High level of calpain1 promotes cancer cell invasion and migration in oral squamous cell carcinoma

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Abstract. Calpain1, termed conventional calpain and a member of the Ca^{2+}-dependent neutral cysteine proteases, is considered to be involved in cancer formation and development. However, the effect of calpain1 on oral squamous cell carcinoma (OSCC) remains poorly understood. The aim of the present study was to evaluate the possibility of calpain1 as a potential molecular target for OSCC diagnosis and therapy. The present study demonstrates that calpain1 was overexpressed in OSCC cell lines and 4/7 of the tumor tissues in paired samples of tumor and noncancerous matched tissues (NCMT). In a cohort of 125 patients with primary OSCC, the high expression of calpain1 was an independent predictor for overall survival in a multivariate analysis (P=0.022). Furthermore, RNA interference-mediated suppression of calpain1 expression reduced the proliferation, migration and invasion potential of the HSC3 and CAL27 OSCC cell lines, but did not increase their apoptosis. These findings indicate that calpain1 may be a useful biomarker for novel prognostic and therapeutic strategies in oral squamous cell carcinoma.

Introduction

Oral squamous cell carcinoma (OSCC) is a highly variable disease with multiple heterogeneous genetic and epigenetic changes that accounts for ~90% of malignant oral lesions (1). Despite the improvement in OSCC treatments, the overall survival of patients has not improved significantly during the past 20 years, with 5-year survival rates of 45-60% (2,3). Local recurrence and progression to metastatic disease are the primary causes of treatment failures, while the cellular and molecular mechanisms underlying OSCC cell biological behaviors remain to be elucidated. Therefore, a comprehensive investigation of the factors and molecular events that contribute to the local recurrence and invasion of OSCC are necessary for the development of novel strategies for diagnosing symptoms and treatment.

Calpains belong to a family of intracellular Ca^{2+}-dependent cysteine proteases and are widely expressed with ubiquitous and tissue specific isoforms in higher organisms (4). The calpain family is classified according to their localization or to the presence or absence of EF-hands, structures that allow calcium binding (5). Calpain1 and Calpain-2, which were named on the basis of the concentration of calcium ions required for their activity *in vitro*, are commonly described and ubiquitously expressed in the majority of tissues (5). The catalytic subunits differing between μ-calpain and m-calpain are formed by calpain1 (encoded by CAPN1) and calpain-2 (encoded by CAPN2), respectively (5-7). The calpain system is involved in numerous cellular functions, including cytoskeletal remodeling, cellular signaling and apoptosis (8). Previously, cumulative evidence has demonstrated that the expression of calpains is associated with more aggressive tumor behavior in a number of tumor types, including breast (9,10), ovarian (11), gastro-esophageal (12) and endometrial cancer (13). However, to the best of our knowledge, the role of calpain isoforms has not been investigated in OSCC.

The aims of the present study were to investigate the pattern and the prognostic impact of calpain1 expression in OSCC. Furthermore, the functional basis of calpain1 as a potential molecular marker was discussed when assessing the effect of knockdown on OSCC cell migration, invasion, cell cycle stage and apoptosis.

Materials and methods

Cell lines and cell culture. HSC-3 cells were purchased from the Cell Bank of Japanese Collection of Research Bioresource (Shinjuku, Japan). CAL27 cells were purchased from American Type Culture Collection (Manassas, USA). CAL33 and HSC6 cells and the NOK-SI normal keratinocyte cell line were

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provided by Professor J. Silvio Gutkind (National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD, USA) subsequent to authentication by polymerase chain reaction (PCR) amplification of short tandem repeats to ensure cell identity. The OSCC cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 mM glucose (cat. no. 11995; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and supplemented with penicillin (5 U/ml), streptomycin (5 μg/ml) and 10% heat-inactivated fetal bovine serum (FBS; cat. no. 10099-141; Gibco; Thermo Fisher Scientific, Inc.). NOK-SI cells were grown in keratinocyte-SFMKSFM (cat. no. 17005-042; Gibco; Thermo Fisher Scientific, Inc.).

Patients. The present study protocol was approved by the Institutional Review Boards of the Hospital of Stomatology, Sun Yat-Sen University (Guangzhou, China), and written informed consent was obtained from all patients in the current study. The study cohort consisted of 125 postoperative patients treated at the Hospital of Stomatology (Sun Yat-Sen University) between January 2008 and January 2010, who had complete clinical and histological data available. All patients were followed-up with interviews and physical examinations every three months during the first year following surgery, every six months in the following four years, and once a year until the study endpoint. A biopsy or positron emission tomography-computed tomography scan was conducted when required, based on any questionable findings (symptoms associated with recurrence). The survival time of each patient was calculated from the day of surgery until the time of mortality from any cause, or end of the follow-up.

Antibodies and western blot analysis. For western blot analysis, four OSCC cell lines and one normal oral keratinocyte cell line were used, along with a total of seven pairs of patient OSCC and corresponding adjacent normal tissues. OSCC specimens were immersed and stored in liquid nitrogen immediately subsequent to surgical resection until examination. The radioimmunoprecipitation assay (RIPA) lysis and extraction buffer (cat. no. 89900; Thermo Fisher Scientific, Inc.) were used for the extraction of proteins. All subsequent manipulations were conducted on ice. The tissue was ground in liquid nitrogen and cells were incubated in RIPA lysis and extraction buffer (25 mM Tris×HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) mix for 30 min at 4˚C and centrifuged at 13,000 x *g* at 4˚C for 20 min, and the resultant supernatant was used for following tests.

