

Calreticulin, an endoplasmic reticulum-resident protein, is highly expressed and essential for cell proliferation and migration in oral squamous cell carcinoma

Wei-Fan Chiang^{a,n}, Tzer-Zen Hwang^{b,n}, Tzyh-Chyuan Hour^c, Lee-Hsin Wang^d, Chien-Chih Chiu^d, Hau-Ren Chen^e, Yu-Jen Wu^f, Chih-Chun Wang^{b,g}, Ling-Feng Wang^{h,i}, Chen-Yu Chien^{h,j}, Jen-Hao Chen^k, Chao-Tien Hsu^{l,m,*}, Jeff Yi-Fu Chen^{d,*}

^a Department of Oral and Maxillofacial Surgery, Chi-Mei Medical Center, Liouying, Taiwan

^b Department of Otolaryngology, E-DA Hospital and I-Shou University, Kaohsiung, Taiwan

^c Institute of Biochemistry, Kaohsiung Medical University, Kaohsiung, Taiwan

^d Department of Biotechnology, Kaohsiung Medical University, Kaohsiung, Taiwan

^e Department of Life Science and Institute of Molecular Biology, National Chung Cheng University, Chia-Yi, Taiwan

^f Department of Beauty Science, Meiho University, Pingtung, Taiwan

^g Institute of Clinical Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan

^h Department of Otolaryngology, Faculty of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan

ⁱ Department of Otolaryngology, Kaohsiung Municipal Ta-Tung Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan

^j Department of Otolaryngology, Kaohsiung Municipal Hsiao-Kang Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan

^k School of Dentistry, College of Dental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan

^l Department of Pathology, E-Da Hospital and I-Shou University, Kaohsiung, Taiwan

^m The School of Chinese Medicine for Post-Baccalaureate, I-Shou University, Kaohsiung, Taiwan

ARTICLE INFO

Article history:

Received 12 October 2012

Received in revised form 30 December 2012

Accepted 5 January 2013

Available online 31 January 2013

Keywords:

Calreticulin (CRT)

Oral squamous cell carcinoma (OSCC)

Cell proliferation

Cell migration

Biomarker

SUMMARY

Objectives: Oral squamous cell carcinoma (OSCC) has emerged as one of the major malignant tumors of the head and neck cancers. However, the molecular mechanism behind tumorigenesis of OSCC is not fully understood. The aim of this study was to investigate the role of calreticulin (CRT), an endoplasmic reticulum-resident protein, in OSCC cells.

Materials and methods: Sixteen paired samples of tumor and non-cancerous matched tissue (NCMT), six OSCC cell lines and normal human oral keratinocytes (NHOKs), and oral tissue microarray were used to reveal the expression of CRT by Western blotting and immunohistochemistry. Later, shRNA-mediated stable knockdown of CRT in OSCC cells was generated. The knockdown cell line was used to analyze cell proliferation, colony formation, anchorage-independent growth and cell migration in vitro.

Results: CRT was differentially expressed in fresh tumor samples and six OSCC cell lines but not adjacent NCMTs and NHOKs. In oral tissue microarray, we showed that there was positive CRT staining in the vast majority of tumor cases (99/103), in sharp contrast to that in NCMT cases (29/92) ($p < 0.001$). Stable knockdown of CRT in oral cancer cells resulted in significantly reduced growth rate, colony-forming capacity and anchorage-independent growth. This may be attributed to the induction of G0/G1 cell cycle arrest when CRT was depleted in the cells. Both horizontal and vertical movements of the CRT-knockdown stable line were markedly impaired. The phosphorylation levels of focal adhesion kinase (FAK), paxillin and ERK1/2 and the activity of matrix metalloproteinase-2 and -9 (MMP-2 and MMP-9) were decreased in the CRT-knockdown cells. These results suggest that CRT can regulate oral cancer cell migration through activation of the FAK signaling pathway accompanied with proteolytic degradation of the extracellular matrix (ECM) by MMP-2 and MMP-9.

Conclusion: Together, this study has defined a novel biological role for CRT in oral cancer. CRT is a potential biomarker and may contribute to the malignant phenotypes of OSCC cells.

© 2013 Elsevier Ltd. All rights reserved.

* Corresponding authors. Addressess: 1, E-Da Road, Jiau-Shu Tsuen, Yan-Chau District, Kaohsiung 824, Taiwan. Tel.: +886 7 615 0011x2917; fax: +886 7 615 0974 (C.-T. Hsu), 100, Shih-Chuan 1st Road, San-Ming District, Kaohsiung 807, Taiwan. Tel.: +886 7 3121101x2730; fax: +886 7 3125339 (J.Y.F. Chen).

E-mail addresses: ed103797@edah.org.tw (C.-T. Hsu), yifuc@kmu.edu.tw (J.Y.F. Chen).

ⁿ These authors contributed equally to this work.

Introduction

Calreticulin (CRT), an endoplasmic reticulum (ER)-resident protein, plays a pivotal role in multiple cellular processes. When localized to the lumen of the ER, CRT acts as a molecular chaperone that ensures correct protein folding.^{1,2} When functioning as a calcium-binding protein, CRT can regulate intracellular calcium

homeostasis and ER calcium storage capacity.^{3,4} Besides, it has become clear that CRT can be localized to non-ER subcellular compartments, such as the plasma membrane and the nucleus, performing diverse and important non-ER functions. For example, CRT on the cell surface was shown to participate in cell adhesion by binding to and stabilizing integrins–extracellular matrix (ECM) complexes.^{5,6} Exposure of CRT on the cell surface has been implicated in adaptive immune responses^{7,8} and clearance of apoptotic cells.⁹ Furthermore, in the nucleus CRT may mediate nuclear export of hormone receptors by interacting with their DNA-binding domains, thus negatively regulating transcriptional activity of the target genes.^{10–12}

Relatively unknown is the role of CRT in cancer. It has been shown that overexpression of CRT correlates with postoperative tumor metastasis in breast cancer patients.¹³ In gastric cancer, CRT was found to promote angiogenesis, proliferation and migration of gastric cancer cells, consistent with its associations with high microvessel density, tumor metastasis and survival in gastric cancer patients.¹⁴ In contrast, previous studies show that CRT inhibits prostate cancer cell growth both in vitro and in vivo, and suppresses lung macrometastasis in subcutaneous xenograft tumor model.¹⁵ Interestingly, numerous recent reports have identified CRT as a key factor on the cell surface that determines immune responses to tumor cells. It has been shown that exposure of CRT presents phagocytic signal for the innate immune system and thus enhances immunogenicity against cancer cells.^{7,16} Therefore the tumorigenic role of CRT remains to be precisely defined.

