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ORIGINAL RESEARCH

The role of *calgranulin B* gene on the biological behavior of squamous cervical cancer in vitro and in vivo

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Objective: The objective of the study was to explore the role of *calgranulin B* gene on the biological behavior of squamous cervical cancer.

Methods: Differential transcription in *calgranulin* gene between human papillomavirus (HPV)-positive and negative cervical cancer groups was identified, and the relationship between *calgranulin B* gene and *matrix metalloproteinase* (*MMP*) genes were explored using The Cancer Genome Atlas database. Subsequently, the role of calgranulin B on the cell proliferation, apoptosis, invasion and migration was investigated, through overexpression and/or underexpression of calgranulin B in cervical cancer cells. In addition, the effect of calgranulin B on the growth of the cervical cancer was studied via constructing xenograft model in BALB/c nude mice that either overexpressed or underexpressed calgranulin B.

Results: *Calgranulin* gene transcription in cervical cancer was highly correlated with the high-risk HPV-16 and HPV-45. In addition, overexpression of calgranulin B increased cell proliferation, invasion and migration, whereas it did not significantly affect cell apoptosis. This effect was also confirmed by calgranulin B knockdown assay. Additionally, we found that the transcription of *calgranulin B* gene was negatively correlated with *MMP15* and *MMP24* genes, but positively associated with *MMP25* genes in cervical cancer. Furthermore, calgranulin B significantly promoted the growth of cervical cancer in vivo.

Conclusion: Calgranulin B promotes cell proliferation, migration and invasion of squamous cervical cancer, possibly via regulation of MMPs. Whether there are synergistic actions between calgranulin B and HPV-16/HPV-45 infection on the squamous cervical carcinogenesis or progression need further study.

Keywords: calgranulin B, cervical cancer, proliferation, migration, invasion

Introduction

Cervical cancer remains the fourth most lethal cancer in women worldwide, with an estimated 527,600 new cases and 265,700 deaths in 2012.[1] In some low-income countries, cervical cancer is one of the most aggressive gynecological malignancies, if failed to be treated in time.[2] In spite of the evidence that persistent infection with some high-risk human papillomavirus (HPV) may cause such disease, infection of HPV alone is not sufficient for carcinogenesis.[3] Cervical cancer is a complex and multifactorial malignancy.[4] Immune, microbial or chemical cofactors and sex hormones could all play a partial role in the development of cervical (pre)neoplastic lesions.[3] In addition, number of sexual partners, smoke habits and oral contraceptives are crucial determinants in the persistent infection of HPV and cervical carcinogenesis, but controversial results are found concerning these risk cofactors.[4]

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Calgranulin B, also called S100A9, is a member of the S100 family of calcium-binding proteins involved in cellular processes translating changes in Ca^{2+} levels into specific cellular responses through binding to target proteins and regulated by zinc.[5,6] *Calgranulin B* gene is located on human chromosome 1q21,[6] a frequent target for chromosomal rearrangements occurring during tumor development.[7] Also, 11 out of 16 *S100* genes including *calgranulin B* were found to be downregulated in esophageal squamous cell carcinoma as compared to the corresponding normal esophageal mucosa.[8] By contrast, the overexpression of calgranulin B has been observed in numerous cancer types including oral tongue squamous cell carcinoma, thyroid carcinoma, lung adenocarcinoma, breast cancer, invasive bladder cancer and ovarian cancer.[9–14] In our previous study, the expression of calgranulin B protein was reported to increase in squamous cervical cancer in comparison with adjacent normal cervical tissues via two-dimensional gel electrophoresis followed by mass spectrometry.[15] Then, we further found that the expression of calgranulin B was observed in 30 chronic cervicitis cases, 50 cervical intraepithelial neoplasia (CIN) cases and 40 squamous cervical cancer cases, gradually increasing as the tumor progressed,[16] suggesting that calgranulin B may play a crucial role in squamous cervical carcinogenesis.

The effect of calgranulin B on the biological behavior of cancer cells is controversial. Calgranulin B recombinant protein was found to significantly inhibit the invasion of gastric cancer cell lines BGC-823.[17] On the other hand, recombinant calgranulin B promoted the migration of human colorectal carcinoma cell lines HCT116 and SW480 via upregulation of the Wnt/β-catenin pathway.[18] However, it still remains unclear regarding the effect of calgranulin B on the biological behavior of squamous cervical cancer.

In the present study, the differential transcription of *calgranulin B* gene between HPV-positive and HPV-negative cervical cancers and the expression of calgranulin B protein in different squamous cervical cancer cell lines were investigated. Subsequently, we used recombinant adenoviruses or RNA interference to overexpress or downregulate the expression of calgranulin B, respectively, in cervical squamous cell carcinoma, in order to elucidate the role of calgranulin B expression on the biological behavior of squamous cervical cancer. We also investigated the relationship between *calgranulin B* gene and *matrix metalloproteinases* (*MMPs*) genes in cervical cancer using The Cancer Genome Atlas (TCGA) database. Additionally, we assessed whether calgranulin B would affect the growth of the xenograft model of cervical cancer in nude mice subcutaneously injected with SiHa cells that either overexpressed or underexpressed calgranulin B.

Methods and materials

Database retrieval

RNA sequencing data from the TCGA database (available online: https://gdc-portal.nci.nih.gov/) were downloaded, including 306 cervical cancer samples (282 HPV positive: 280 high-risk HPV and 2 low-risk HPV; 23 HPV negative; 1 HPV indeterminate) and 3 matched normal samples. The number of other HPV-positive samples was following: HPV-31 (6); HPV-33 (10); HPV-35 (7); HPV-52 (9); HPV-58 (7); HPV-59 (4); HPV-43 (2). Limma and edgeR package in R language were used to identify the genes' differential transcriptions. Only the genes with *p*-value <0.05 and log2 fold change (logFC) >1 were considered to have statistical difference. "cor.test" in R language was used to evaluate the relation between *calgranulin B* gene and *MMP* genes. *p*-value had been adjusted by the Bonferroni correction, and thus the *p*-value less than 0.05/23 (0.00217) should be considered to have statistical difference.

Cell lines and cell culture

Four squamous cervical cancer cell lines (SiHa, Caski, C33A and MS751) were obtained from Shanghai Cell Biology Medical Research Institute, Chinese Academy of Sciences. Caski cells contained both integrated HPV-16 and HPV-18. MS751 cells contained both HPV-18 and HPV-45. SiHa cells only contained HPV-16, whereas C33A cells were negative for *HPV* gene. Caski was cultured in RPMI-1640 (Gibco; Invitrogen, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco; Invitrogen). SiHa, C33A and MS751 cells were maintained in Dulbecco's modified Eagle's medium (Gibco; Invitrogen) with 10% FBS.

Immunocytochemical analysis

The expression and localization of calgranulin B in squamous cervical cancer cells were detected by immunocytochemistry. Briefly, cells were fixed and permeabilized for 20 min, respectively. Endogenous peroxidase activity of cells was blocked with 0.3% hydrogen peroxide in methanol. Cells were incubated with anti-calgranulin B rabbit antibody (1:100; Abcam, Cambridge, MA, USA) overnight at 4 °C after blocking with 5% normal goat serum for 30 min. Then cells were treated with biotinylated goat anti-rabbit antibody for 30 min and streptavidin peroxidase for 10 min at 37 °C and visualized with diaminobenzidine. Immunostaining of

the negative control was incubated with phosphate-buffered saline (PBS) in the absence of the primary antibody.

Reverse transcription-polymerase chain reaction

The transcription of calgranulin B mRNA in four cervical cancer cells was measured by reverse transcription-polymerase chain reaction (RT-PCR). Trizol reagent was used to isolate total RNA from cervical cancer cells according to the manufacturer's instructions (Invitrogen). The RNA pellets were dissolved in diethylpyrocarbonate-treated H_2O and then stored at −80 °C. The RNA preparations were quantified by measuring absorbance at 260 nm, and the purity of RNA was determined by the value of A260/A280. Isolated RNA was reverse transcribed by using reverse transcription kit (Thermo Fisher Scientific, Waltham, MA, USA) using the following reaction composition: 1 μg of total RNA; 11 μL of nuclease-free water; 1 μL of RevertAid M-MuL reverse transcriptase; 2 μL of 10 mM dNTP mix; 1 μL of riboLock RNase inhibitor; 4 μL of 5× reaction buffer; 1 μL of Oligo (dT) 18 primer. The primers sequences used were as follows: calgranulin B primer (forward: 5′-ATCAACACCTTCCACCAATACTC-3′ reverse: 5′-GACCTTTTCATTCTTATTCTCCTTC-3′); GAPDH primer (forward: 5′GAAATCCCAT[illegible]A CATCTTCCAG-3′ reverse: 5′-ATGAGTCCTTCC[illegible]C-GATACCAAA-3′). GAPDH was used as an i[illegible]al cont[illegible] PCR was performed for 30 cycles (for c[illegible]granuli[illegible]B) or 2[illegible] cycles (for GAPDH) of 94 °C denatur[illegible]ng for[illegible]58 °C annealing for 30 s and extension [illegible]72 °C [illegible]50 s; the final extension step was performe[illegible]72 °C for [illegible]min. Then, 4 μL of RT-PCR product of calgran[illegible]in B was mixed with 4 μL of RT-PCR produ[illegible]of GAPDH, a[illegible]then separated by electrophoresis in 2[illegible]agarose gel. The results were recorded by using Quantity [illegible]ne [illegible]nsitometry software package (Bio-Rad, H[illegible]es, C[illegible]USA)[illegible]l experiments were performed i[illegible]triplica[illegible]s. *Calg[illegible]ulin B* gene expression level was ex[illegible]ssed a[illegible]ulin B/GAPDH pixel signal ratio.