The protein concentration of each cell sample was determined with the bicinchoninic acid protein assay reagent (Thermo Fisher Scientific, Inc.). Samples were denatured in sodium dodecyl sulfate sample buffer (cat. no. 9173; Takara Biotechnology Co., Ltd., Dalian, China) and loaded onto 10% polyacrylamide gels. Subsequent to electrophoresis, the proteins were transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA) and immunoblotted with rabbit anti-human monoclonal anti-calpain 1 (H-65) antibody (cat. no. sc-13990; dilution, 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), in universal antibody dilution buffer (cat. no. U3635; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) at 4˚C overnight. Incubation with biotin-conjugated goat anti-rabbit immunoglobulin G secondary antibody (cat. no. BA1003; 0.1 μg/ml; Wuhan Boster Biological Technology, Ltd.) was conducted, and the signals were visualized with an enhanced chemiluminescence kit (Luminata Western HRP Substrate; Merck Millipore). The expression of protein was determined by western blotting using anti-GAPDH antibodies (cat. no. 2118; dilution, 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) as loading controls. All kits were completed according to the manufacturer's protocols. The result was analyzed using ImageJ software version 1.5 (National Institutes of Health, Bethesda, MD, USA) for the comparison of relative target protein expression.

RNA isolation and quantitative PCR (qPCR). Total RNA from all cell lines was isolated with TRIzol® reagent (Takara Biotechnology Co., Ltd.). In brief, 1 ml TRIzol® reagent was added to each tube, and the tubes were incubated for 5-10 min on ice to ensure complete dissociation of the nucleoprotein complexes. A total of 0.2 ml chloroform was then added to each RNA specimen and this was agitated for 30 sec, followed by incubation at room temperature for 15 min. Following centrifugation at 13,000 x *g* for 15 min at 4˚C, each RNA specimen was divided into three layers and the aqueous phase containing RNA (upper layer) was removed and transferred into a fresh RNase free 1.5 ml Eppendorf tube. Isopropanol (0.5 ml) was added and the tubes were agitated for 30 sec, followed by incubation at room temperature for 10 min. The Eppendorf tubes were then centrifuged at 13,000 x *g* for 10 min at 4˚C to pellet the precipitated RNA. Taking care not to disturb the RNA pellet, the supernatant was removed and the pellet was subsequently washed by the addition of 1 ml 75% ethanol and vortexed.

Following centrifugation at 13,000 x *g* for 5 min at 4˚C, supernatant was removed (this wash step was repeated). The RNA pellet was allowed to air-dry for 5-10 min and then resuspended in 15 μl diethylpyrocarbonate-treated water. The RNA isolated from cell line specimens was pooled, and the quantity and quality of extracted RNA was assessed by reading absorbance at 260, 280 and 230 nm using a NanoDrop ND-1000 (NanoDrop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). The first-strand cDNA was synthesized from 1 μg total RNA using Oligo (T) primer and the GoScript™ Reverse Transcription system (Roche Applied Science, Penzberg, Germany), according to the manufacturer's protocol. qPCR was performed (LightCycler® 480; Roche Applied Science) with the LightCycler® FastStart DNA Master SYBR-Green I kit (cat. no. 03003230001; Roche Diagnostics, Basel, Switzerland), according to the manufacturer's protocol. The primer sequences for CAPN1 were as follows: Forward, 5'-AACTTCCTCATCACCAAC-3' and reverse, 5'-CTCATC CTCCTCATCCTC-3'. The cycling conditions were 95˚C for 5 min, followed by 45 cycles of 95˚C for 10 sec, 60˚C for 20 sec and 72˚C for 30 sec. The cDNA content was determined using the $2^{-\Delta\Delta Cq}$ method (14). The results are presented as relative expression normalized to that of the internal control, GAPDH (forward, 5'-GGTGGTCTCCTCTGACTT CAAC-3' and reverse, 5'-TCTCTCTTCCTCTTGTGTTCT TG-3'). All primer sequences were designed and synthesized by Invitrogen (Thermo Fisher Scientific, Inc.). All experiments were performed in triplicate.

Tissue microarray and immunohistochemistry. The tissue microarray (TMA) was prepared by placing duplicate 1.5-mm tissue cores of the tumor, which was identified by a pathologist, into a single-recipient paraffin block. Sections of the TMA (4 μm) were mounted on poly-L-lysine coated slides. Sections were deparaffinized, rehydrated and treated using Dako REAL peroxidase blocking solution (cat. no. S202386; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA). Heat-based antigen retrieval was completed prior to incubation of the tissue sections with a rabbit anti-human monoclonal anti-calpain1 (H-65) antibody (cat. no. sc-13990; dilution, 1:50; Santa Cruz Biotechnology, Inc.) at 4˚C overnight. Bound antibodies were visualized using an EnVision+ kit (Dako North America, Inc.; Agilent Technologies, Inc.) according to the manufacturer's protocol.

Subsequent to the development of the expected stain intensity, the sections were lightly counterstained with hematoxylin. Sections treated without primary antibodies were used as negative controls. Each block of TMA staining results were confirmed by each tissue slide obtained from the same patient. Immunostained cells were evaluated over 8 visual fields at a magnification of x400 under a light microscope (Olympus Corporation, Tokyo, Japan). For the statistics of the prognostic value in the OSCC cohort, two independent observers performed microscopy analysis. The staining was scored using a 3-point scale on the basis of the product of the staining intensity (weak, 1; moderate, 2; strong, 3).

RNA interference. The calpain1 small interfering RNA (siRNA) sequences were 5'-CCACGGAACUGCUGUCAA AdTdT-3' and 5'-dTdTGGUGCCUUGACGACAGUUU-3'. The scrambled control siRNA sequences and all siRNAs were synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The HSC3 and CAL27 OSCC tongue cancer cell lines were used for RNA interference. Cells were plated ($2x10^5$/well) onto 6-well plates and allowed to grow for 24 h, until they reached 70% confluency. Cells were then transfected with 100 nmol/l of calpain1 siRNA or negative control using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific Inc.), according to the manufacturer's protocol. Following 6 h of incubation, the medium was replaced with serum-enriched medium and the cells were cultured for an additional 48 h. Subsequently, the transfected cells were collected and processed for qPCR, western blot analysis, proliferation, cell cycle stage, apoptosis, migration and invasion assays.