Oral cancer, or oral squamous cell carcinoma (OSCC), has emerged as one of the major cancers worldwide and is frequently associated with environmental carcinogens.¹⁷ Recent epidemiological data show that OSCC is ranked fourth in both cancer incidence and mortality among Taiwanese men.¹⁸ Due to its predisposition to lymph node metastasis, OSCC is characterized by poor prognosis and reduced five-year survival rate.^{19,20} Integrative approaches including the high-throughput omics technologies have been used to identify potential biomarkers for OSCC and unravel the molecular mechanism of OSCC pathogenesis.²¹ In particular, there are accumulating proteomics-based studies on OSCC in recent years.^{22–24} However, most of the candidate biomarkers require validation on biological functions and associations with clinical parameters. The aim of this study was to identify cancer-associated proteins by comparing the proteomes of OSCC and adjacent non-cancerous matched tissues (NCMTs). After identification of candidate proteins by LC/MS/MS, CRT was found to be up-regulated in OSCC specimens. Expression of CRT in clinical tumor samples and the functions of CRT in OSCC cell lines were further examined.

Materials and methods

Cell lines and cell culture

Oral cancer cell lines SCC-9, FaDu and CAL-27 were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA), while HSC-3, SAS and Ca9-22 were from Health Science Research Resources Bank (Osaka, Japan). These cells were routinely maintained in Dulbecco's modified Eagle medium supplemented with nutrient mixture F-12 (DMEM/F12) (GIBCO, Invitrogen Corporation) and with 10% FBS, and had been used in our recent studies.^{25,26} Primary cultures of normal human oral keratinocytes (NHOKs) were established from oral mucosa obtained from dental surgery and cultured in Defined Keratinocytes-SFM (GIBCO, Invitrogen Corporation) supplemented with growth factors and bovine pituitary extract.²⁷ NHOKs at the third passage were used in Western blot analysis.

Fresh tissues and tissue microarrays

Fresh oral tissue lysates were obtained as previously described.²⁵ The tissue microarrays included samples from 110 male patients with head and neck squamous cell carcinoma who underwent primary surgical resection at E-Da Hospital between 2008 and 2010. Paired sets of tumor tissues and NCMTs were procured during surgery. For tissue microarray construction, the surgical specimens were fixed in formalin and embedded in paraffin. Four cylindrical cores, each with a diameter of 0.6 mm, were obtained from every donor block using a tissue microarray workstation (MTA-1; Beecher Instruments, Silver Spring, MD, USA). Three tumors and two control tissues of each case were arrayed in a new paraffin block. The use of the specimens was approved by the Ethics and Clinical Research Committee of E-Da Hospital, and the informed consent was obtained from the patients or from their legal guardians.

Antibodies and Western blot analysis

Mouse monoclonal anti-CRT antibody was purchased from Stressgen (Ann Arbor, MI). Antibody of FAK was purchased from GeneTex (Irvine, CA), paxillin from Upstate Biotechnology (Lake Placid, NY), ERK1/2 from Cell Signaling Technology (Danvers, MA) and Src from Santa Cruz Biotechnology (Santa Cruz, CA). Immunoblotting was performed as previously described.²⁷ Incubation with the primary antibody (1:1000 dilution in 5% milk) was done at 4 °C overnight. Detection of the phosphoproteins using antibodies (all from Cell Signaling) of p-FAK, p-paxillin, p-ERK1/2 and p-Src was done the same as above with the exception of 3% BSA used in primary antibody incubation.

Immunohistochemistry

Immunohistochemical studies on the tissue microarrays were performed according to our previous protocol.²⁵ CRT staining intensity was graded into four categories based on the percentage of positive epithelial cells: negative, <5%; weak, 5–35%; moderate, 35–70%; strong, >70%.

Transfection and generation of CRT-knockdown stable line

SAS cells were seeded into 10-cm culture dishes at a density of 1×10^6 cells per dish. After attachment, the cells were transfected with 4 μ g CRT-shRNA vector (purchased from National RNAi Core Facility, Academia Sinica, Taiwan) or the control vector using FuGene6 transfection reagent (Roche Applied Science, Indianapolis, IN). Two days after transfection, the cells were trypsinized and re-plated in a 15-cm culture dish with medium containing 0.5 μ g/ml puromycin. Two weeks later, single colonies were isolated by cloning cylinders and seeded initially into each well of the 96-well plates, followed by progressive expansion of the cultures in large culture dishes. Two months later, the puromycin-resistant stable line was established and maintained in medium with 2 μ g/ml puromycin.

RNA isolation, semi-quantitative RT-PCR and real-time PCR

Total RNA was isolated from 1×10^7 of the CRT-knockdown stable line using TRIzol reagent (Invitrogen). Five micro gram of total RNA was reverse transcribed into cDNA by SuperScript III First-Strand Synthesis Supermix (Invitrogen). PCR reactions were carried out using ten-fold cDNA diluents and primers as follows: 5'-GAATTCATGCTGCTATCCGTGCC-3' (forward) and 5'-GAATTCCTACAGCTCGTCTTGG-3' (reverse). The thermal cycling conditions were 94 °C for 5 min, followed by 28 cycles of 94 °C for 1 min,

57 °C for 1 min and 72 °C for 1.5 min. For real-time PCR, 125 ng of cDNA was mixed with Power SYBR Green PCR Master Mix and amplified in StepOne Real-Time PCR System (Applied Biosystems). The primer sequences were 5'-AACTTCCTCATCACC AAC-3' (forward) and 5'-CTCATCTCTCATCTCT-3' (reverse). The cycling conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The cDNA content was determined using a comparative C_T method of $2^{-\Delta\Delta C_T}$. The results are shown as relative expression normalized to that of the internal control, GAPDH.

MTT cell proliferation assay

The CRT-knockdown stable transfectants were seeded into 96-well plates at a density of 1×10^4 in each well. After culturing for 24, 48, 72 or 96 h, cell growth studies were conducted by MTT assay (Chemicon International Inc., CA). MTT (0.01 ml) was added to each well and incubated at 37 °C for 4 h. The cells were lysed by 0.1 ml isopropanol with 0.04 N HCl. The optical density was measured at 570 nm as a test wavelength and 630 nm as a reference wavelength.

Colony formation assay

The CRT-knockdown stable transfectants were seeded in a 6-well plate at a density of 200 cells per well. After incubation for 15 days, the colonies were fixed with methanol for 10 min and stained with 0.1% crystal violet for 15 min. The number of colonies was counted and quantitated by Image-Pro Plus software.