Western blot analysis

The cervical cancer cells were cultured in 6-well plates (5×10^5 cells per well). Then, the expression of calgranulin B protein was measured with Western blot. The cell lysates were prepared and analyzed for protein concentration. Then, electrophoresis and transfer were performed, followed by blocking with 5% milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 2 h at room temperature. The membrane was incubated overnight at 4 °C with primary antibodies against calgranulin B (rabbit antibody, 1:1,000) and 1:2,000 dilution of mouse anti-tubulin, respectively. After washing in TBST, membranes were probed with goat anti-rabbit or goat anti-mouse secondary antibody at room temperature for 2 h. The Logene-i YG2006 image acquisition system (Langjia, Wuxi, People's Republic of China) was used to detect and quantify the immunoreactive bands. All experiments were performed in triplicate.

Enzyme-linked immunosorbent assay

Detection of calgranulin B antibody in cell culture supernatants was performed using the [illegible]yme-linked immunosorbent assay (ELISA) calc[illegible]ulin B [illegible]tibody test kit (USCN, Wuhan, People's Re[illegible]blic of Chin[illegible] according to the manufacturer's manu[illegible]. Detec[illegible]n limit w[illegible]s 15.6 pg/mL. The cell culture sup[illegible]natants were c[illegible]e[illegible]ed by centrifugation at 3,000 *g* for [illegible]min. [illegible]ash solution, standards, Detection Reagent [illegible]and De[illegible]tion Re[illegible]gent B were diluted into required c[illegible]ntration. Fiv[illegible]s were prepared for standard and one well fo[illegible]lank. Then, 100 μL standard and samples we[illegible]ed into eac[illegible]well of the ELISA plate, respectively, [illegible]d then sealed with closure plate membrane for incubation at [illegible]7 °C for 2 h. [illegible]fterward, the liquid in the plate was discarded a[illegible]d 100 μL [illegible]f prepared Detection Reagent A was added. Then, [illegible]liquid in the plate was discarded again following [illegible]bation for 1 h at 37 °C. The plates were washed with 350 μL of diluted wash solution for three times. After this, 100 μL of prepared Detection Reagent B was added into each well to incubate for 30 min at 37 °C. The discard/wash process was repeated for a total of 5 times as conducted already. Then, 90 μL TMB (3,3′,5,5′-tetramethylbenzidine) was added into each well in a dark room to incubate for 20 min at 37 °C, and then stopped with adding 50 μL of stop solution. Reading of optical density was performed using a microplate reader (Bio-Rad) at 450 nm.

Construction of recombinant adenoviruses

To prepare calgranulin B-overexpressing adenovirus, the human *calgranulin B* gene was amplified by PCR using calgranulin B cDNA and was cloned into the NotI and NsiI sites of pHBAd-MCMV-RFP vector (Hanbio, Shanghai, People's Republic of China) in vitro. After confirmation using gene sequencing, the pHBAd-MCMV-RFP-calgranulin B plasmid and plasmid pHBAd-BHG (Hanbio) were transfected into HEK293 cells for packaging by using LipofiterTM (Hanbio). Cells infected by pHBAd-MCMV-RFP-calgranulin B (Ad-calgranulin B) were used to validate the efficiency of infection. The pHBAd-MCMV-RFP (Ad-RFP) vector

(Hanbio) was used as a negative control. The cells without treatment were used as naïve control group.

RNA interference

Calgranulin B siRNA and control siRNA were obtained from GenePharma (Shanghai, People's Republic of China). The sequences of calgranulin B siRNA was as follows: 5′-CCUUGAACUCUAUCGACGUCUA-3′. A control siRNA was synthesized for use as the control. The sequence of control siRNA was as follows: 5′-UUCUCCGAACGUGUCACGUdTdT-3′. The cells were cultured in 6-well plates when the cells reached 30–50% confluence, and then the cells were transfected with calgranulin B siRNA (5 μL) or control siRNA (3 μL) in serum-free medium using LipofectamineTM 2000 (Invitrogen). Medium with 10% FBS replaced the serum-free medium after 6 h and the cells were incubated for another 42 h. Then, protein lysates were collected from cells and analyzed with Western blot analysis.

Cell counting kit-8 (CCK-8) assay

Cell counting kit-8 assay was performed for detecting the effect of calgranulin B expression on the proliferation ability of cervical cancer cells. The cervical cancer cells were seeded in 96-well plates (1×10^4 cells per well). After transfection or transduction, cells were incubated at 37 °C in 5% CO_2 humidified incubator with 10 μL/well CCK-8 solution for 2 h. The absorbance value at 450 nm was quantified using a microplate reader (Bio-Rad). The viability of cells was calculated according to the following formula: cell viability = (OD450 of test well − OD450 of blank well)/(OD450 of control well − OD450 of blank well). The experiments were repeated three times.

Flow cytometry

The cervical cancer cells were cultured in 6-well plates (2×10^5 cells per well). After transfection or transduction, cells were digested by 0.25% trypsin and dissociated into single cell. Then the cells were double stained with Annexin V-fluorescein isothiocyanate and propidium iodide or Annexin V-allophycocyanin (APC)/7-amino-actinomycin D, according to the manufacturer's instructions (BD, Franklin Lakes, NJ, USA). The percentage of apoptotic cells were detected by flow cytometry (BD) after staining. The experiment was repeated three times.

Transwell invasion and migration assay

The polycarbonate filter membrane (diameter 6.5 mm, pore size 8 μm; Corning, New York, NY, USA) at the bottom of the Transwell chamber was coated with 50 μL Matrigel (BD) and air dried in a laminar hood overnight for invasion assay. The cells were suspended in serum-free medium and the concentration of cells was adjusted to 1×10^5/mL after transfection or transduction. Then, medium containing 10% FBS (600 μL per well) was placed in the lower chamber, and suspended cells (200 μL per well) were plated into the upper chamber. After 24 h, cells that did not penetrate the polycarbonate membrane at the bottom of the chamber were removed with a cotton swab. Then cells were washed with PBS. The cells that had invaded through the membrane to the lower surface were fixed and stained with 1% crystal violet for 10 min. The number of cells that penetrated the membrane was counted by randomly selecting five vision fields under a microscope (Nikon, Tokyo, Japan). In the Transwell migration assay, the upper chamber was not coated with Matrigel (BD) and the other steps were the same as the invasion assay.

Construction of calgranulin B-silenced SiHa cells

The co-transduction of a pLenti vector (control-pGFP-C-shLenti or calgranulin B-pGFP-C-shLenti) and lentiviral packaging mix (Cyagene, Rockville, MD, USA) with human embryonic kidney HEK293T cells was used to produce sh-calgranulin B lentivirus, according to the manufacturer's manual. After HEK293T cells were transfected for 48 h, lentivirus-containing medium was collected, filtered through 0.45 μm filter to remove cell debris and stored at −80 °C. In total, 1 mL of pGFP-C-sh-control or pGFP-C-sh-calgranulin B lentiviruses were incubated with SiHa cells in the presence of 8 μg/mL polybrene for 48 h at 37 °C in 5% CO_2 humidified incubator for viral infection. Then puromycin (final concentration of 1 μg/mL) was used to screen cells for target expression for 1–2 weeks. The selected puromycin-resistant cells were continued to be cultured in regular complete medium with pen-strep and the antibiotic for subsequent experiments. Verification of target cells was conducted by Western blot analysis.

Construction of recombinant lentivirus

Lentiviral constructs that express calgranulin B were prepared by using pLVX-IRES-ZsGreen1 vectors (donated by the Department of Inspection; Wenzhou Medical University, Wenzhou, People's Republic of China). Calgranulin B cDNAs were prepared by RT-PCR. Primers for preparing the coding sequence of calgranulin B were: 5′-TTACTCGAGATGACTTGCAAA-3′ (forward) and 5′-TAAGCGGCCGCTTAGGGGGTGCC-3′ (reverse). Calgranulin B open reading

frame sequences were inserted into the vector through BamHI and XhoI sites to get pLVX-IRES-ZsGreen1-calgranulin B constructs. Then the pLVX-IRES-ZsGreen1 vectors (control and calgranulin B) were transfected into HEK293T cells with Lipofectamine 3000 in the presence of the psPAX2 and pMD2.G vector for lentiviral particles packaging. Lentiviral particles were harvested from cell culture medium transduction by filtering through 0.45 μm filter. Then lentiviral particles were incubated with SiHa cells to produce stable cell lines overexpressing calgranulin B or only the negative control vector. Cells infected by pLVX-IRES-ZsGreen1-calgranulin B were used to validate the efficiency of infection. Fluorescence-activated cell sorting was used to sort out infected cells. Verification of target cells overexpressing calgranulin B was confirmed by Western blot analysis.

