorts, multivariate analysis showed that HSP47 overexpression is an independent prognostic marker associated with OSCC outcomes.

Chern et al.,[5] exploring two public databases derived of colorectal cancers (The Cancer Genome Atlas database and the curatedCRCData), revealed that HSP47 overexpression is significantly associated with poor overall survival of patients with colorectal cancer. In this same year, Mori et al.[24] showed that HSP47 expression is as an independent predictive marker for poor overall survival and disease-free survival by applying multivariate analyses in 139 patients with colorectal cancer. The negative impact of HSP47 overexpression on survival of patients with cancer was also reported in gastric cancer,[9] esophageal squamous cell carcinoma,[22] and renal cell carcinoma.[23] Although the literature has consistently demonstrated a HSP47 gain of expression across multiple cancers compared to corresponding normal tissues and a significant poorer survival in patients with high HSP47 expression levels than in patients with low levels in different types of cancers, Song et al.[26] demonstrated in 50 samples of laryngeal squamous cell carcinoma that HSP47 expression in normal laryngeal tissues is higher than that found in cancer tissues, and that lower HSP47 expression levels were associated with poor overall survival. It is not still clear whether these discrepant findings are due to tissue-specific expression, because no additional studies have explored the expression of HSP47 in laryngeal tumors. Interestingly, cancers from esophagus and oral cavity, which have demonstrated similar pattern of HSP47 expression and role in tumor

progression, and from larynx belong to the group of the head and neck cancers. It is also important to mention that surgical pieces of laryngeal tumors are commonly decalcified due to the presence of cartilage, which is necessary for the histological procedure, and immunohistochemical expression in decalcified tissues need to be carefully interpreted.

The present study further revealed that the expression of HSP47 controls the proliferation, migration and invasion of OSCC cells, with clearer effects on SCC9 cells, which show a higher expression of HSP47 than HSC3 cells. The knockdown of HSP47, which was expressed in high levels in TE-8 cells, an esophageal cancer cell line, significantly inhibited proliferation and colony formation, conferring an oncogenic potential on esophageal squamous cell carcinoma.[22] Loss-of-function studies based on shRNA against HSP47 revealed that HSP47 contributes to proliferation, migration, and invasion of lung cancer cells.[10] In gastric cancer, HSP47-silenced cells demonstrated reduction in WNT/β-catenin signaling pathway proteins, including Snail1, Slug, and TWIST, which control epithelial–mesenchymal transition, culminating in decreased cell migration and invasion.[9] Our data did not demonstrate that HSP47 knockdown alters OSCC cell survival or sensitize cells to cisplatin, in spite of HSP47 levels has been correlated to tumor progression and response to chemotherapy in glioblastomas,[27] and overexpression of HSP47 conferred chemoresistance to 5-fluorouracil in colorectal cancer[5] and to gemcitabine, a nucleotide analog included in the multidrug chemotherapy regimen in many tumor types, in pancreatic cancer.[6] Further studies should explore the connection between HSP47 expression levels and chemoresistance, and between HSP47 and immunotherapy, which has become more frequent for OSCC, because chemotherapy has been shown to have immunomodulatory effects in multiple cancer types, and the addition of immunotherapy to chemotherapy has demonstrated overcome the chemoresistance promoted by some drugs, such as cisplatin, used alone.

OSCC is a major health problem worldwide, particularly in undeveloped countries, due to its increasing incidence and high mortality rates.[28] Toward the precision medicine era, the characterization of biomarkers ensuring prevention, early diagnosis, personally optimized treatments and prognostic prediction for OSCC is utmost desirable. As OSCC is considered a complex and very heterogeneous tumor in terms of genomic and molecular alterations, even for those displaying clinical and pathological similarities, and different biomarkers may display variable sensitivity and specificity, multiple biomarkers, specially covering different aspects of tumor, should be more effective than a single marker. The results of this study lead to the conclusion that HSP47 overexpression may induce poor prognosis in OSCC by regulating proliferation, migration, and invasion of the tumor cells and may represent a potential therapeutic target. Regarding this later potential, an important advantage is that HSP47 has procollagen as its exclusive target, though it is important to consider that HSP47 is expected to locate in the cytosol, cycling between ER compartments and Golgi complex, and an effective delivery system is needed. However, previous studies have demonstrated that cell lines derived from head and neck cancers may express HSP47 in the cell surface anchored to CD9.[16,29]

AUTHOR CONTRIBUTIONS

Bruno Cesar da Costa: Conceptualization; data curation; formal analysis; investigation; methodology; writing—original draft; writing—review and editing. **Mauricio Rocha Dourado:** Conceptualization; data curation; formal analysis; investigation; methodology; writing—review and editing. **Everton Freitas de Moraes:** Conceptualization; data curation; formal analysis; investigation; methodology; writing—original draft; writing—review and editing. **Luana Marí Panini:** Conceptualization; data curation; formal analysis; investigation; methodology; writing—review and editing. **Amr Elseragy:** Data curation; formal analysis; writing—review and editing. **Fábio Haach Téo:** Data curation; formal analysis; writing—review and editing. **Gustavo Narvaes Guimarães:** Data curation; formal analysis; writing—review and editing. **Renato Assis Machado:** Data curation; formal analysis; writing—review and editing. **Maija Risteli:** Data curation; formal analysis; writing—review and editing. **Clarissa Araujo Gurgel Rocha:** Data curation; formal analysis; writing—review and editing. **Lívia Máris Ribeiro Paranaíba:** Data curation; formal analysis; writing—review and editing. **Wilfredo Alejandro González-Arriagada:** Data curation; formal analysis; writing—review and editing. **Sabrina Daniela da Silva:** Data curation; formal analysis; writing—review and editing. **Ana Lucia Carrinho Ayroza Rangel:** Data curation; formal analysis; writing—review and editing. **Marcelo Rocha Marques:** Data curation; formal analysis; writing—review and editing. **Tuula Salo:** Conceptualization; formal analysis; funding acquisition; writing—review and editing. **Ricardo D. Coletta:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; supervision; writing—original draft; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

PEER REVIEW

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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