Cell invasion assay. Chambers (cat. no. 353096; BD Biosciences, Franklin Lakes, NJ, USA) were uniformly coated with 60 μl Matrigel (BD Biosciences) diluted with DMEM to ~20-30% and incubated at 37˚C for 2-4 h. OSCC cells ($1x10^5$/well) in serum-free DMEM (0.1 ml) were placed into the upper chamber. The 10% FBS-containing DMEM (used as a chemoattractant, 0.5 ml) was placed in the lower chamber. Serum-free DMEM (0.5 ml) was used for the control. Cells were allowed to invade for 24 h at 37˚C in a 5% CO_2 atmosphere. Subsequent to the incubation, non-invading cells were removed from the top of the wells with a cotton swab, and the upper chamber was washed twice with PBS. Cells that had transferred to the inverse surface of the membrane were fixed by 4% paraformaldehyde at room temperature and stained for 10 min with 0.2% crystal violet.

Cell migration assay. Cells ($1.5x10^5$/well) in serum-free DMEM (0.1 ml) were plated in the top chamber, whereas the bottom chambers were filled with 500 μl DMEM supplemented with 10% FBS. Subsequent to incubation at 37˚C in a 5% CO_2 atmosphere, the cells were fixed and stained. All experiments were conducted in triplicate; cells were counted under a microscope at a magnification of x200, and cell numbers were counted in ≤5 fields/well.

MTT assay. The calpain1 knockdown transfectants were seeded into 96-well plates at a density of $5x10^3$/well. Subsequent to culturing for 24, 48, 72 or 96 h, cell growth studies were conducted using an MTT assay (cat. no. M5655; Sigma-Aldrich; Merck Millipore). MTT (0.01 ml, 5 mg/ml) was added to each well and incubated at 37˚C for 3-4 h. The medium was then removed and the cells were lysed using 0.1 ml isopropanol with 0.04 N HCl. The absorbance of the converted dye was determined by a microplate reader (Model Elx808; BioTek Instruments, Inc. Winooski, VT, USA) at a wavelength of 570 nm, with background subtraction at 630-690 nm, and the optical density at 570 nm of the control (CAL27 or HSC3 cells treated without supplement) was considered to be 100%.

Cell cycle analysis. CAL27 and HSC3 cell lines transfected with CAPN1 siRNA were collected as aforementioned, and then resuspended in 70% ethanol overnight at 4˚C. Subsequently, cells were stained with 500 μl PI/RNase Staining Buffer (cat. no. 556463; BD Pharmingen, San Diego, CA, USA), according to the manufacturer's protocol. Finally, the analysis was conducted using flow cytometry (Beckman Coulter, Inc., Brea, CA, USA) and FlowJo software version 10.0 (Tree Star, Inc., Ashland, OR, USA).

Apoptosis analysis. Apoptosis was detected by flow cytometry 48 h after OSCC cells were transfected with calpain1 siRNA, using Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit (BD Biosciences; cat. no. 556547), according to the manufacturer's protocol. Cells were harvested, washed twice in PBS, and resuspended in Annexin V-FITC binding buffer, following the addition of 5 μl Annexin V-FITC and 5 μl propidium iodide (PI), and mixed gently. Cells were then stained at room temperature in the dark for 15 min. Finally, 400 μl buffer was added to each tube and the cell samples were analyzed by flow cytometry. The experiment was conducted in triplicate.

Statistical analysis. Survival analysis was determined by the Kaplan-Meier method, and the differences were compared on the basis of the category of the variables using the log-rank test. To determine the hazard ratio (HR), the univariate cox proportional-hazards model was used. The multivariate cox proportional-hazards model was then used to assess the association of overall survival (OS) rates with the suggested variables ($P<0.20$) identified by the univariate analysis. The variables in the final model were adjusted with respect to age (as a continuous variable) and gender. Statistical analyses were performed using SAS software, version 9.3 (SAS Institute Inc., Cary, NC, USA). Unless stated otherwise, two-sided values of $P<0.05$ were considered to indicate a statistically significant difference.

Results

Patient cohort. The present cohort included 24 patients with clinical stage I, 39 patients with stage II, 37 patients with stage III and 25 patients with stage IV cancer. A total of 88 patients were male and 37 were female. Pretreatment clinically positive nodes were present in 41 patients. Tumor differentiation was classified using the World Health Organization criteria (15) (88 patients, well differentiated; 32 patients, moderately differentiated; 5 patients, poorly differentiated). Adjuvant postoperative radiation was added for patients with histologically positive regional lymph nodes. Resected specimens were used to create a tissue microarray and slides for immunohistological analysis. Clinical and histological variables assessed included T classification, nodal status, primary tumor differentiation and clinical stage. Patient factors analyzed included age, gender, smoking status (57 patients, never smokers; 55 patients, past smokers; 13 patients, current smokers) and alcohol use (57 patients, never drinkers; 57 patients, past drinkers; 11 patients, current drinkers). The demographics of the patient cohort are presented in Table I. All patients were regularly followed up. The last assessment of vital status of all patients was in May 2014; therefore, the median follow-up time for these patients was 3.58 years.