Subcutaneous xenograft cervical cancer model in nude mice

Female BALB/c nude mice (5- to 7-week-old) xenotransplanted with SiHa cells that either overexpressed or underexpressed calgranulin B were injected with 4×10^6 cells (either pLVX-IRES-ZsGreen1-calgranulin B, pLVX-IRES-ZsGreen1-control, pGFP-C-sh-calgranulin B or pGFP-C-sh-control) in 100 μL PBS into the back of right armpit of mice subcutaneously. Tumor volume measurements were performed every 2–3 days and calculated with the formula: $V = \text{length} \times \text{width}^2/2$. Then 5 weeks after cell injection, mice were killed and tumor tissues were removed and weighed.

Ethical approval

National Research Council's guide for the Care and Use of Laboratory animals was followed. Ethical and legal approval was obtained from the Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University prior to the commencement of the study.

Statistical analysis

Data in the text and figures were continuous variables and of normal distribution, which was shown as mean ± standard deviation, with a 2-sided *p*-value of less than 0.05 considered statistically significant. The differences between multi-groups were analyzed by using one-way analysis of variance followed by the least significance difference method or Dunnett's T3 test. Statistical comparisons between two groups were performed by unpaired Student's *t* test. Statistical analysis was performed by using SPSS 17.0 statistical software.

Results

Calgranulin B gene was potentially associated with high-risk HPV genes in cervical cancer

Using the TCGA database, a total of 2,086 differentially transcribed (logFC >1) genes were found in cervical cancers between HPV-positive and HPV-negative groups, with 551 upregulated genes (including *calgranulin B*) and 1,535 downregulated genes in the HPV-positive group. *Calgranulin B* (logFC = 1.453) was further studied for the relation with HPV subtypes in 306 cervical cancer samples (282 HPV positive: 280 high-risk HPV, 172 HPV-16-positive, 41 HPV-45-positive, 24 HPV-18-positive, 2 low-risk HPV, 23 HPV negative). The expression of *calgranulin B* gene was related to high-risk HPV, especially related to HPV-16 and HPV-45, as shown in Table 1.

Calgranulin B protein was located in the nuclei and cytoplasm of cervical squamous cancer cells

The localization of calgranulin B protein in four cervical squamous cancer cells (SiHa, C33A, Caski and MS751) is illustrated in Figure S1. Caski cells contained both HPV-16 and HPV-18. MS751 cells contained both HPV-18 and HPV-45. SiHa cells were only HPV-16 positive, whereas C33A cells were negative for *HPV* gene. Calgranulin B protein expression was mainly found in the cytoplasm and occasionally in the nuclei among four cell lines. The cytoplasm of all SiHa, Caski and MS751 cells showed moderate or intense positive staining for calgranulin B. Some C33A cells cytoplasm showed weak positive staining. Part of SiHa and MS751 cell nuclei showed moderate positive staining for calgranulin B. Moderate or intense positive staining for calgranulin B was observed

Table 1 The relationship between *calgranulin B* gene and HPV in cervical cancer from the TCGA database

***Calgranulin B* in cervical cancer**	**logFC**	***p*-value[a]**
HPV-positive (282) vs. HPV-negative (23)	1.453	0.006[a]
High-risk HPV-positive (280) vs. high-risk HPV-negative (26)	1.477	0.006[a]
HPV-16-positive (172) vs. HPV-negative (23)	1.577	0.003[a]
HPV-45-positive (41) vs. HPV-negative (23)	1.265	0.035[a]
HPV-18-positive (24) vs. HPV-negative (23)	0.884	0.156

Notes: [a]$p < 0.05$ was considered statistically significant. The number of other HPV-positive samples was: HPV-31 (6); HPV-33 (10); HPV-35 (7); HPV-52 (9); HPV-58 (7); HPV-59 (4); HPV-43 (2).
Abbreviations: HPV, human papillomavirus; logFC, log2 fold change; TCGA, The Cancer Genome Atlas.

in individual Caski cell nuclei. Weak positive staining for calgranulin B was also shown in individual C33A cell nuclei.

Calgranulin B mRNA level and protein expression was heterogeneous in cervical squamous cancer cell lines

Calgranulin B mRNA was found in the four cervical squamous cancer cell lines (C33A: HPV [−]; Caski: HPV-16 [+]/–18 [+]; SiHa: HPV-16 [+]; MS751: HPV-18 [+]/–45 [+]). The level of calgranulin B mRNA was lowest in C33A cells and highest in Caski cells ($p < 0.05$). There was no significant difference in the level of calgranulin B mRNA between SiHa cells and MS751 cells ($p > 0.05$) (Figure 1A and C).

Similarly, the expression of calgranulin B protein was consistent with that of mRNA in four cells. The expression of calgranulin B protein was lowest in C33A cells and highest in Caski cells ($p < 0.05$). No difference was found in the level of calgranulin B protein in SiHa cells and MS751 cells ($p > 0.05$) (Figure 1B and D).

Alteration of calgranulin B protein expression in human cervical squamous cancer cells at different culture times

In this study, the changes of calgranulin B protein level in four cells were investigated after culturing for 12, 24, 36, 48 and 72 h, respectively. As shown in Figure 1E–G, there was no significant change in expression of calgranulin B protein in MS751 cells at different culture time. When cultured for 24 h, calgranulin B protein level in SiHa cells reached the peak, and then decreased with extending of time. Data also showed the lowest expression of calgranulin B protein when C33A cells were cultured for 24 h. Then, calgranulin B protein levels increased and reached the peak when cells were cultured for 48 h, decreased with extending time. In view of Caski cells, the expression of calgranulin B protein had been increasing when cells were cultured from 12 to 72 h, reaching the peak until cultured for 72 h. Additionally, calgranulin B protein expression in Caski cells was the highest with different times (36, 48, 72 h). The expression of calgranulin B protein in C33A was the lowest when cultured for 24 h. Therefore, Caski cells were transfected with calgranulin B siRNA after being cultured for 72 h, and C33A cells were transfected with the Ad-calgranulin B after being cultured for 24 h in the subsequent studies.

The level of calgranulin B protein secreted into the supernatant in four cervical carcinoma cell lines at different culture time points was also measured by ELISA. Calgranulin B protein was detected at 46 pg/mL when Caski cells were cultured for 72 h. However, the secretion of calgranulin B protein was not seen in the supernatant of the other three cells at all time points and Caski cells at all time points except for 72 h (the data were all negative), indicating that the expression of secretory calgranulin B in human cervical carcinoma cell line SiHa, C33A, Caski and MS751 is very little. These data suggest that calgranulin B is mainly expressed in cells, but not secreted into supernatant. Thus, we chose the calgranulin B recombinant adenoviruses or calgranulin B siRNA to change the expression of calgranulin B instead of adding recombinant calgranulin B protein or calgranulin B protein inhibitors to explore the effect of calgranulin B on the biological behavior of cervical cancer cells.

Calgranulin B promoted proliferation of cervical cancer cells

To investigate the function of calgranulin B in cervical cancer cells, we chose C33A cell lines in which the expression of calgranulin B was lowest for further study after transduction with the pHBAd-MCMV-RFP-calgranulin B (Ad-calgranulin B). The transduction efficiency of calgranulin B recombinant adenovirus observed was high, as shown in Figure 2. C33A cells were divided into three groups: Ad-calgranulin B group, pHBAd-MCMV-RFP (Ad-RFP) group and control group. Transduction of C33A cells with Ad-calgranulin B markedly increased calgranulin B protein levels (Figure 3A and B). Meanwhile, Caski cells with the highest expression level of calgranulin B were chosen to downregulate the expression of calgranulin B by transfecting with calgranulin B siRNA. The expression of calgranulin B protein in the calgranulin B siRNA group was decreased, as compared to that in the control siRNA group (Figure 3C and D).

Results revealed that overexpression of calgranulin B could enhance the proliferation of C33A cells significantly when compared with the Ad-RFP group ($p < 0.05$) (Figure 4A). When compared with the control siRNA group, calgranulin B knockdown inhibited the proliferation of Caski cells ($p < 0.05$) (Figure 4B).