Soft agar assay

Five thousand cells of the stable transfectants were mixed in the 0.3% top agarose (SeaPlaque) and plated onto the 0.5% bottom agarose in each well of a 6-well plate. Colonies were photographed directly or visualized by Giemsa stain and quantitated as above three weeks after incubation.

Assessment of cell cycle distribution

The flow cytometer-based analysis of cell cycle distribution was conducted as described.²⁸ In brief, 5×10^5 cells of CRT-knockdown stable transfectants or the control were starved with serum-free medium for 24 h. After starvation, 10% FBS was added into the medium and incubated for 48 h. Cells were then harvested, washed twice with PBS and fixed in 70% ethanol overnight. After centrifugation at 3000 rpm for 5 min at 4 °C, the cell pellets were stained with 10 μ g/ml propidium iodide (PI) (Sigma, St. Louis, MO, USA) and 10 μ g/ml RNase A in PBS buffer for 20 min at 37 °C in the dark. The cells were analyzed using a FACScan flow cytometer (Becton-Dickinson, Mansfield, MA, USA) and the results were evaluated using the WinMDI 2.9 software (written by Joseph Trotter, Scripps Research Institute, La Jolla, CA).

Transwell migration assay

3×10^4 of the CRT-knockdown stable transfectants were assayed for cell migration as mentioned previously.²⁹

Wound healing assay

The CRT-knockdown stable transfectants were plated in a 6-well plate (8×10^5 /well). After the cells grew to confluence, a scratch/wound was made with a pipette tip in each of the wells. Cells were washed with PBS and refreshed with FBS-containing medium. Microscopic fields were photographed at the initial point (0 h) and thereafter.

Transient siRNA knockdown

siGENOME SMARTpool siRNA against CRT was purchased from Dharmacon (Lafayette, CO). Transfection of siRNA (50 nM) was performed according to our previous protocols.²⁶ Knockdown of CRT expression was verified by Western blot analysis.

Gelatin zymography

To analyze the secretion of MMP2/MMP9 in culture supernatants, cells were incubated in serum-free medium for 24 h and then collected and concentrated by a speed vacuum. The samples were separated on 10% SDS-PAGE containing 1 mg/ml gelatin (Merck) under nonreducing conditions. The gels were washed in wash buffer containing 2.5% Triton X-100 in 50 mM Tris-HCl, pH 7.4, followed by incubation in reaction buffer (0.2 M NaCl, 0.02% NaN₃, 1 μ M ZnCl₂, 2% Triton-X 100, 5% Brij-35 in 50 mM Tris-HCl, pH 7.4) at 37 °C for 20 h. To visualize the gelatinolytic bands, the gels were stained with Coomassie Blue and then washed in destaining buffer. The gels were dried and quantified by densitometry.

Statistical analysis

The results of MTT assay, colony formation assay, soft agar assay, migration assay and cell cycle distribution were analyzed by Student's *t*-test. **P* < 0.05 was considered statistically significant.

Results

Up-regulation of CRT in paired tumor tissue specimens, OSCC cell lines and tissue microarrays

To identify differentially expressed proteins in OSCC, we investigated the proteomes of tongue tumors and adjacent non-cancerous matched tissues (NCMTs) by two-dimensional electrophoresis (2-DE). Several protein spots with differential expression were yielded and identified by mass spectrometry (LC/MS/MS), among which CRT was up-regulated in tumor tissues compared to the NCMTs (Supplementary data). To confirm the proteomic results, expression of CRT in sixteen fresh tumors and the paired NCMTs was examined by Western blot analysis. We found that CRT was highly expressed in the tumor areas of thirteen NCMT/tumor pairs (13/16 or 81.25%) (Fig. 1A). In addition, expression of CRT was significantly higher in six OSCC cell lines than in primary cultures of NHOKs (Fig. 1B). Furthermore, we analyzed CRT expression in oral tissue microarrays by immunohistochemistry (IHC) and demonstrated that 96.1% (99/103) of tumor specimens had positive CRT staining. In sharp contrast, only 31.5% (29/92) of the NCMTs had detectable CRT expression (*p* < 0.001 by χ^2 test). Fig. 1C shows the representative results of IHC. These results suggest that CRT is selectively expressed in cancerous lesions but not in normal mucosa of the oral cavity.

Establishment of stable CRT-knockdown in oral cancer cells

To study the function of CRT in oral cancer cells, we generated a stable CRT-knockdown cell line. Oral cancer cells, SAS, were transfected with CRT-shRNA vectors and selected with puromycin (0.5 μ g/ml). After two weeks, single colonies were formed and isolated by trypsin digestion in cloning cylinders. The puromycin-resistant colonies were amplified for two months in medium containing puromycin (2 μ g/ml). Effective silence of CRT expression was confirmed by real-time PCR, semi-quantitative RT-PCR and Western blot analysis (Fig. 2A and B). We checked the