Expression pattern of calpain1 in OSCC cell lines and tissues. To identify differing protein expression of calpain1 in OSCC, four OSCC cell lines and seven fresh tumor tissues together with the paired NCMTs were examined by western blot analysis. The present study found that calpain1 was highly expressed in HSC3, HSC6, CAL33 and CAL27 OSCC cell lines, in comparison with normal oral keratinocytes (Fig. 1A; HSC3, P=0.029; CAL27, P=0.0023; CAL33, P=0.0002; HSC6, P=0.0007). In addition, calpain1 was highly expressed in the tumor areas of 4 NCMT/tumor pairs (Fig. 1B; P=0.0008). Increased calpain1 expression in tumor tissues is also presented in Fig. 2A, with representative images of normal border epithelium, precancerous epithelium and infiltrative carcinoma on the same tissue slide. Expression of calpain1 was located in the cytoplasm, with a certain degree of heterogeneity within samples. Typical staining patterns, which vary between weak, moderate and strong, are presented in Fig. 2A.

Association between calpain1 expression and survival analysis. Immunohistochemical analysis was completed to assess the expression of calpain1 in 125 OSCC tissue blocks. Overall, 73 of the 125 tumor samples (58.4%) exhibited high expression of calpain1 (immunoreactivity intensity scale, 3), whereas 17 samples (13.6%) had low expression (immunoreactivity intensity scale, 1). The other 35 samples (28%) exhibited moderate calpain1 expression (immunoreactivity intensity scale, 2; Table I).

Kaplan-Meier analysis and the log-rank test were used to analyze the effect of the calpain1 expression on OSCC survival. The expression of calpain1 was assessed for association with a number of clinicopathological variables. In the univariate analysis, patient age (β=-0.022; 95% HR, 0.959-0.999; P=0.037), cell differentiation (β=-0.561; 95% HR, 0.345-0.945; P=0.029) and calpain1 expression (β=0.414; 95% HR, 1.039-2.204; P=0.031) were significantly associated with overall survival. Radiotherapy and chemotherapy (pre- and post-surgery) were not associated with overall survival.

Table I. Common characteristics of the study population (n=125).

Variables	Number, n (%)
Age, years	
≥60	67 (53.60)
<60	58 (46.40)
Gender	
Female	37 (29.60)
Male	88 (70.40)
Smoking	
Never	57 (45.60)
Past	55(44.00)
Current	13(10.40)
Drinking	
Never	57 (45.60)
Past	57 (45.60)
Current	11 (8.80)
T-stage	
1	34 (27.20)
2	52 (41.60)
3	22 (17.60)
4	17 (13.60)
Lymphatic metastasis	
No	84 (67.20)
Yes	41 (32.80)
Differentiation	
Well	88 (70.40)
Moderate	32 (25.60)
Poor	5 (4.0)
Clinical stage	
1	24 (19.20)
2	39 (31.20)
3	37 (29.60)
4	25 (20.00)
Surgical method[a]	
1	53 (42.40)
2	65 (52.00)
3	7 (5.60)
Radiotherapy (Yes)	9 (7.20)
Preoperative chemotherapy (Yes)	8 (6.40)
Postoperative chemotherapy (Yes)	62 (49.60)
Calpain1 staining intensity	
Low	17 (13.60)
Median	35 (28.00)
High	73 (58.40)

[a]1, lesion resection only; 2, lesion resection and unilateral neck dissection; 3, lesion resection and bilateral neck dissection.

Table II. Univariate and multivariate analyses of overall survival among patients with oral squamous cell carcinoma.

	Univariate analyses			Multivariate analyses		
Variable	β	95% HR	P-value	β	95% HR	P-value
Age	-0.022	0.959-0.999	0.037[a]	-0.337	0.387-1.316	0.28
Gender	0.26	0.772-2.178	0.326	0.137	0.646-2.036	0.64
Smoking history	-0.384	0.417-1.108	0.681			
Drinking history	-0.084	0.562-1.505	0.738			
Cell differentiation	-0.561	0.345-0.945	0.029[a]	-1.807	0.023-1.187	0.073
Tumor size	0.119	0.878-1.445	0.348			
Lymphatic metastasis	0.258	0.808-2.075	0.283			
Clinical TNM stage	0.217	0.971-1.59	0.085			
Radiotherapy	-1.74	0.024-1.267	0.085			
Chemotherapy before surgery	-0.682	0.123-2.075	0.506			
Chemotherapy after surgery	-0.234	0.477-1.314	0.366			
Calpain1	0.414	1.039-2.204	0.031[a]	0.455	1.069-2.322	0.022[a]

[a]P<0.05; β, partial regression coefficient; HR, hazard ratio; TNM, tumor nodal metastasis.