Calgranulin B did not induce apoptosis of cervical cancer cells

There was no statistically significant difference in the apoptosis percentage of C33A cells between Ad-calgranulin B group (1.90 ± 0.56%), Ad-RFP group (2.47 ± 0.49%) and control group (2.35 ± 0.21%) ($p > 0.05$) (Figure 5A and B). In addition, no statistically significant difference was found

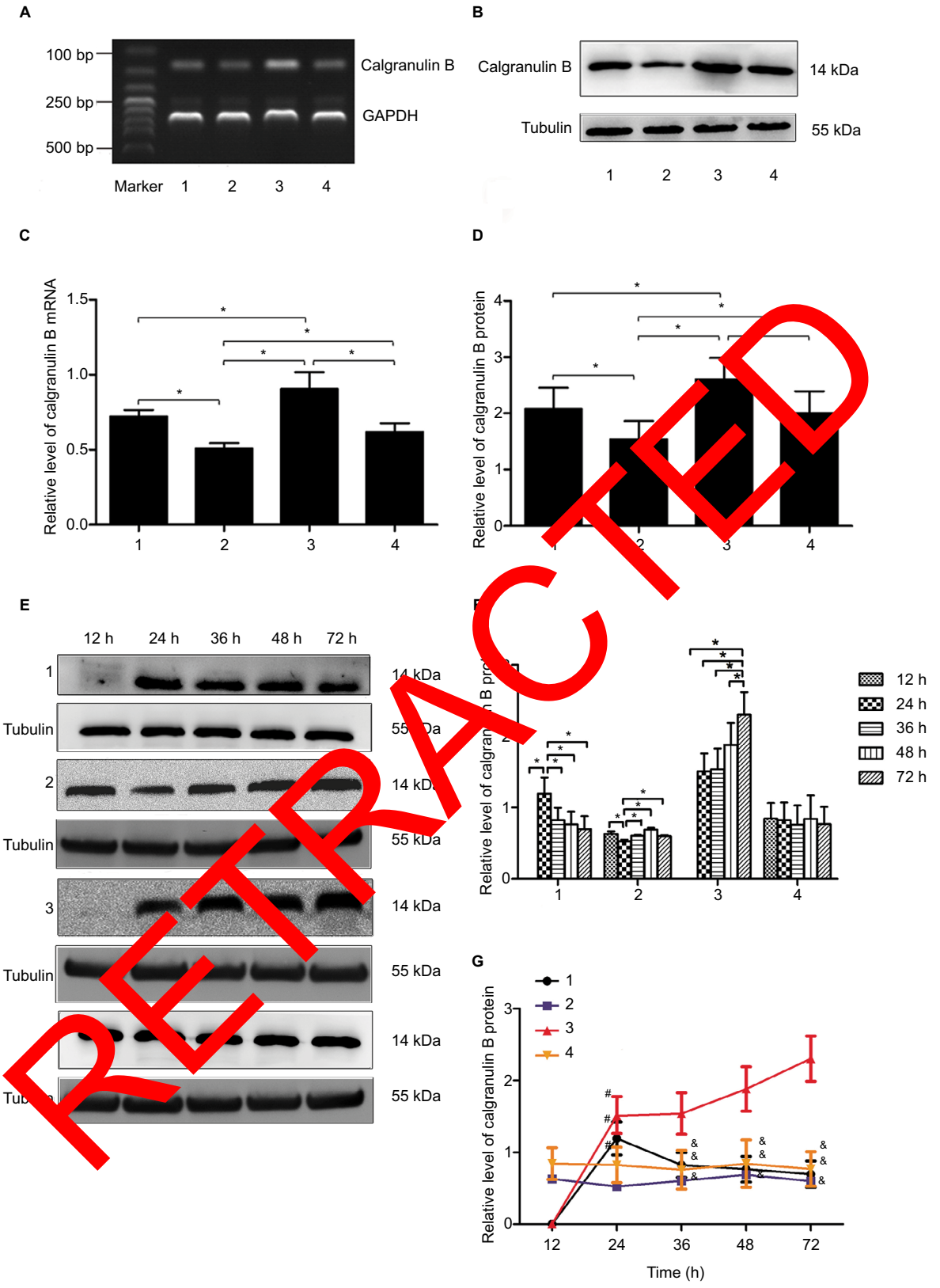

Figure 1 The expression of calgranulin B mRNA and protein in cervical squamous cancer cells. (**A**) The expression of calgranulin B mRNA in four cells was detected by RT-PCR. 1: SiHa; 2: C33A; 3: Caski; 4: MS751. (**B**) The expression of calgranulin B protein in four cells was detected by Western blot. 1: SiHa; 2: C33A; 3: Caski; 4: MS751. (**C** and **D**) Densitometer analysis results for calgranulin B mRNA and protein. Data are expressed as mean ± standard deviation from triplicate experiments (*$p < 0.05$). (**E**) The expression of calgranulin B protein in cervical cancer cells at different culture times. 1: SiHa; 2: C33A; 3: Caski; 4: MS751. (**F** and **G**) The data are presented as relative expression level of calgranulin B protein. Data are expressed as mean ± standard deviation from triplicate experiments (*$p < 0.05$; #$p < 0.05$ vs. C33A; and &$p < 0.05$ vs. Caski).
Abbreviation: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction.

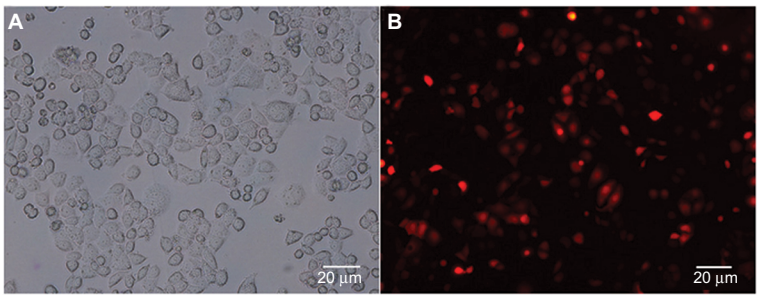

Figure 2 The transduction efficiency of Ad-calgranulin B after transfecting C33A cells for 48 h. (**A**) Image of C33A cells at normal light (×100 magnification). (**B**) Image of C33A cells transduced with calgranulin B recombinant adenovirus at fluorescent. The transduction efficiency of calgranulin B recombinant adenovirus was 54 ± 1.25%. The data are from triplicate experiments.

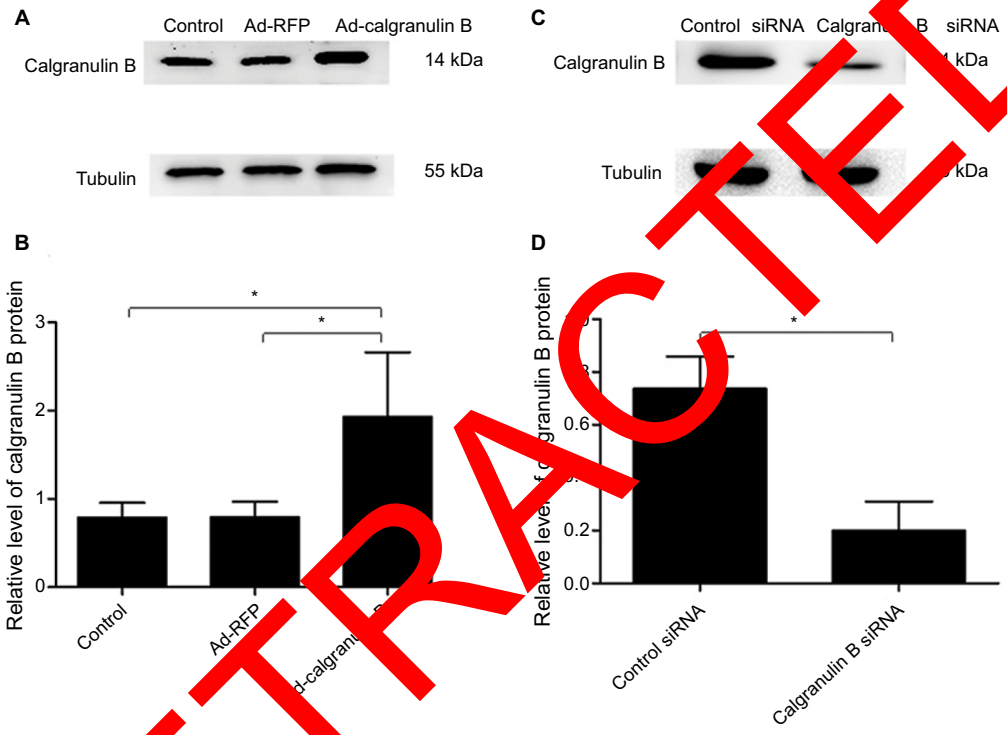

Figure 3 The expression of calgranulin B protein. (**A** and **B**) Calgranulin B protein expression in C33A cells transduced with Ad-calgranulin B was detected by Western blot. Control: naïve control group. (**C** and **D**) Calgranulin B protein expression in Caski cells transfected with calgranulin B siRNA. Data are expressed as mean ± standard deviation from triplicate experiments (*p < 0.05).
Abbreviation: RFP, red fluorescent protein.

between that of Caski cells between calgranulin B siRNA group and control siRNA group (4.40 ± 0.78% vs. 2.97 ± 1.17%, $p > 0.05$) (Figure 5C and D).

Calgranulin B promoted invasion and migration of cervical cancer cells

The role of calgranulin B on cervical cancer cells migration was assessed by determining the number of migrated cells using Transwell migration assay. Migration assay demonstrated that the migrated number of C33A cells in the Ad-calgranulin B group was increased when compared with the Ad-RFP group (70.80 ± 3.70 vs. 46.40 ± 2.70, $p < 0.05$) (Figure 6A). Furthermore, calgranulin B knockdown significantly reduced the migrated number of Caski cells in comparison with the control siRNA group (35.60 ± 6.80 vs. 45.60 ± 3.57, $p < 0.05$) (Figure 6B).