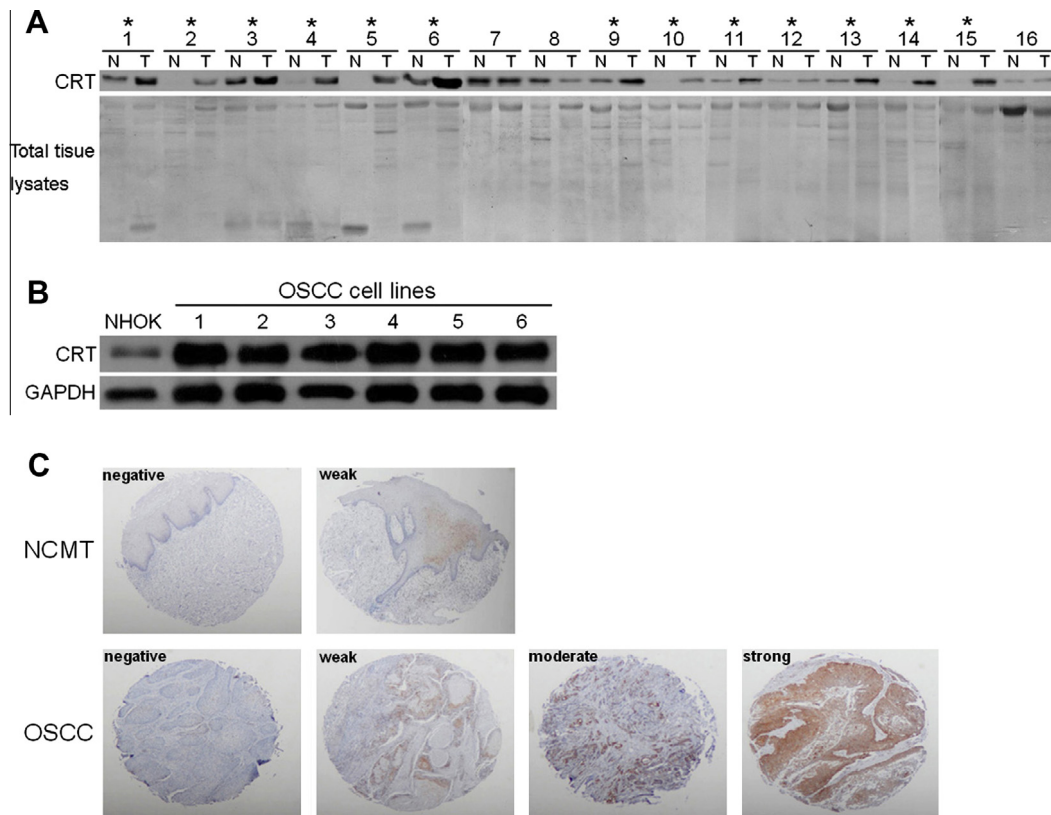


Figure 1 Differential expression of CRT in OSCC tissues and oral cancer cell lines. (A) Tissue extracts of 16 paired NCMT (N)/OSCC (T) samples were analyzed by immunoblotting using anti-CRT antibody. The lower panel shows the total tissue lysates stained with Coomassie Blue and used as the loading control. “*” represents overexpression of CRT in 13 of 16 tissue pairs. (B) Expression of CRT in six oral cancer cell lines and normal human oral keratinocyte (NHOK) was examined by immunoblotting. The six OSCC cell lines: 1, Ca9-22; 2, CAL-27; 3, HSC-3; 4, SCC-9; 5, SAS; 6, FaDu. (C) Immunohistochemical studies of CRT expression in tissue microarrays that contain 103 OSCC and 92 NCMT sections. The representative results show negative or weak staining in NCMTs, and negative, weak, moderate or strong staining in OSCC. Magnification: 40 \times . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

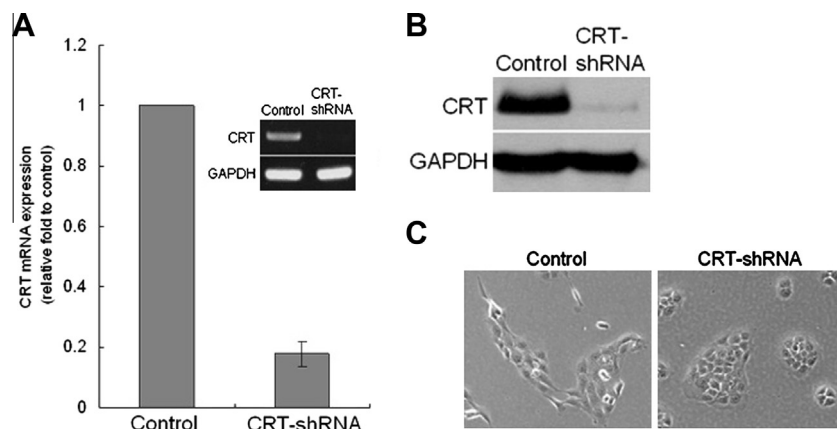


Figure 2 Down-regulation of CRT expression and morphological alterations in the CRT-knockdown oral cancer cell line. (A) CRT mRNA expression in the control or the CRT-knockdown stable line (CRT-shRNA) was confirmed using real-time PCR or semi-quantitative RT-PCR (upper right) that amplified a 1.25 Kb fragment of CRT coding sequence. Experiments in real-time PCR were done in triplicates and the results were presented as fold decrease in the stable line relative to the control (deliberately set to 1). (B) Effective depletion of CRT protein expression in the CRT-knockdown stable line was confirmed by Western blot analysis. (C) Cell morphology of the CRT-knockdown stable line at two months after the initial selection was photographed under bright-field microscopy. Magnification: 100 \times .

morphology of the stable transfectants and found distinct morphological changes between the control and the CRT-knockdown stable line. Unlike the scattered spindle-shaped cells in the control, the CRT-knockdown cells are smaller, round-shaped and have the propensity to cluster together (Fig. 2C).

Depletion of CRT induces cell cycle arrest and inhibits proliferation of oral cancer cells

We asked whether CRT was involved in cancer cell growth in vitro. Using MTT assay, we found that the CRT-knockdown