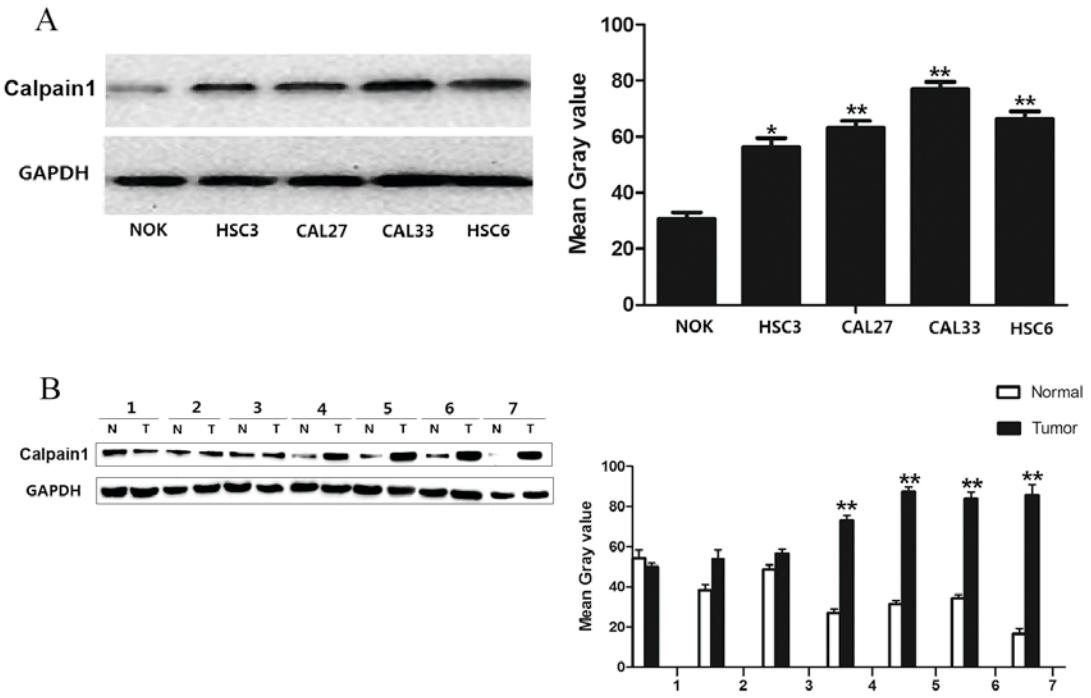

Figure 1. Expression of calpain1 in OSCC cell lines and tissues by western blot analysis. (A) Expression of calpain1 in 4 oral cancer cell lines (HSC3, CAL27, CAL33 and HSC6) and in a normal oral keratinocyte (NOK-SI) cell line. The graph represents the mean grey value of the average data of five samples from 3 independent experiments (*P<0.05, Student's *t*-test). HSC3, CAL33, CAL27 and HSC6 cell lines exhibited higher protein expression levels, compared with NOK. (B) Expression of calpain1 in 7 paired NCMT (N)/OSCC (T) samples. The graph represents the mean grey value of the average data of 7 paired samples (**P<0.01, Student's *t*-test). Calpain1 was highly expressed in the tumor areas of 4 NCMT/tumor pairs. OSCC, oral squamous cell carcinoma; NCMT, noncancerous matched tissues; N, normal; T, tumor.

Furthermore, multivariate analysis revealed that calpain1 expression was an independent predictor for the overall survival of OSCC patients (β=0.455; 95% HR, 1.069-2.322; P=0.022; Table II). With respect to overall survival, marked separation in curves between patients with low, median and high calpain1 expression level was observed in the present cohort (Fig. 2B).

Effect of calpain1 gene silence on the migration and invasion of OSCC cells. To determine the effect of calpain1 on the invasion and migration potential of tumor cells, the present study transfected HSC3 and CAL27 cells with calpain1 siRNA and conducted Transwell migration and invasion assays. As observed in Fig. 3A and B, the protein and mRNA expression of calpain1 in cancer cells was downregulated subsequent