To explore the effect of calgranulin B on the cervical cancer cells invasion, the number of invasive cells was determined via Transwell invasion assay. Overexpression of calgranulin B could significantly increase the invasive number of C33A cells as compared to Ad-RFP group (70.40 ± 3.85 vs. 46.40 ± 2.70, $p < 0.05$) (Figure 6C). Additionally, compared

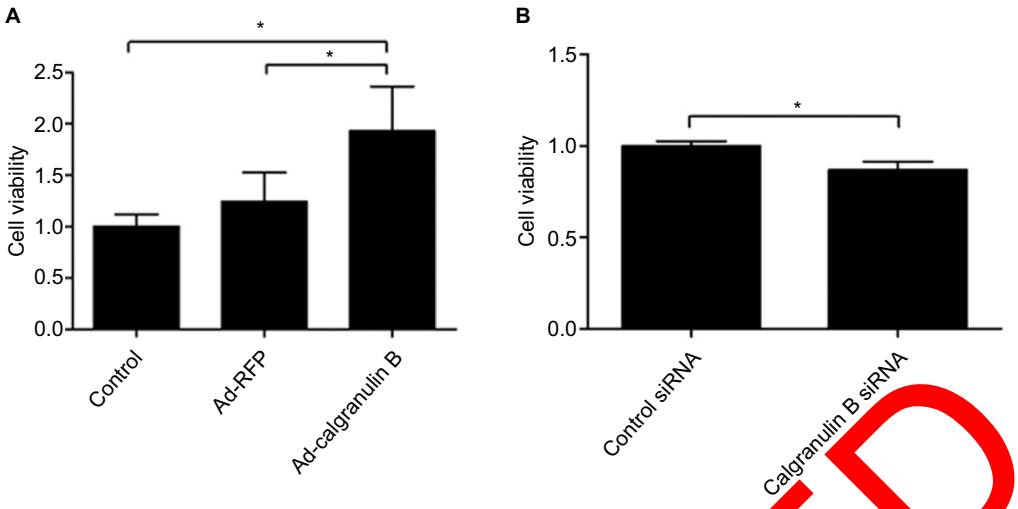

Figure 4 The effect of calgranulin B on the proliferation of cervical cancer cells. (**A**) The effect of Ad-calgranulin B o[n] the proliferation o[f] 3A[...]s evaluated by CCK-8 assay. Control: naïve control group. (**B**) The effect of calgranulin B siRNA on the proliferation of Caski cells. Data a[re] expressed [as] mean ± sta[...]d deviation from triplicate experiments (*$p < 0.05$).
Abbreviations: CCK-8, cell counting kit-8; RFP, red fluorescent protein.

with the control siRNA group, knockdown of calgranulin B markedly reduced the invasive number of Caski cells (55.80 ± 4.81 vs. 40.20 ± 3.27, $p < 0.05$) (Figure 6D).

The relationship between *Calgranulin B* gene and *MMP* genes in cervical cance[r]

As is shown in Table 2, the expression of *calgranulin B* g[ene] was positively correlated with *MMP25* g[enes]. [H]owever, a negative correlation was found betwe[en] the le[v]el of *calgranulin B* gene expression and *MMP15* a[nd] *MMP24* ge[nes].

Calgranulin B promo[te]d [g]rowth o[f] cervical cancer c[e]lls in vivo

To further evaluate t[he] effects of calgran[u]lin B on cervical carcinogenesis in [v]o, we established a xenograft model in BALB/c nude mice, [in] which S[i]a cells that transfected with the sh-calgr[a]nulin [le]ntivirus or ectopic expression lentivi[r]s of calg[a]nulin B were utilized (Figure 7A and D). Compare[d] with control group, tumor growth was inhibited in the sh-calgranulin B group, but was dramatically increased in calg[a]nulin B overexpressed group from day 17 to till sacrifice (Figure 7B). The final tumor volume of the sh-calgranulin B group (1101.1 ± 112.2 mm^3) was smaller than the control group (2243.1 ± 311.2 mm^3) (Figure 7B). Tumor weight in the sh-calgranulin B group was lower than in the control group (0.94 ± 0.23 g vs. 1.91 ± 0.24 g, $p < 0.05$) (Figure 7C). The calgranulin B overexpressed group had a significant increase of tumor volume and weight as compared with the control group (938.58 ± 130.25 mm^3 vs. 451.85 ± 83.26 m[m]^3, $p < 0.05$; 0.89 ± 0.15 g vs. 0.40 ± 0.09 g, $p < 0.05$) (Figure 7E and F).

Discussion

Calgranulin B, with multiple ligands and post-translation modifications, is a calcium and zinc binding protein involved in carcinogenesis and inflammatory events.[19] Researches exploring the effects of calgranulin B proteins on different gynecologic tumors are contradictory. The expression of calgranulin B was shown to be significantly increased in the plasma of stage II ovarian cancer patients in comparison with that in normal subjects.[14] Calgranulin B was overexpressed in breast cancer tissues as compared to its normal counterparts.[12] With regard to cervical cancer, a study of 243 samples included 124 cervical squamous cancer tissues, 33 CIN III, 25 CIN II, 31 CIN I and 30 normal cervical tissues, showing that the positive staining rate for calgranulin B decreased from normal cervical tissue to intraepithelial neoplasia and to squamous cervical cancer gradually.[20] In contrast, *calgranulin B* gene transcription was upregulated in the cervical squamous carcinoma specimens as compared with the surrounding normal tissue counterparts by gene expression profiles in another study.[21] In our previous study, calgranulin B protein expression was observed as gradually increasing from chronic cervicitis to cervical intraepithelial neoplasia and to squamous cervical cancer.[16] When compared with moderately and poorly differentiated tumors, the immunostaining scores of calgranulin B were found to be significantly higher in well-differentiated tumors.

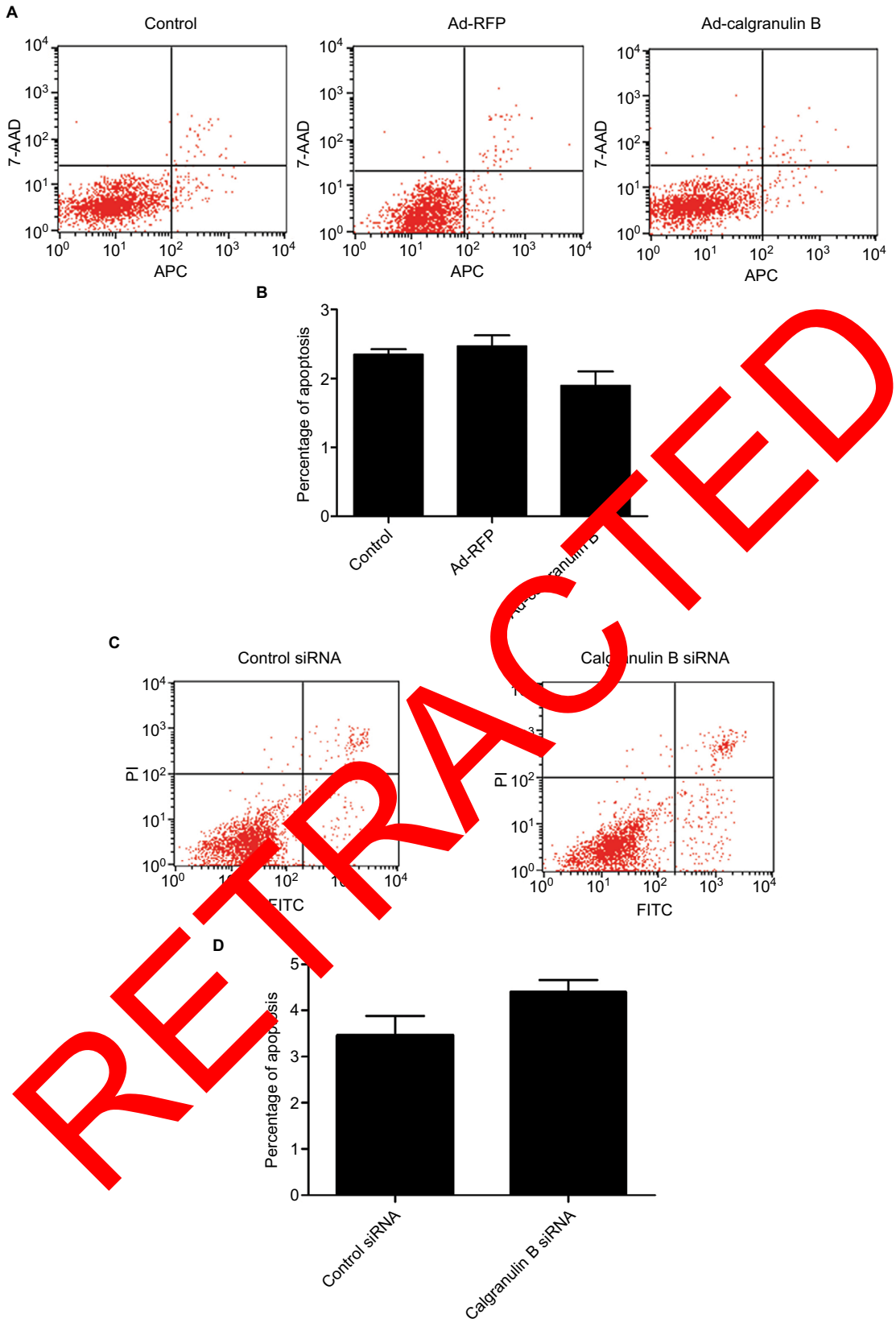

Figure 5 The effect of calgranulin B on the apoptosis of cervical cancer cells. (**A** and **B**) C33A cells were transfected with Ad-calgranulin B. Apoptosis percentage was analyzed by Annexin V-APC/7-AAD staining. Control: naïve control group. (**C** and **D**) Caski cells were transfected with calgranulin B siRNA. Apoptosis percentage was analyzed by Annexin V-FITC/PI staining. Each bar represents mean ± standard deviation of triplicate experiments.
Abbreviations: APC, allophycocyanin; AAD, amino-actinomycin D; FITC, fluorescein isothiocyanate; PI, propidium iodide; RFP, red fluorescent protein.