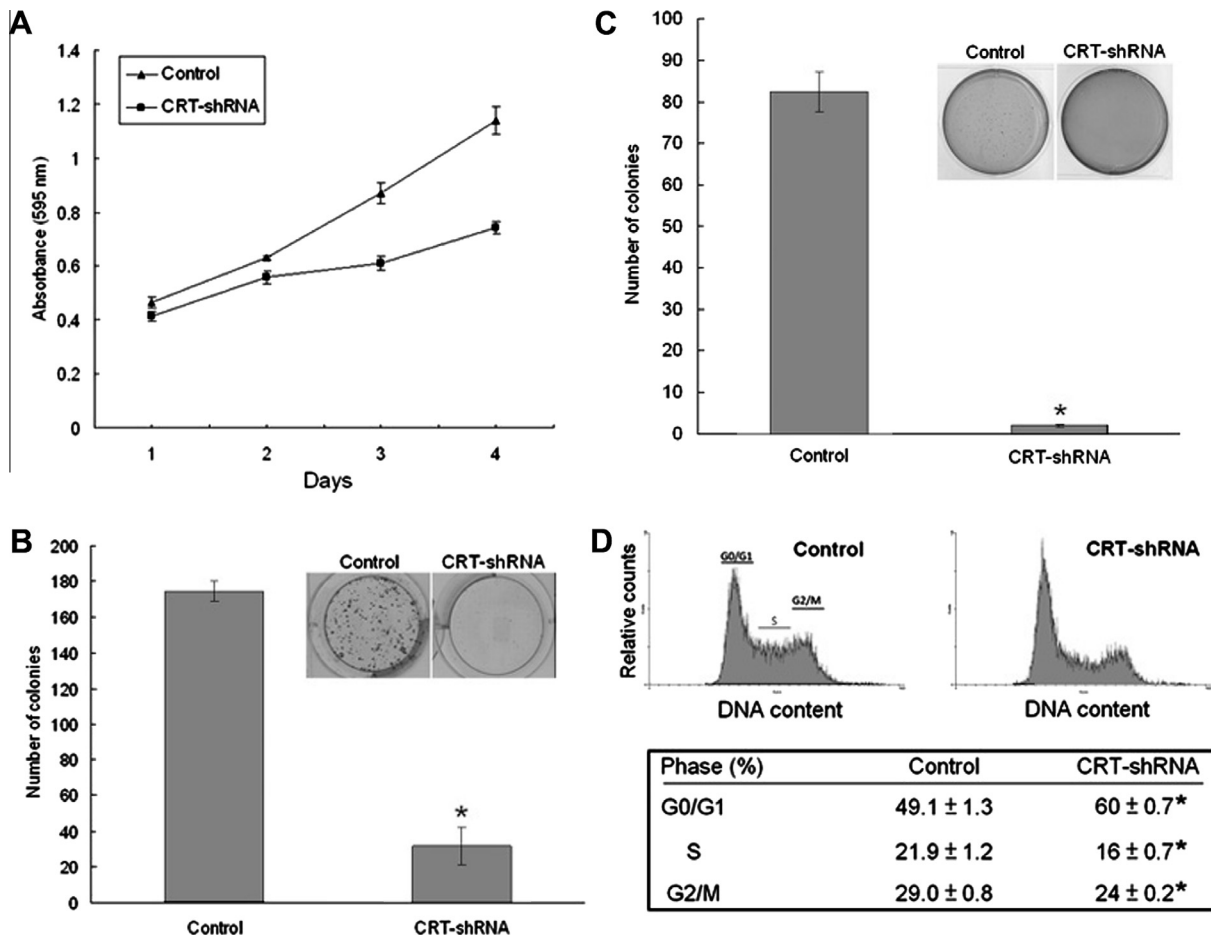


Figure 3 Cell growth assay and cell cycle analysis of the CRT-knockdown stable line. (A) The growth rate of the CRT-knockdown stable line and the control during a 4-day period was evaluated by MTT assay. (B) Representative photos and quantitative data of colony formation assay in monolayer cultures after two weeks growth. (C) Representative photos and quantitative data of soft agar assay after three weeks growth. (D) Cell cycle phase distribution was analyzed by flow cytometry. The CRT-knockdown stable line exhibited impaired cell cycle progression as indicated by more cells accumulated in G0/G1 phase. The table shows the percentages of cell distribution in different phases of the cell cycle. All experiments were done in triplicates and the results were presented as means \pm SD. * $P < 0.01$ when compared with the control.

stable line had a reduced growth rate during the 4-day growth period (Fig. 3A). We then tested colony-forming ability of the knockdown cell line on a solid support or in soft agar. In both conditions, there was a marked decrease in colony formation when CRT expression was depleted ($p < 0.01$) (Fig. 3B and C). Moreover, analysis of cell cycle-phase distribution by flow cytometry revealed a significant increase of cells in G0/G1 phase (from 49.1% to 60%) ($p < 0.01$) and a concomitant decrease of cells in S phase (from 21.9% to 16%) and G2/M phase (from 29% to 24%) ($p < 0.01$) in the knockdown cell line (Fig. 3D). This altered cell cycle progression is presumed to be responsible for the observed decreased cell proliferation. In addition, there was no evident sub-G0/G1 peak, excluding apoptotic cell death in the CRT-knockdown stable line.

Depletion of CRT inhibits migration of oral cancer cells presumably due to decreased activity of FAK and MMP-2/MMP-9

Cell motility of the CRT-knockdown stable line was examined by wound healing (horizontal) and transwell (vertical) migration assays. Compared to the parental control, the CRT-knockdown cells displayed a much impaired ability to move both horizontally and vertically ($p < 0.01$), strongly suggesting that CRT is required for oral cancer cell migration (Fig. 4A and B). Focal adhesion kinase (FAK) and matrix metalloproteinases (MMPs) are the key players in integrin-mediated cell migration. Indeed, phosphorylation of

FAK (pY397) and paxillin (pY118), the known phosphorylation target of FAK, was decreased in the knockdown stable line (Fig. 4C). Given that ERK1/2 is a downstream effector of FAK, our data also indicated a decreased phosphorylation level of ERK1/2 in the knockdown stable line. Consistent results were obtained from another oral cancer cell line, Ca9-22, with transient knockdown of CRT by specific siRNA oligos (Fig. 4C). On the other hand, using gelatin zymography and Western blot analysis, we showed that both the secreted level and the activity of MMP-2 and MMP-9 were significantly attenuated in the CRT-knockdown stable line (Fig. 4D). These data suggest that CRT may regulate cell migration through the FAK/paxillin/ERK1/2 signaling pathway coupled with the proteolytic degradation of extracellular matrix (ECM) in oral cancer cells.

Discussion

Although CRT has been implicated in diverse normal cellular processes, its role in cancer development has not been fully elucidated. In particular, it remains unknown whether CRT has important functions in oral cancer cells and may serve as a biomarker for OSCC patients. In an attempt to identify differential protein expression in OSCC tissues by proteomic analysis, we found that CRT was overexpressed in tongue cancerous tissues. Verification of CRT expression in sixteen NCMT/OSCC pairs and six OSCC cell