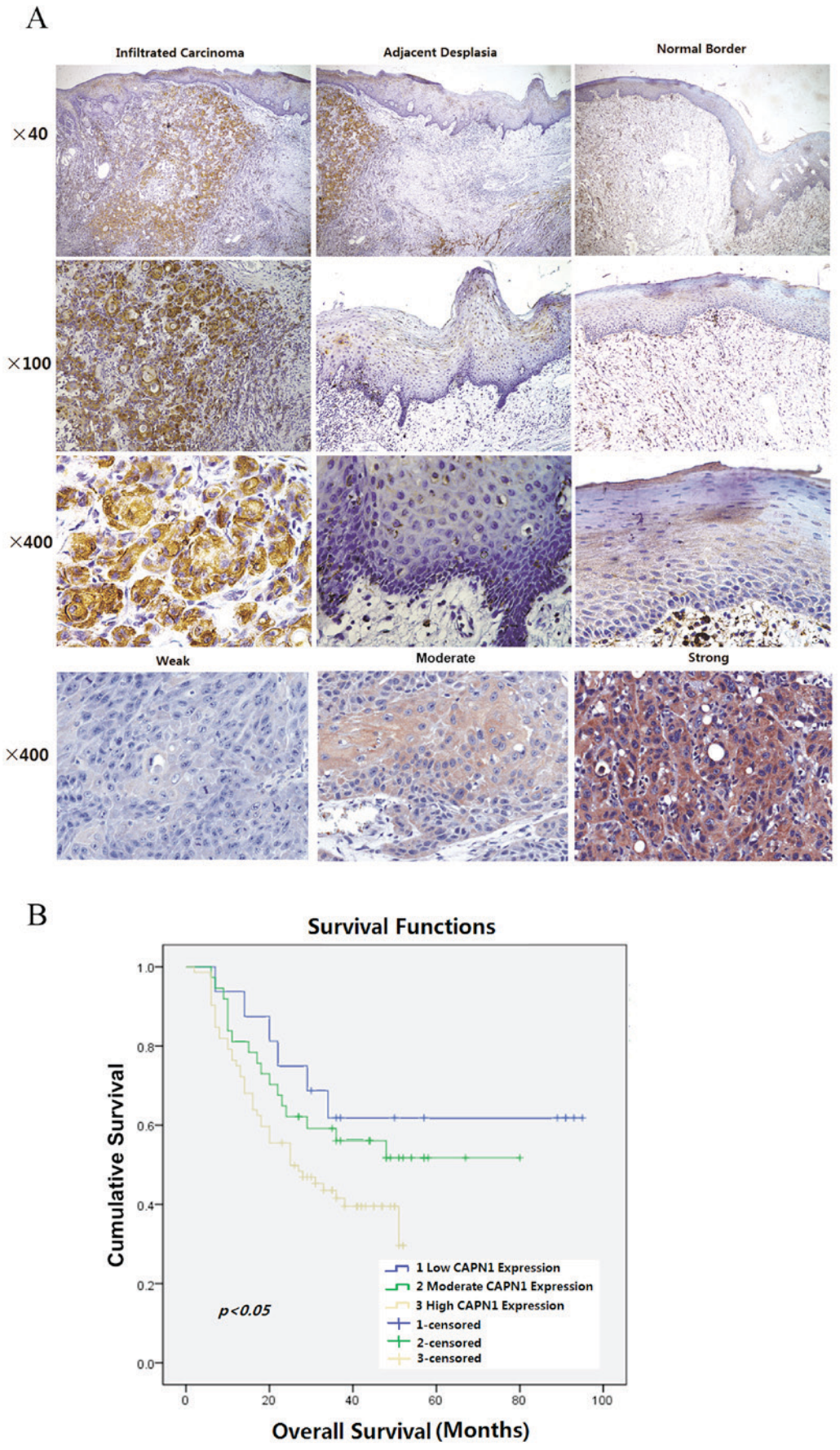

Figure 2. Immunohistochemical analysis of calpain1 expression in OSCC tissue microarrays. (A) The representative results exhibit staining patterns from normal to hyperplasia epithelium and infiltrative carcinoma tissues in one slide at the indicated magnification. Representative images of weak, morderate and strong staining intensity in OSCC are also provided. Magnification, x400. (B) Kaplan-Meier analysis of cumulative survival revealing the impact of low and high calpain1 expression in the 125 patient-cohort, with significance determined using the log-rank test. OSCC, oral squamous cell carcinoma; CAPN, calpain.

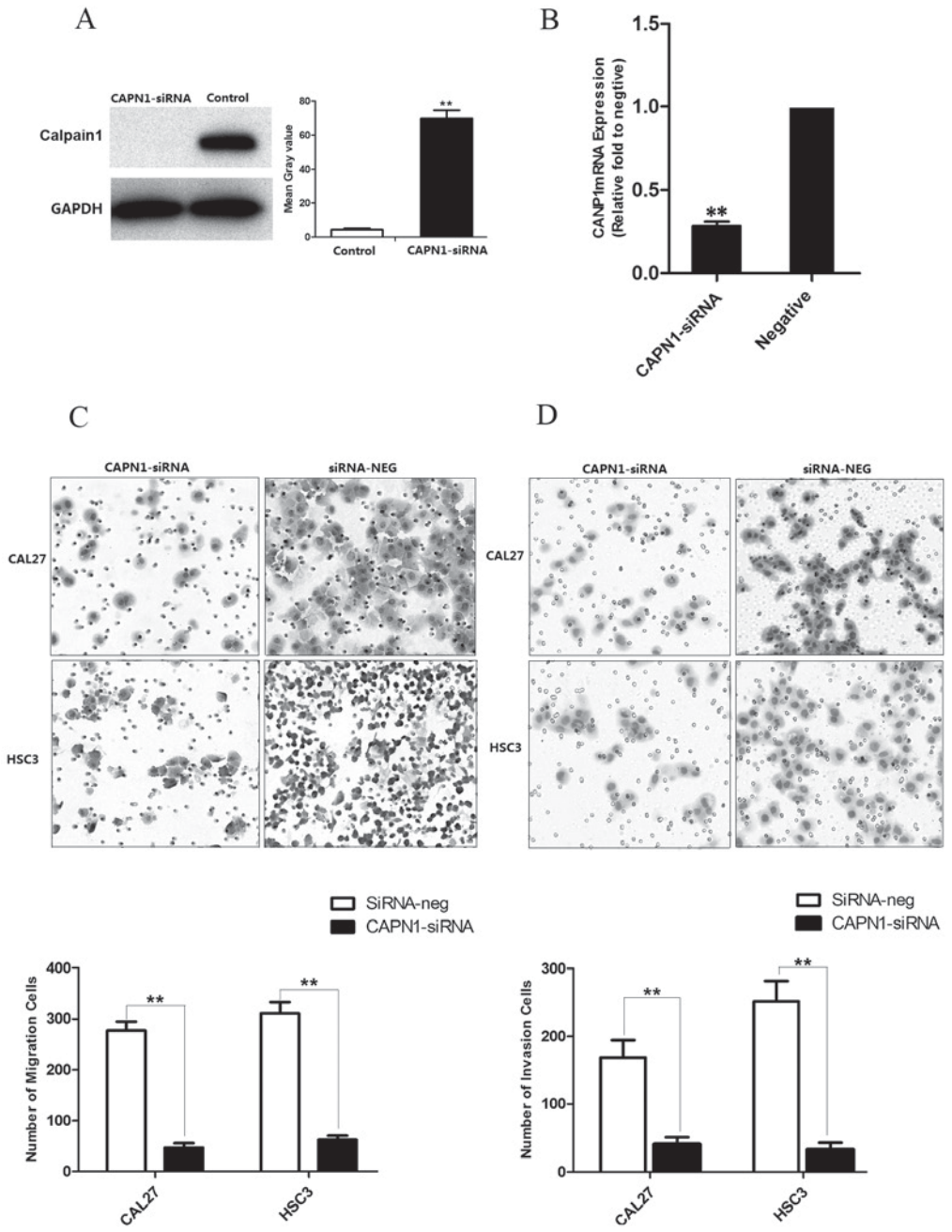

Figure 3. Calpain1 silencing decreased the migration and invasion capacity of OSCC cells. (A) Effective interference of calpain1 expression in the two OSCC cell lines was demonstrated by western blot analysis (**P<0.01, compared with the negative control group). (B) Effective interference of calpain1 expression in OSCC cell lines was demonstrated by qPCR (**P<0.01, compared with the negative control group). (C) Cell migration was decreased by silencing calpain1 in OSCC cell lines (CAL27, HSC3), as revealed by Transwell migration assay at 24 h. Cells that had migrated to the bottom of the chamber were counted in 5 fields at x20 magnification. Representative images and quantitative data are presented. Data are expressed as the mean ± standard deviation (**P<0.01, compared with the negative control group). (D) Cell invasion was decreased by silencing calpain1 in OSCC cell lines, as revealed by invasion assay at 24 h (right panels). Cells that had invaded to the bottom of the chamber were counted in five fields x20 magnification. Representative images and quantitative data are presented. Data are expressed as the mean ± standard deviation (**P<0.01, compared with the negative control group). OSCC, oral squamous cell carcinoma; CAPN1, calpain1; siRNA, small interfering RNA; neg, negative.