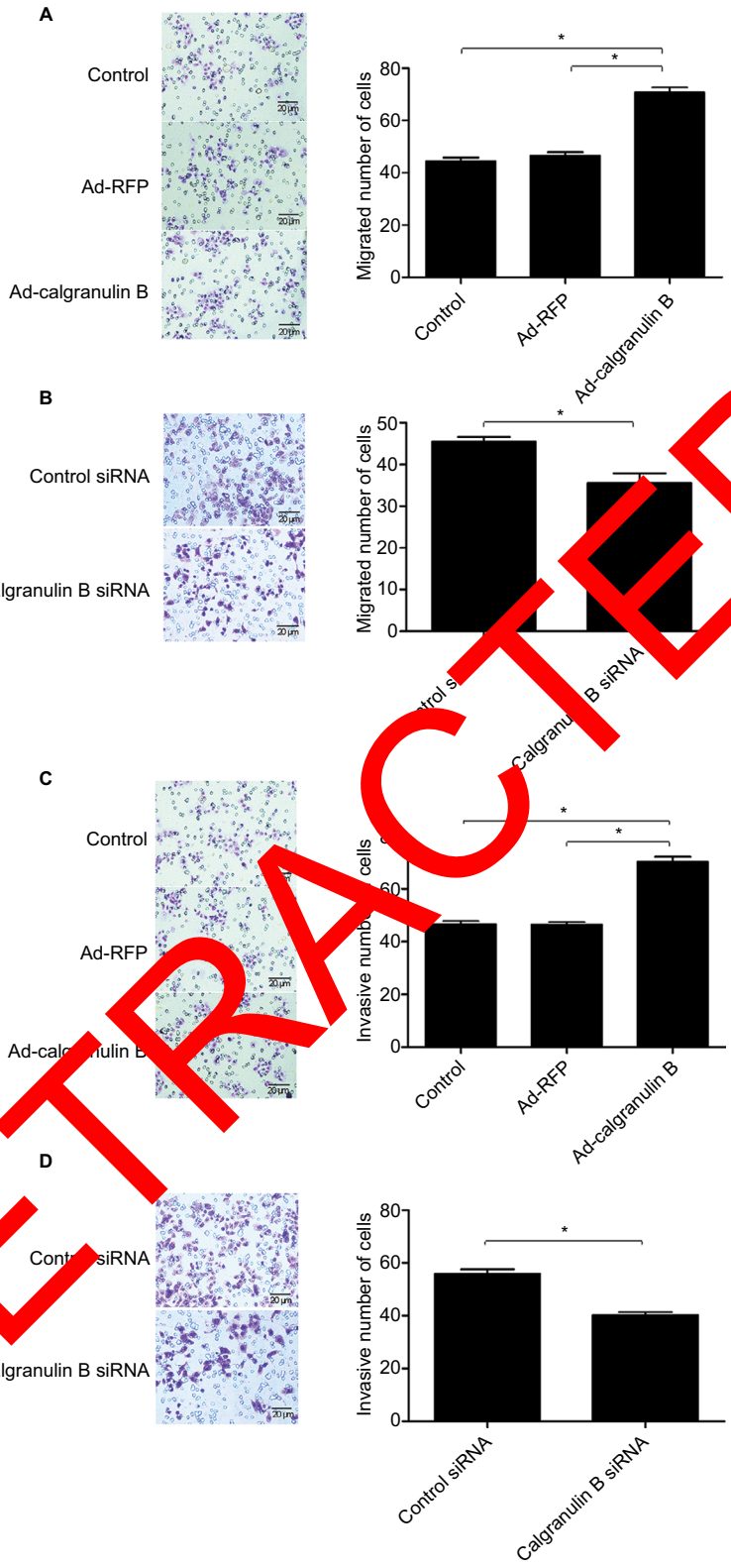

Figure 6 The effect of calgranulin B on cervical cancer cell migration and invasion. (**A**) C33A cells were transfected with Ad-calgranulin B. Migration of C33A cells was analyzed by Transwell migration assay (×200 magnification). Control: naïve control group. (**B**) Caski cells were transfected with calgranulin B siRNA. Migration of Caski cells was analyzed by Transwell migration assay (×200 magnification). Data are presented as mean ± standard deviation of triplicate experiments (*$p < 0.05$). (**C**) The invasion of C33A cells was evaluated by Transwell invasion assay after the cells were transfected with Ad-calgranulin B (×200 magnification). Control: naïve control group. (**D**) Caski cells were transfected with calgranulin B siRNA and the invasion of Caski cells was analyzed by Transwell invasion assay (×200 magnification). Data are presented as mean ± standard deviation from triplicate experiments.

Abbreviation: RFP, red fluorescent protein.

Table 2 The relationship between *calgranulin B* gene and *MMP* genes in cervical cancer

Calgranulin B in cervical cancer	Pearson correlation coefficient	*p*-value[a]
MMP1	–0.03361	0.5561
MMP2	–0.11942	0.0358
MMP3	0.04566	0.4238
MMP7	–0.07216	0.2059
MMP8	0.13982	0.0139
MMP9	0.06639	0.2446
MMP10	–0.04639	0.4165
MMP11	–0.12277	0.0310
MMP12	0.06640	0.2445
MMP13	–0.06264	0.2724
MMP14	0.02692	0.6373
MMP15	–0.23194	0.0000[a]
MMP16	–0.15133	0.0077
MMP17	–0.03884	0.4963
MMP19	–0.00793	0.8895
MMP20	–0.05530	0.3326
MMP21	–0.15977	0.0049
MMP23	–0.02190	0.7014
MMP24	–0.21198	0.0002[a]
MMP25	0.24846	0.0000[a]
MMP26	–0.04377	0.4433
MMP27	0.04706	0.4097
MMP28	0.13098	0.0213

Note: [a]$p < 0.00217$ was considered statistically significantly.
Abbreviation: MMP, matrix metalloproteinase.

In the present study, using the TCGA database,[22] *calgranulin B* gene was found to be upregulated in cervical cancer with positive HPV compared to HPV-negative group. The transcription of *calgranulin B* gene was related to high-risk HPV, especially related to HPV-16 and HPV-45. In addition, we explored the transcription and expression of calgranulin B in four squamous cervical cancer cells (SiHa, Caski, C33A and MS751) by RT-PCR and Western blot analysis. Expression of calgranulin B mRNA and protein was observed to be the lowest in C33A cells and highest in Caski cells among the four cell lines. The expression of calgranulin B gradually increased from high-risk type HPV-negative cells (C33A), HPV-18 or HPV-16 or HPV-45-positive cells (MS751 and SiHa), to both HPV-16 and HPV-18-positive cells (Caski), indicating that calgranulin B expression is related to the type and number of high-risk type HPV in cells. However, the involvement of synergistic effects of calgranulin B and high-risk type HPV on the cervical carcinogenesis or progression needs further study. In addition, calgranulin B protein is mainly expressed in squamous cervical cancer cells, but not in the supernatant. Based on these findings, we used calgranulin B recombinant adenovirus to upregulate the expression of calgranulin B in C33A cells, and calgranulin B siRNA to downregulate calgranulin B expression in Caski cells. As the level of calgranulin B protein in SiHa cells was the medium among the four cell lines, we chose such cell line to upregulate or knockdown the expression of calgranulin B by using lentivirus transduction in vivo study.

Intracellular recombinant expression of calgranulin B suppressed the growth of MCF7 breast cancer cell line.[23] However, emerging studies have shown that calgranulin B acts as a risk factor by enhancing cell proliferation of colorectal carcinoma, osteosarcoma and hepatocellular carcinoma.[18,24,25] Treatment with recombinant calgranulin B proteins promoted the viability of normal cells or colorectal carcinoma partially mediated by the Wnt/β-catenin signaling pathway.[18] It was documented that exogenous calgranulin B induced the proliferation of Hep-2 hepatocellular carcinoma cells in vitro via partially activating the mitogen-activated protein kinase signaling pathway. This effect was also confirmed in vivo by tumorigenicity assays in nude mice.[25] In view of cervical cancer, it was demonstrated that calgranulin B had no effect on the cell viability of SiHa and C33A cells in previous study.[9] By contrast, the present study demonstrated that overexpression of calgranulin B by calgranulin B recombinant adenovirus promoted proliferation of C33A cells, and that knockdown of calgranulin B could suppress the proliferation of Caski cells. These findings indicate that calgranulin B can enhance the proliferation of cervical cancer cells. Using a xenograft model of cervical cancer, we also observed an increase of growth of tumors formed by calgranulin B-overexpressed SiHa cells, and a decrease of tumor growth in animal xenotransplanted with calgranulin B-underexpressed SiHa cells, consistent with our in vitro data. The underlying mechanism responsible for the effects of calgranulin B on the growth of cervical cancer cells remains to be further clarified.