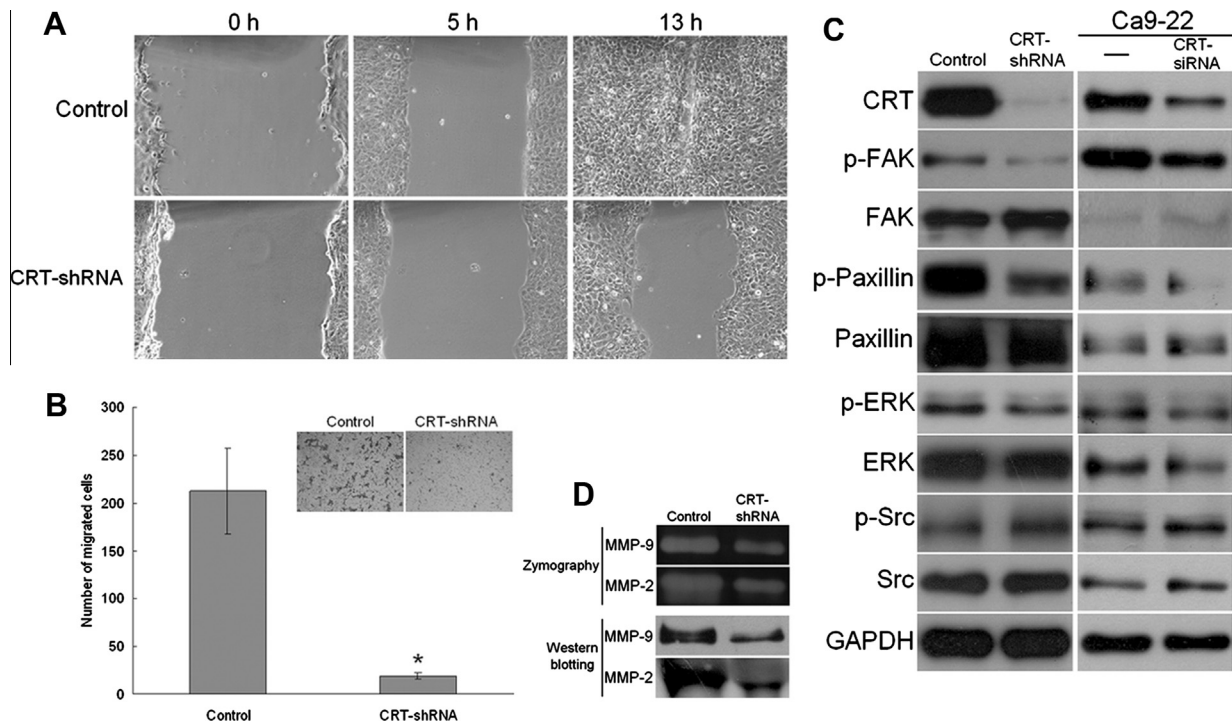


Figure 4 Cell motility of the CRT-knockdown stable line and its correlation with the activity of FAK pathway and MMP-2/MMP-9. (A) Horizontal cell movement was examined by wound healing assay. After the wounds were created, the representative results were photographed at time 0, 5 and 13 h. (B) Transwell migration assay was used to assess the vertical movement of the cells. The migrated cells were stained with Giemsa and counted under bright-field microscopy at 100 \times magnification. Representative photos and quantitative data are shown. The experiment was done in triplicates and the data were expressed as means \pm SD. * $P < 0.01$ when compared with the control. (C) Phosphorylation levels of FAK (p-FAK), paxillin (p-paxillin), ERK1/2 (p-ERK) and Src (p-Src) were revealed by Western blot analysis in the CRT-knockdown stable line (left panel) and Ca9-22 cells with transient CRT knockdown by siRNA (right panel). Levels of p-FAK, p-paxillin and p-ERK but not p-Src were down-regulated when CRT expression was knocked down in both cell lines. (D) The secretion (Western blotting) and activity (zymography) of MMP-2 and MMP-9 were decreased in the culture medium of the CRT-knockdown stable line.

lines revealed its selective expression in cancerous lesions of the oral cavity. To evaluate whether CRT can be a biomarker for OSCC, we investigated the expression of CRT in a large cohort of clinical samples using oral tissue microarrays and IHC. We found that, among a total of 103 OSCC tissue sections, there was a striking percentage of CRT-positive OSCC cases (96.1%). Moreover, the majority of the positive cases (55/99 or 55.6%) exhibited moderate to strong staining intensity. On the contrary, only minor NCMT cases had positive staining (31.5%) of mostly weak staining intensity, consistent with the CRT expression in primary cultures of NHOKs. These findings suggest that CRT is a potential biomarker for diagnosing oral cancer, which correlates with previous studies by others that soluble CRT level in sera and urine may be used as a biomarker for prediction and diagnosis of lung and bladder cancer, respectively.^{30,31} However, at this point the clinicopathological correlation of CRT expression was not statistically significant (data not shown). This may be due to an insufficient follow-up period (2008–2010) and thus a lower mortality rate. Clinicopathological data are continuously accumulated for better prediction of clinical outcomes in the future.

To study the cellular effects of CRT in oral cancer cells, we established an oral cancer cell line with stable CRT-knockdown, and asked how this could influence cell growth and migration. We showed that, in addition to morphological alterations, CRT knockdown by shRNA vectors resulted in significantly reduced growth rate, colony-forming capacity and anchorage-independent growth, suggesting the requirement of CRT for oral cancer cell proliferation. This is consistent with several previous reports that forced expression of CRT enhanced cell proliferation in gastric cancer cells¹⁴, whereas knockdown of CRT suppressed cell growth rate in bladder cancer cells.³² However, it is still unknown how forced expression

of CRT may affect proliferation of the normal counterparts of gastric and bladder cancer cells. Nor is it clear how exogenous expression of CRT may have an impact on the physiological functions of primary NHOKs. To understand the mechanism behind the growth inhibition in the CRT-knockdown stable line, we examined cell cycle distribution by flow cytometry. Our results demonstrated that CRT knockdown triggered a marked accumulation of cells in G0/G1 phase without the induction of sub-G0/G1 peak. These findings suggest that the depletion of CRT caused G0/G1 cell cycle arrest but not apoptosis, at least in part accounting for the slow growth of the CRT-knockdown stable line. Furthermore, our preliminary results indicate marked elevation p21^{Cip1/Waf1} level in the CRT-knockdown cells (data not shown). Because p21^{Cip1/Waf1} is well known as a negative regulator of cell cycle and an inducer of cellular senescence, it seems plausible that the G0/G1 arrest may lead to senescence by the up-regulation of p21^{Cip1/Waf1} in the CRT-knockdown oral cancer cells.

The effect of CRT knockdown on oral cancer cell motility was further investigated by migration assays. We used wound healing and transwell migration assays to assess horizontal and vertical cell movements, respectively. In both cases, the CRT-knockdown stable line had a significant decrease in cell movement compared to the control cells, indicating that CRT is necessary for cell motility in vitro. These results correlate with the role of CRT in other cancer types, in which reduced CRT expression impairs cell migration of esophageal³³, bladder³² and gastric¹⁴ cancers. Since CRT has been shown to regulate focal adhesion^{34,35} and the activity of matrix metalloproteinases (MMPs)³⁶, we suspected that focal adhesion kinase (FAK), MMP-2 and MMP-9 may participate in CRT-mediated oral cancer cell migration. Indeed, the activity, or the phosphorylation level, of FAK was down-regulated in the CRT-knockdown

stable line. Moreover, the phosphorylation level of paxillin and ERK1/2, the known downstream effectors of FAK, was decreased in the knockdown cells. Although Src has been shown to phosphorylate FAK in integrin-FAK signaling³⁷, the activity of Src (SrcpY416) was not decreased in the CRT-knockdown stable line. Thus, it is possible that CRT-mediated oral cancer cell migration involves the FAK pathway but is independent of the Src pathway. To support these results, we demonstrated the involvement of the FAK signaling in CRT-mediated cell migration using another oral cancer cell line (Ca9-22) with transient knockdown of CRT. On the other hand, we showed that the CRT-knockdown stable line had a dramatic reduction of secretion and activity of MMP-2 and MMP-9 in the medium. Interestingly, the FAK-ERK signaling pathway has been implicated in the regulation of MMPs secretion in various carcinoma cells.^{38–41} Besides, mounting evidence indicates up-regulation of MMP-2 and/or MMP-9 expression in OSCC tissues^{42–45}, and higher ratios of MMP-2 and MMP-9 activation in malignant neoplasms than in their adjacent normal tissues⁴⁶, correlating with the invasive and metastatic potential and poor prognosis in OSCC patients. Thus, the observed reduced motility in the CRT-knockdown cells may be due to down-regulation of the FAK-ERK-MMP-2/MMP-9 signaling pathway. However, how CRT may modulate the activity of this signaling pathway and the resulting cell migration is not clear and deserves detailed investigations.