to transfection with calpain1 siRNA, as compared with the negative control (P<0.0001). The induced downregulation of calpain1 expression resulted in apparent CAL27 and HSC3 cell decrease in number of invading and migrating cells, compared with the siRNA negative controls at 24 h following seeding (P=0.00057 for migration; P=0.0001 for invasion; Fig. 3C and D). The results indicated that knockdown of calpain1 expression was able to suppress the mobility of CAL27 and HSC3 cells *in vitro*.

Effect of calpain1 knockdown on the cell proliferation. To assess the effect of siRNA-mediated calpain1 knockdown on the proliferation of OSCC cells, the cell viability of two cell lines was determined by MTT assay. In Fig. 4A, relative cell growth rate was significantly reduced in the calpain1 siRNA group following transfection, at 72 and 96 h, by comparison with that of the siRNA-negative groups (72 h, P=0.0027 for CAL27, P=0.009 for HSC3; 96 h, P=0.0012 for CAL27, P=0.0001 for HSC3). The flow cytometry analysis revealed

Table III. The percentages of cell in various phases of the cell cycle.

Groups	Cell-cycle phase (%)		
	G_0/G_1	S	G_2/M
CAL27			
Negative control	43.21±4.40	46.12±2.36	9.70±2.71
CAPN1-siRNA	71.33±1.02[a]	20.41±1.42[a]	7.08±0.78
HSC3			
Negative control	33.50±1.55	54.24±1.92	9.56±2.05
CAPN1-siRNA	69.42±1.63[a]	23.99±2.14[a]	5.77±0.43

[a]P<0.01, compared with the negative control. These data were analyzed by one-way analysis of variance. All experiments were performed in triplicate and the results were presented as mean ± standard deviation. CAPN1, calpain1 gene; siRNA, small interfering RNA.

that cells transfected with caplain-1 siRNA were blocked in the G_0/G_1 phase (47.73-70.44% for CAL27, P=0.0028; 34.77-67.79% for HSC3, P=0.0014; Fig. 4B; Table III). The analysis also revealed a concomitant decrease of cells in the S phase in the knockdown cell line (44.67-20.83% for CAL27, P=0.006; 53.44-25.88% for HSC3, P=0.0046). These findings suggest that the downregulation of calpain1 resulted in cell cycle arrest and therefore inhibited the proliferation of OSCC cells. The results indicated that calpain1 may promote OSCC cell cycle progression.

Effect of calpain1 knockdown on the apoptosis of OSCC cells. In order to determine whether CAPN1 knockdown may induce apoptosis in OSCC cell lines, Annexin V/PI staining was examined by flow cytometry analysis. As presented in Fig. 4C, the proportion of Annexin V and PI positive cells was 12±0.16% in calpain1 siRNA transfected cells and 14±0.51% in siRNA-negative cells. The two populations of Annexin V-positive cells and of Annexin V plus PI-positive cells in 2 groups exhibited no significant difference (P=0.8593 for CAL27; P=0.8993 for HSC3; Fig. 4C). These data suggest that calpain1 may have no significant effect on apoptosis in OSCC cell lines.