Apoptosis plays a vital role in embryogenesis and cellular homeostasis, and is recognized as a protective mechanism against cancer progression by removing mutated, infected or damaged cells.[26] The development and progression of cancer is related to abnormal proliferation and apoptosis. Calgranulin B could induce apoptosis of acute promyelocytic leukemia cells NB4 cells.[27] It was reported that calgranulin B induced apoptosis in human neuroblastomas cells SHEP by mediating selective release of Omi/HtrA2 and Smac/DIABLO from mitochondria and downregulating the expression of Bcl-2 and Bcl-X_L.[28] It was shown that calprotectin (heterodimer of calgranulin A [S100A8] and B [S100A9]) induces apoptosis of Caski cells, whereas calgranulin B proteins had no apoptotic

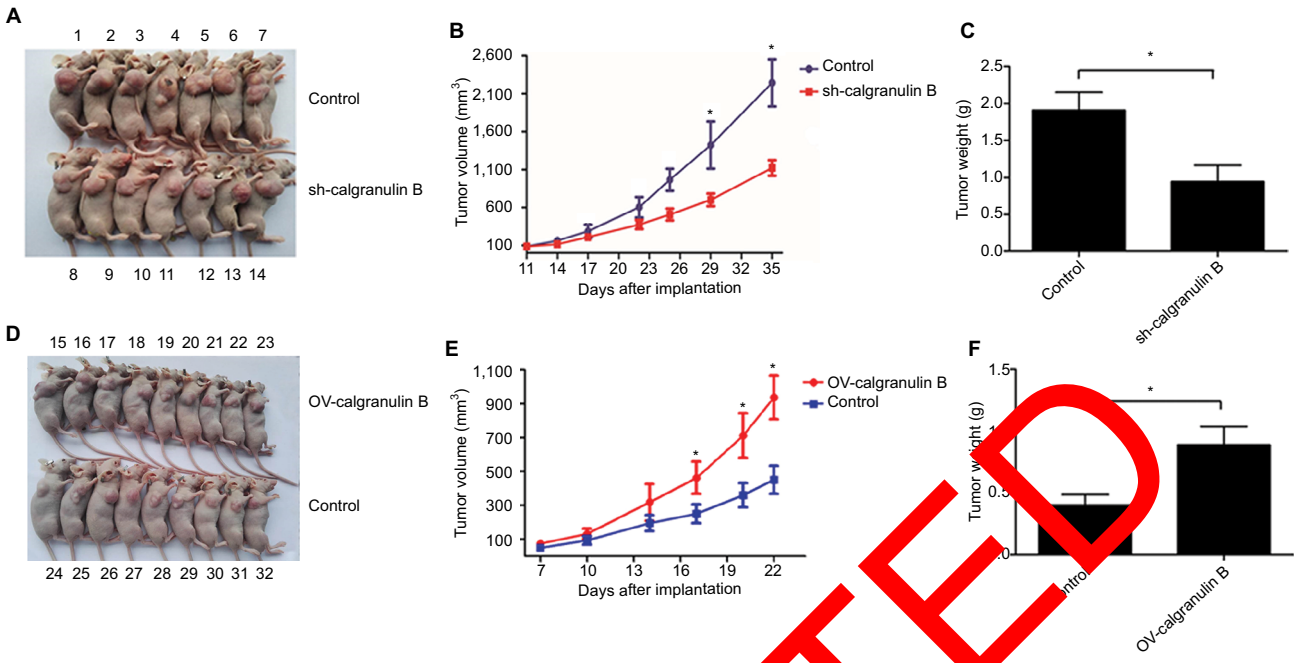

Figure 7 The effect of calgranulin B on growth of cervical cancer in vivo. (**A**) BALB/c nude mice were subcutaneously transplanted with SiHa cells that were transfected with pGFP-C-shcalgranulin B or pGFP-C-shControl. Control group: pGFP-C-sh-Control group; sh-calgranulin B group: pGFP-C-sh-calgranulin B group. The number of mice was labeled in (**A**). (**B** and **C**) The tumor volume was measured every 2–3 days after transplantation and mice were sacrificed 35 days after implantation. The volume of each tumor was calculated as the length × width²/2. Tumor weight was calculated after mice were sacrificed. Control group: pGFP-C-sh-Control group; sh-calgranulin B group: pGFP-C-sh-calgranulin B group. Data are presented as mean ± standard deviation. *$p < 0.05$ mean control group differs from sh-calgranulin B group. (**D–F**) Nude mice were transplanted with SiHa cells that were transfected with pLVX-IRES-ZsGreen calgranulin B or pLVX-IRES-ZsGreen1-control. The number of mice was labeled in (**D**). Control group: pLVX-IRES-ZsGreen1-control group; OV-calgranulin B group: pLVX-IRES-ZsGreen-calgranulin B group. The tumor volume was calculated every 2–3 days after transplantation. Tumor weight was measured after mice were sacrificed. Data are shown as mean ± standard deviation. *$p < 0.05$ differs from control group.
Abbreviation: GFP, green fluorescent protein.

effect on the Caski cells.[29] Consistently, calgranulin B was also found not to have any effect on the apoptosis of C33A or Caski cells in the present study.

Metastasis is a multistep process and the process of invasion is characterized by the interaction with matrix involving the adhesion, decomposition of the matrix components and migration of cancer cells.[30] Calgranulin B was documented to inhibit the invasion of gastric cancer cells.[17] However, most researches have shown that calgranulin B can promote the invasion and migration of cancers, for example, colorectal cancer, hepatocellular carcinoma and gastric cancer, etc.[18,25,31] It was shown that exogenous calgranulin B enhanced migration and invasion of hepatocellular carcinoma cell via the activating the mitogen-activated protein kinase pathway.[25]

In the view of gynecological cancer, endogenous calgranulin B suppressed breast cancer cell invasion and metastasis through modulating the promalignant focal adhesion kinase-1 signaling cascade activity.[23] Calprotectin was reported to inhibit migration of Caski cells, but there was no difference in cell migration between the control group and calgranulin B group.[29] Knocking down calgranulin B also significantly promoted cell invasion of C33A cells, but had no effect on the invasion of SiHa cells.[20] By contrast, we observed that calgranulin B promoted invasion and migration of Caski and C33A cells. Calgranulin B has been reported to exert contradictory effect on breast cancer cells: extracellular calgranulin B induced cells proliferation, whereas the intracellular one suppressed cells growth.[23] In view of such observation, as the calgranulin B investigated in the previous study was exogenous,[29] it is plausible that the endogenous calgranulin B of the present study could exert a different, or even opposite effect, on cervical cancer cells. In the present study, we chose Caski cells to knock down calgranulin B, whereas the previous study downregulated the expression of calgranulin B in C33A cells.[20] Caski and C33A cells were different in the type and number of high-risk type HPV, suggesting that calgranulin B may exert differential effects depending on cell types. The present study showed that the level of calgranulin B protein expression was the least in C33A cells and the highest in Caski, and in view of such, we overexpressed the expression of calgranulin B in C33A cells. Contrarily, the previous one knocked down calgranulin B in C33A cells,[20] which would result in an extremely low level of target protein. Depending on the respective native expressions

of protein within different cells, artificially manipulating the protein level (overexpress or underexpress protein) could produce distinct changes in cell biological behaviors. It was documented that calprotectin promoted gastric cancer cell migration and invasion through upregulation of MMP2 and MMP12.[31] Calgranulin B was demonstrated to increase the expression of MMP7, enhancing the migration of CNE1 nasopharyngeal carcinoma cells.[32] However, in the present study, the transcription of *calgranulin B* gene was found to be negatively correlated with *MMP15* and *MMP24* genes in cervical cancer. Furthermore, a positive relation was found between *calgranulin B* gene and *MMP25* genes, indicating that calgranulin B-induced migration and invasion of cervical cancer cells may be involved in the regulation of *MMPs*, except *MMP2*. When compared with calgranulin B, calprotectin has different effects on the metastasis of Caski cells.[29] However, the underlying mechanism responsible for the different role in the metastasis of cervical cancer cells warrants to be further studied.

Conclusion

Calgranulin B expression in cervical cancer was significantly related to high-risk HPV-16 and HPV-45. In addition, overexpression of calgranulin B promoted the proliferation, invasion and migration of C33A cells. Calgranulin B knockdown inhibited the proliferation, invasion and migration of Caski cells. *Calgranulin B* gene was demonstrated to be significantly and positively correlated with *MMP25* gene but negatively correlated with *MMP15* and *MMP24* genes in cervical cancer. The in vivo study has provided the evidence that calgranulin B can promote the growth of SiHa cervical cancer xenografts. Taken together, our findings suggest that calgranulin B can promote the cervical cancer cell proliferation confirmed by *in* vivo and in vitro studies invasion and migration, probably through regulating the expression of MMPs. However, the molecular mechanism responsible for the role of calgranulin B in such malignancy and the potential of synergistic actions between calgranulin B and HPV-16/HPV-45 on squamous cervical carcinogenesis or progression need further investigation. An in vivo study focused on the role of calgranulin B in the metastasis of cervical cancer should be further conducted.