In summary, this study indicates that CRT is a potential biomarker and contributes to the malignant phenotypes of OSCC. Although CRT appears to have oncogene-like functions in vitro in oral cancer cells, CRT was shown to behave like a tumor suppressor that inhibited growth and metastasis of prostate cancer cells.¹⁵ It is therefore surmised that whether CRT plays an oncogenic or a tumor suppressive role is cancer cell type-dependent. Furthermore, our findings provide the first implication that CRT is important for proper cell cycle progression, and regulates cell motility through the FAK signaling pathway accompanied with concomitant activation of MMP-2 and MMP-9 in OSCC cells.

Conflicts of interest statement

None declared.

Acknowledgments

This work was supported by National Science Council (Grant 98-2311-B-037-002-MY3), the Chi-Mei Medical Center and Kaohsiung Medical University Research Foundation (100CM-KMU-05) and by E-DA hospital (Grant EDAHI99001).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.oraloncology.2013.01.003>.

References

- Nauseef WM, McCormick SJ, Clark RA. Calreticulin functions as a molecular chaperone in the biosynthesis of myeloperoxidase. *J Biol Chem* 1995;**270**(9):4741–7.
- Vassilakos A, Michalak M, Lehrman MA, Williams DB. Oligosaccharide binding characteristics of the molecular chaperones calnexin and calreticulin. *Biochemistry* 1998;**37**(10):3480–90.
- Gelebart P, Opas M, Michalak M. Calreticulin, a Ca²⁺-binding chaperone of the endoplasmic reticulum. *Int J Biochem Cell Biol* 2005;**37**(2):260–6.
- Michalak M, Mariani P, Opas M. Calreticulin, a multifunctional Ca²⁺ binding chaperone of the endoplasmic reticulum. *Biochem Cell Biol* 1998;**76**(5):779–85.
- Coppolino M, Leung-Hagesteijn C, Dedhar S, Wilkins J. Inducible interaction of integrin alpha 2 beta 1 with calreticulin. Dependence on the activation state of the integrin. *J Biol Chem* 1995;**270**(39):23132–8.
- Coppolino MG, Woodside MJ, Demareux N, Grinstein S, St-Arnaud R, Dedhar S. Calreticulin is essential for integrin-mediated calcium signalling and cell adhesion. *Nature* 1997;**386**(6627):843–7.
- Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini JL, et al. Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nat Med* 2007;**13**(1):54–61.
- Tesniere A, Apetoh L, Ghiringhelli F, Joza N, Panaretakis T, Kepp O, et al. Immunogenic cancer cell death: a key-lock paradigm. *Curr Opin Immunol* 2008;**20**(5):504–11.
- Gardai SJ, McPhillips KA, Frasch SC, Janssen WJ, Starefeldt A, Murphy-Ullrich JE, et al. Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. *Cell* 2005;**123**(2):321–34.
- Holaska JM, Black BE, Love DC, Hanover JA, Leszyk J, Paschal BM. Calreticulin is a receptor for nuclear export. *J Cell Biol* 2001;**152**(1):127–40.
- Holaska JM, Black BE, Rastinejad F, Paschal BM. Ca²⁺-dependent nuclear export mediated by calreticulin. *Mol Cell Biol* 2002;**22**(17):6286–97.
- Roderick HL, Campbell AK, Llewellyn DH. Nuclear localisation of calreticulin in vivo is enhanced by its interaction with glucocorticoid receptors. *FEBS Lett* 1997;**405**(2):181–5.
- Eric A, Juranic Z, Milovanovic Z, Markovic I, Inic M, Stanojevic-Bakic N, et al. Effects of humoral immunity and calreticulin overexpression on postoperative course in breast cancer. *Pathol Oncol Res* 2009;**15**(1):89–90.
- Chen CN, Chang CC, Su TE, Hsu WM, Jeng YM, Ho MC, et al. Identification of calreticulin as a prognosis marker and angiogenic regulator in human gastric cancer. *Ann Surg Oncol* 2009;**16**(2):524–33.
- Alur M, Nguyen MM, Eggener SE, Jiang F, Dadras SS, Stern J, et al. Suppressive roles of calreticulin in prostate cancer growth and metastasis. *Am J Pathol* 2009;**175**(2):882–90.
- Chao MP, Jaiswal S, Weissman-Tsukamoto R, Alizadeh AA, Gentles AJ, Volkmer J, et al. Calreticulin is the dominant pro-phagocytic signal on multiple human cancers and is counterbalanced by CD47. *Sci Transl Med* 2011;**2**(63):63ra94.
- Choi S, Myers JN. Molecular pathogenesis of oral squamous cell carcinoma: implications for therapy. *J Dent Res* 2008;**87**(1):14–32.
- Chen YJ, Chang JT, Liao CT, Wang HM, Yen TC, Chiu CC, et al. Head and neck cancer in the betel quid chewing area: recent advances in molecular carcinogenesis. *Cancer Sci* 2008;**99**(8):1507–14.
- Liu SY, Lu CL, Chiou CT, Yen CY, Liaw GA, Chen YC, et al. Surgical outcomes and prognostic factors of oral cancer associated with betel quid chewing and tobacco smoking in Taiwan. *Oral Oncol* 2010;**46**(4):276–82.
- Lo WL, Kao SY, Chi LY, Wong YK, Chang RC. Outcomes of oral squamous cell carcinoma in Taiwan after surgical therapy: factors affecting survival. *J Oral Maxillofac Surg* 2003;**61**(7):751–8.
- Wu RQ, Zhao XF, Wang ZY, Zhou M, Chen QM. Novel molecular events in oral carcinogenesis via integrative approaches. *J Dent Res* 2011;**90**(5):561–72.
- Lo WY, Tsai MH, Tsai Y, Hua CH, Tsai FJ, Huang SY, et al. Identification of over-expressed proteins in oral squamous cell carcinoma (OSCC) patients by clinical proteomic analysis. *Clin Chim Acta* 2007;**376**(1–2):101–7.
- Turhani D, Krapfenbauer K, Thurnher D, Langen H, Fountoulakis M. Identification of differentially expressed, tumor-associated proteins in oral squamous cell carcinoma by proteomic analysis. *Electrophoresis* 2006;**27**(7):1417–23.
- Wang Z, Jiang L, Huang C, Li Z, Chen L, Gou L, et al. Comparative proteomics approach to screening of potential diagnostic and therapeutic targets for oral squamous cell carcinoma. *Mol Cell Proteomics* 2008;**7**(9):1639–50.
- Chiang WF, Ho HC, Chang HY, Chiu CC, Chen YL, Hour TC, et al. Overexpression of Rho GDP-dissociation inhibitor alpha predicts poor survival in oral squamous cell carcinoma. *Oral Oncol* 2011;**47**(6):452–8.
- Chiu CC, Chen BH, Hour TC, Chiang WF, Wu YJ, Chen CY, et al. Betel quid extract promotes oral cancer cell migration by activating a muscarinic M4 receptor-mediated signaling cascade involving SFKs and ERK1/2. *Biochem Biophys Res Commun* 2010;**399**(1):60–5.
- Chen JY, Chang YL, Yu YC, Chao CC, Kao HW, Wu CT, et al. Specific induction of the high-molecular-weight microtubule-associated protein 2 (hmw-MAP2) by betel quid extract in cultured oral keratinocytes: clinical implications in betel quid-associated oral squamous cell carcinoma (OSCC). *Carcinogenesis* 2004;**25**(2):269–76.
- Chen JY, Hwang CC, Chen WY, Lee JC, Fu TF, Fang K, et al. Additive effects of C(2)-ceramide on paclitaxel-induced premature senescence of human lung cancer cells. *Life Sci* 2010;**87**(11–12):350–7.
- Tseng MY, Liu SY, Chen HR, Wu YJ, Chiu CC, Chan PT, et al. Serine protease inhibitor (SERPIN) B1 promotes oral cancer cell motility and is over-expressed in invasive oral squamous cell carcinoma. *Oral Oncol* 2009;**45**(9):771–6.
- Kageyama S, Isono T, Iwaki H, Wakabayashi Y, Okada Y, Kontani K, et al. Identification by proteomic analysis of calreticulin as a marker for bladder cancer and evaluation of the diagnostic accuracy of its detection in urine. *Clin Chem* 2004;**50**(5):857–66.
- Liu R, Gong J, Chen J, Li Q, Song C, Zhang J, et al. Calreticulin as a potential diagnostic biomarker for lung cancer. *Cancer Immunol Immunother* 2012;**61**(6):855–64.
- Lu YC, Chen CN, Wang B, Hsu WM, Chen ST, Chang KJ, et al. Changes in tumor growth and metastatic capacities of j82 human bladder cancer cells suppressed by down-regulation of calreticulin expression. *Am J Pathol* 2011;**179**(3):1425–33.
- Du XL, Yang H, Liu SG, Luo ML, Hao JJ, Zhang Y, et al. Calreticulin promotes cell motility and enhances resistance to anoikis through STAT3-CTTN-Akt pathway in esophageal squamous cell carcinoma. *Oncogene* 2009;**28**(42):3714–22.