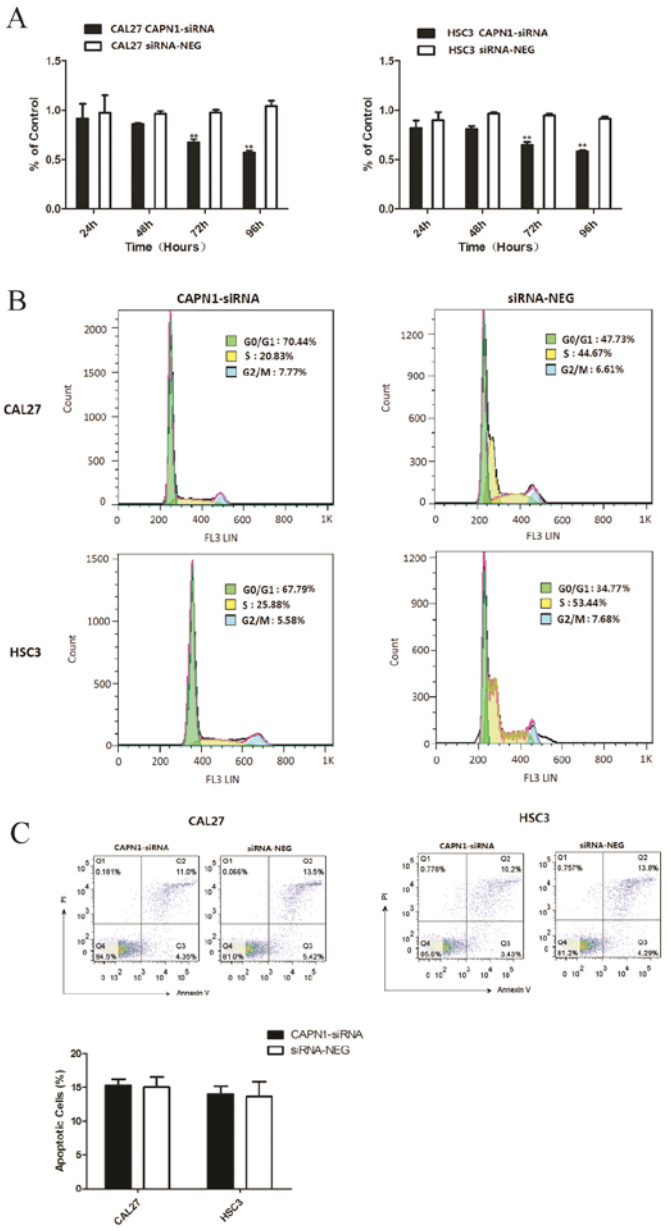

Figure 4. Calpain1 silencing resulted in cell cycle arrest but had no effect on apoptosis. (A) The relative cell growth rate of the calpain1 knockdown OSCC cells and the negative control cells during a 4-day period was evaluated using an MTT assay (*P<0.05, **P<0.01, compared with the negative control group). (B) The calpain1 knockdown cell line exhibited impaired cell cycle progression as indicated by more cells accumulated in G_0/G_1 phase and a concomitant decrease of cells in S phase. (C) Apoptosis was detected by flow cytometry. Quantitative determination of data from fluorescence activated cell sorting analysis revealed that calpain1 siRNA transfection did not result in a significant difference in the number of cells undergoing apoptosis (AV+) in two groups. OSCC, oral squamous cell carcinoma; CAPN1, calpain1; siRNA, small interfering RNA.

Discussion

The role of calpains in cancer pathology has been widely discussed (8). Among the different members of the calpain family, calpain1 and -2 are most widely implicated in the development and progression of cancer (16). However, little is understood about the biological significance of calpains in OSCC. The present study demonstrated the expression of calpain1 in OSCC cell lines and tissues. Additionally, the high expression of calpain1 was significantly associated with the poor prognosis of patients with OSCC. Accordingly, obstruction of calpain1 by specific siRNAs hindered the progression of OSCC cells by affecting migration, invasion and proliferation, but not apoptosis. To the best of our knowledge, these results are the first observations to demonstrate that calpain1 may potentially be important in OSCC progression, and may be associated with poor patient prognosis.

In the present study, it was observed that calpain1 was overexpressed in OSCC cell lines and paired OSCC tissues. Its role as a significant predictor of poor outcome was indicated in the study. The current findings are consistent with the results of previous studies that have demonstrated the association between elevated calpain1 levels and poor survival rates in breast (10) and ovarian cancer (11). As the calpain isoforms

possess tumor-type specific patterns, the opposite observations have been made in gastro-esophageal adenocarcinoma (12). In the present study, the majority of the common used patient variables, including epidemiological and clinicopathological factors, were included in the regression model, and only calpain1 expression remained a significant predictor for poor outcome ($P<0.05$).

To study the effects of calpain1 in the development of oral cancer cells, the present study selected two oral cancer cell lines with transient calpain1 knockdown, and investigated how this gene may affect cell invasion and migration. It was revealed that, in addition to morphological alterations, deletion of calpain1 may be associated with a decrease in the migratory and invasive ability of OSCC cells. Certain studies have demonstrated that calpain1 is able to modulate the expression and secretion of MMPs (17,18), which are proteinases that are able to degrade numerous extracellular matrix components, making the invasion of neoplastic cells possible. Therefore, targeting the activity of calpain with specific inhibitors may be a feasible way to suppress the dissemination of tumor cells. However, treatments targeting calpain activity exhibit no effects on invasiveness in the case of amoeboid migration and invasion (19), calpain inhibition is even able to promote HT1080 cell invasion that is sensitive to a Rho-associated protein kinase inhibitor, which suppresses amoeboid invasion (19). Therefore, further analysis of the biological functions of calpain family members within complex cellular or *in vivo* systems is important.

The calpains were notably shown to regulate the cell cycle, particularly the transition between the G_1 and S phases through underlying mechanisms that include several crucial regulators, including cyclin D1 and cyclin E, which are substrates of calpain1 and calpain2 (20,21). In cancer cells, calpains have been demonstrated to proteolyze two inhibitors of cyclin-dependent kinases (Cdk), $p21^{Cip1}$ and $p27^{Kip1}$, leading to the activation of the complexes cyclinD-Cdk4 and cyclinE-Cdk2 (22,23). By regulating these proteins, calpains are therefore strongly involved in the progression of the cell cycle and in cellular proliferation. The present results also demonstrated that calpain1 knockdown resulted in a significantly reduced growth rate in two OSCC cell lines and triggered a marked accumulation of cells in G_0/G_1 phase and a concomitant decrease of cells in S phase. These findings suggest that the depletion of calpain1 may account for the slow growth of the calpain1 transfected cell line.

To additionally understand the effect of calpain1 activity on OSCC cell apoptosis, the current study conducted an apoptosis assay. The results demonstrated that calpain1 knockdown did not induce marked cell apoptosis, compared with that in the negative control group ($P>0.05$). The functions of calpain in the perturbed cell apoptosis of cancer remain to be established. Certain studies indicate that calpain is able to cleave wild-type p53, regulating protein stability to prevent p53-dependent apoptosis (24,25). However, in other studies, calpain activity is able to promote apoptosis in neurodegenerative disorders (26), and calpain1 was demonstrated to suppress the differentiation of neural stem cells (27). The present findings suggest that calpain1 knockdown may have no significant effect on apoptosis in OSCC cell lines. At present, there is no clear explanation for these inconsistent results; the detailed underlying molecular mechanisms require investigation and further studies are necessary in a number of models.

In conclusion, the present study indicates that calpain1 may provide important prognostic information in OSCC and contributes to the malignant progression of OSCC. As part of future studies, multi-center, non-selected patients with OSCC must be investigated to test if determining calpain1 expression may be of clinical benefit. Additional studies are warranted to enable a good understanding of the function and mechanism of calpain1 *in vivo*, which may provide a potential therapeutic target for OSCC.

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