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Disclosure

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References

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin.* 2015;65(2):87–108.
2. Segovia-Mendoza M, Jurado R, Mir R, M... Prado-Garcia H, Garcia-Lopez P. Antihormonal agents as strategy to improve the effect of chemo-radiation in cervical cancer in vitro and in vivo study. *BMC Cancer.* 2015;15:21.
3. Delvenne P, Herman L, Khol... N, et al. Role of hormone cofactors in the human papillomavirus-induced carcinogenesis of the uterine cervix. *Mol Cell Endocrinol.* 20...;264(1–2):...–5.
4. Matos A, Moutinho J, Pint... D, Medeiros R. The influence of smoking and other cofactors on the time to onset to cervical cancer in a southern European pop...on. *Eur J Cancer Prev.* 2005;14(5):485–491.
5. Zimmer DB, Wrig... Sadosky P, W...er DJ. Molecular mechanisms of S100-target protein interactions. *Microsc Res Tech.* 2003;60(6):552–...
6. M...enholz I, Heizmann C W, Fritz G. S100 proteins in mouse and man: from evolution to function and pathology (including update of the nomenclature). *Biochem Biophys Res Commun.* 2...4;322(4):111...1122.
7. Schä... BW, ...cki R, Engelkamp D, Mattei MG, Heizmann CW. Isolation of a YAC clone covering a cluster of nine S100 genes on human chromosome 1q21: rationale for a new nomenclature of the S100 calcium-binding protein family. *Genomics.* 1995;25(3):638–643.
8. Ji J, Zhao L, Wang X, et al. Differential expression of S100 gene family in human esophageal squamous cell carcinoma. *J Cancer Res Clin Oncol.* 2004;130(8):480–486.
9. He QY, Chen J, Kung HF, Yuen AP, Chiu JF. Identification of tumor-associated proteins in oral tongue squamous cell carcinoma by proteomics. *Proteomics.* 2004;4(1):271–278.
10. Ito Y, Arai K, Nozawa R, et al. S100A8 and S100A9 expression is a crucial factor for dedifferentiation in thyroid carcinoma. *Anticancer Res.* 2009;29(10):4157–4161.
11. Su YJ, Xu F, Yu JP, Yue DS, Ren XB, Wang CL. Up-regulation of the expression of S100A8 and S100A9 in lung adenocarcinoma and its correlation with inflammation and other clinical features. *Chin Med J (Engl).* 2010;123(16):2215–2220.
12. Cross SS, Hamdy FC, Deloulme JC, Rehman I. Expression of S100 proteins in normal human tissues and common cancers using tissue microarrays: S100A6, S100A8, S100A9 and S100A11 are all overexpressed in common cancers. *Histopathology.* 2005;46(3):256–269.
13. Kim WJ, Kim SK, Jeong P, et al. A four-gene signature predicts disease progression in muscle invasive bladder cancer. *Mol Med.* 2011;17(5–6):478–485.
14. Shield-Artin KL, Bailey MJ, Oliva K, et al. Identification of ovarian cancer-associated proteins in symptomatic women: a novel method for semi-quantitative plasma proteomics. *Proteomics Clin Appl.* 2012;6(3–4):170–181.
15. Zhu X, Lv J, Yu L, et al. Proteomic identification of differentially-expressed proteins in squamous cervical cancer. *Gynecol Oncol.* 2009;112(1):248–256.
16. Zhu X, Jin L, Zou S, et al. Immunohistochemical expression of RAGE and its ligand (S100A9) in cervical lesions. *Cell Biochem Biophys.* 2013;66(3):843–850.

17. Fan B, Zhang LH, Jia YN, et al. Presence of S100A9-positive inflammatory cells in cancer tissues correlates with an early stage cancer and a better prognosis in patients with gastric cancer. *BMC Cancer.* 2012;12:316.
18. Duan L, Wu R, Ye L, et al. S100A8 and S100A9 are associated with colorectal carcinoma progression and contribute to colorectal carcinoma cell survival and migration via Wnt/β-catenin pathway. *PLoS One.* 2013;8(4):e62092.
19. Markowitz J, Carson WE 3rd. Review of S100A9 biology and its role in cancer. *Biochim Biophys Acta.* 2013;1835(1):100–109.
20. Zhu H, Wu TC, Chen WQ, et al. Roles of galectin-7 and S100A9 in cervical squamous carcinoma: clinicopathological and in vitro evidence. *Int J Cancer.* 2013;132(5):1051–1059.
21. Chao A, Wang TH, Lee YS, et al. Molecular characterization of adenocarcinoma and squamous carcinoma of the uterine cervix using microarray analysis of gene expression. *Int J Cancer.* 2006;119(1):91–98.
22. Cerami E, Gao J, Dogrusoz U, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov.* 2012;2(5):401–404.
23. Cormier K, Harquail J, Ouellette RJ, Tessier PA, Guerrette R, Robichaud GA. Intracellular expression of inflammatory proteins S100A8 and S100A9 leads to epithelial-mesenchymal transition and attenuated aggressivity of breast cancer cells. *Anticancer Agents Med Chem.* 2014;14(1):35–45.
24. Cheng S, Zhang X, Huang N, Qiu Q, Jin Y, Jiang D. Down-regulation of S100A9 inhibits osteosarcoma cell growth through inactivating MAPK and NF-κB signaling pathways. *BMC Cancer.* 2016;16:253.
25. Wu R, Duan L, Ye L, et al. S100A9 promotes the proliferation and invasion of HepG2 hepatocellular carcinoma cells via the activation of the MAPK signaling pathway. *Int J Oncol.* 2013;42(3):1001–1010.
26. Lowe SW, Lin AW. Apoptosis in cancer. *Carcinogenesis.* 2000;21(3):485–495.
27. Zhu Y, Zhang F, Zhang S, et al. Regulatory mechanism and functional analysis of S100A9 in acute promyelocytic leukemia cells. *Front Med.* 2017;11(1):87–96.
28. Ghavami S, Kerkhoff C, Chazin WJ, et al. S100A8/9 induces cell death via a novel, RAGE-independent pathway that involves selective release of Smac/DIABLO and Omi/HtrA2. *Biochim Biophys Acta.* 2008;1783(2):297–311.
29. Qin F, Song Y, Li Z, Zhao L, Zhang Y, Geng L. S100A8/A9 induces apoptosis and inhibits metastasis of Cas[illegible]man cervical cancer cells. *Pathol Oncol Res.* 2010;16(3):353–[illegible].
30. Aznavoorian S, Murphy AN, [illegible]tler-Stevens[illegible]WG, Liotta LA. Molecular aspects of tumor cell invasion and metastasis. *Cancer.* 1993;71(4):1368–1383.
31. Kwon CH, Moon HJ, Park HJ, Choi JH, [illegible]k DY, [illegible]00A8 and S100A9 promotes invasion and migration through p[illegible]togen-activated protein kinase-dependent NF-κB activation in gastric cancer cells. *Mol Cells.* 2013;35(3):22[illegible]234.
32. Yan LL, Hu[illegible]g YJ, Yi X, et al. Effect of silencing S100A8 and S100A9 with small interfering RNA on the migration of CNE1 nasopharyngeal carcinoma cells. *Oncol Lett.* 2015;9(6):2534–2540.

Supplementary material

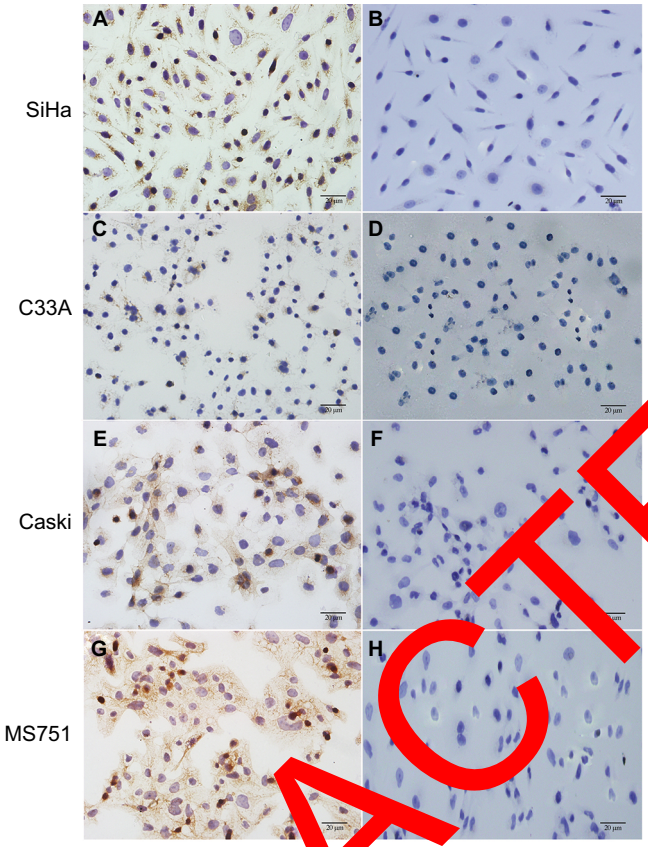

Figure S1 Immunocytochemical staining of four cervical cancer cell lines for the expression of calgranulin B protein. (**A**) Positive staining of calgranulin B protein in SiHa cells (SP staining, ×400). (**B**) Negative control in SiHa cells. (**C**) Positive staining of calgranulin B protein in C33A cells. (**D**) Negative control in C33A cells. (**E**) Positive staining of calgranulin B protein in Caski cells. (**F**) Negative control in Caski cells. (**G**) Positive staining of calgranulin B protein in MS751 cells. (**H**) Negative control in MS751 cells.
Abbreviation: SP, streptavidin peroxidase.