34. Villagomez M, Szabo E, Podcheko A, Feng T, Papp S, Opas M. Calreticulin and focal-contact-dependent adhesion. *Biochem Cell Biol* 2009;**87**(4):545–56.
35. Yan Q, Murphy-Ullrich JE, Song Y. Structural insight into the role of thrombospondin-1 binding to calreticulin in calreticulin-induced focal adhesion disassembly. *Biochemistry* 2010;**49**(17):3685–94.
36. Wu M, Massaeli H, Durston M, Mesaeli N. Differential expression and activity of matrix metalloproteinase-2 and -9 in the calreticulin deficient cells. *Matrix Biol* 2007;**26**(6):463–72.
37. Miranti CK, Brugge JS. Sensing the environment: a historical perspective on integrin signal transduction. *Nat Cell Biol* 2002;**4**(4):E83–90.
38. Hu B, Jarzynka MJ, Guo P, Imanishi Y, Schlaepfer DD, Cheng SY. Angiopoietin 2 induces glioma cell invasion by stimulating matrix metalloproteinase 2 expression through the alphavbeta1 integrin and focal adhesion kinase signaling pathway. *Cancer Res* 2006;**66**(2):775–83.
39. Lu W, Zhou X, Hong B, Liu J, Yue Z. Suppression of invasion in human U87 glioma cells by adenovirus-mediated co-transfer of TIMP-2 and PTEN gene. *Cancer Lett* 2004;**214**(2):205–13.
40. Mon NN, Hasegawa H, Thant AA, Huang P, Tanimura Y, Senga T, et al. A role for focal adhesion kinase signaling in tumor necrosis factor-alpha-dependent matrix metalloproteinase-9 production in a cholangiocarcinoma cell line, CCKS1. *Cancer Res* 2006;**66**(13):6778–84.
41. Xu HY, Qian AR, Shang P, Xu J, Kong LM, Bian HJ, et al. siRNA targeted against HAb18G/CD147 inhibits MMP-2 secretion, actin and FAK expression in hepatocellular carcinoma cell line via ERK1/2 pathway. *Cancer Lett* 2007;**247**(2):336–44.
42. de Vicente JC, Fresno MF, Villalain L, Vega JA, Hernandez Vallejo G. Expression and clinical significance of matrix metalloproteinase-2 and matrix metalloproteinase-9 in oral squamous cell carcinoma. *Oral Oncol* 2005;**41**(3):283–93.
43. Imanishi Y, Fujii M, Tokumaru Y, Tomita T, Kanke M, Kanzaki J, et al. Clinical significance of expression of membrane type 1 matrix metalloproteinase and matrix metalloproteinase-2 in human head and neck squamous cell carcinoma. *Hum Pathol* 2000;**31**(8):895–904.
44. Katayama A, Bandoh N, Kishibe K, Takahara M, Ogino T, Nonaka S, et al. Expressions of matrix metalloproteinases in early-stage oral squamous cell carcinoma as predictive indicators for tumor metastases and prognosis. *Clin Cancer Res* 2004;**10**(2):634–40.
45. Kurahara S, Shinohara M, Ikebe T, Nakamura S, Beppu M, Hiraki A, et al. Expression of MMPs, MT-MMP, and TIMPs in squamous cell carcinoma of the oral cavity: correlations with tumor invasion and metastasis. *Head Neck* 1999;**21**(7):627–38.
46. Patel BP, Shah PM, Rawal UM, Desai AA, Shah SV, Rawal RM, et al. Activation of MMP-2 and MMP-9 in patients with oral squamous cell carcinoma. *J Surg Oncol* 2005;**90**(2):81–8.