STEAP2, as an oncogene, enhanced the invasion ability of cervical caner cells and promoted EMT through PI3K/AKT/mTOR pathway

Mei Wang
Shandong Provincial Hospital Affiliated to Shandong First Medical University
Na Li
Shandong Provincial Hospital Affiliated to Shandong First Medical University
Chunrun Yang (ycr911@163.com)
Shandong Provincial Hospital Affiliated to Shandong First Medical University
Fei Wang
Shandong Provincial Hospital Affiliated to Shandong First Medical University

Research Article

Keywords: Cervical cancer, EMT, Invasiveness, Proliferation, PI3K signal transduction pathway, STEAP2

Posted Date: June 15th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1732498/v1

Abstract

Despite the established pivotal role of STEAP2 (six-transmembrane epithelial antigen of prostate 2) in the progression of prostate cancer, its prognostic value and functionality in cervical carcinoma have not been comprehensively analyzed. The STEAP2 expressions in healthy and cancerous cervical tissues, as well as in five varying cervical carcinoma cells (Ca Ski, HeLa, SiHa, C33A and HT-3) were checked by immunohistochemistry and western blotting. The association of STEAP2 with the outcome was explored by the Kaplan-Meier method among the cervical carcinoma population. Through the in-vivo and in-vitro lentiviral transfection and cellular functionality assays, we examined how the STEAP2 elevation and decline influenced the biological properties of cervical carcinoma cells. Furthermore, the effects of STEAP2 knockdown or over-expression on the crucial EMT (epithelial–to–mesenchymal transition) hallmarks and matrix metalloproteinases (MMPs) were also determined by real time RT-qPCR in conjunction with western-blot. As demonstrated the results, high STEAP2 level in cancerous cervical tissues was associated positively with the clinico-pathological traits of malignant cervical cancer and the poor prognosis of patients. Through the PI3K/AKT/mTOR axis deactivation, the STEAP2 knockdown could significantly inhibit cellular proliferation and invasion abilities, reduce the MMP-1 plus MMP-13 levels, and impede the EMT event. Meanwhile, STEAP2 over-expression had the opposite effects. All in all, STEAP2, as an oncogene, enhanced the invasion ability of cervical caner cells and promoted EMT through PI3K/AKT/mTOR axis, which thus probably acts as a therapeutic target for suppressing the cervical carcinoma cell invasion.

Introduction

Despite advances in the prevention, detection and treatment of cervical cancer (CC) diseases over the past decade, CC still remains the 4th most frequently occurring female malignancy globally,[1] with a 6.6% prevalence in 2018.[2] It was estimated that there were 570,000 diagnoses and 311,000 deaths worldwide in 2018.[3] Chemotherapy and radiotherapy are recognized as the main interventions for controlling disease progression in patients with advanced CC, but at this time, there are severer adverse effects, with a five-year survival of below 20%.[4] Gene-based targeted therapy as a less-invasive promising approach of cancer treatment has been reported with encouraging preclinical results, but at present these therapies have not yet shown significant clinical benefits.[5] The possible reason is insufficient insights into the proliferative and metastatic mechanisms of cancer at the molecular level. Hence, it is very important to study the occurrence, development and invasion and metastasis mechanism of cervical cancer biological behavior with the purpose of finding new treatment strategies and improve prognosis.

STEAP (six-transmembrane epithelial antigen of the prostate) was cloned for the first time by Hubert RS et al. in 1999, which encoded a 339-amino acid protein comprising 6 potential transmembrane regions, with hydrophilic carboxyl- and amino-terminal domains at two sides.[6] The STEAP family in humans is constituted by 5 (at least) homologous members, i.e. STEAP1-4 and STEAP1b. Their similar structures indicated their potential function as channels or transporters, which were involved in a broad spectrum of

biological events, including the endocytotic and exocytotic transport of molecules, and regulation of cellular multiplication and apoptosis.[7, 8] Diverse stimuli are responsible for modulating their expressions, such as hormones and cytokines, whose role in cellular secretion and differentiation is crucial, therefore, they are associated with inflammatory and metabolic conditions and carcinomas. [9, 10] With location on chromosomal 7q21.13 in the STEAP1 and STEAP4 vicinities, the STEAP2 gene, called STAMP1 (six-transmembrane protein of the prostate 1), encodes a 2.2 kb mRNA and a 490 amino acids protein.[11, 12] Like STEAP1, expression of STEAP2 was also high in prostatic carcinomas,[13] which could drive aggressive characterization of prostatic carcinoma cells through the multiplication, migration and infiltration facillitation.[14] As indicated by the differential expression of STEAP1 and STEAP2 in healthy and cancerous tissues, as well as their assignment in cellular membrane, they probably exert crucial effects on the carcinogenesis and progression. Currently, STEAP1 has been extensively explored as an encouraging alternative therapeutic target,[15] however, for STEAP2, studies are just beginning. In cervical cancer, the STEAP2 expression in cancer tissue and cells, and the direct molecular mechanism underlying cancer progressions have poorly been understood.

Materials And Methods

Tissue Specimens

With the patient's informed consent, from 2008 and 2018, 388 samples of human cervical tissues were gathered from the Gynecology and Obstetrics Department, Shandong First Medical University Affiliated Provincial Hospital, including 248 cervical cancer, 80 CIN (cervical intraepithelial neoplasia), as well as 60 healthy cervical tissues. All cervical cancer patients were diagnosed as per the modified FIGO (International Federation of Gynecology and Obstetrics) staging system. The present work was approved by the Shandong First Medical University’s Medical Ethics Committee, and all procedures were implemented following the relevant regulations and guidelines.

Cell Lines

Human cervical carcinoma (Ca Ski, C-33A, HT-3, HeLa and SiHa) cells were acquired from the CAS Shanghai Institute for Biological Sciences. Ca Ski, HeLa and SiHa cells were cultured with complete cell culture RPMI-1640 (Gibco) involving 10% FBS (fetal bovine serum; Gibco) plus 1% penicillin-streptomycin solution. The HT-3 cells were cultivated using McCOY's 5A medium (Gibco) involving 10% FBS plus 1% penicillin-streptomycin, while cultivation of the C-33A cells was accomplished with minimum Eagle's medium (MEM, Gibco) also involving 10% FBS plus 1% penicillin-streptomycin. The foregoing cells were all cultivated in a 37°C incubator including 5% CO_2 and subcultured with 0.25% trypsin (Gibco) digestion.

Immunohistochemistry (Ihc) And Immunocytochemistry (Icc)

A standard SP (streptavidin-biotin-peroxidase) staining procedure was adopted for IHC and ICC, with which the STEAP2 expressions in human cervical tissues and carcinoma cells were examined. Sequential baking, deparaffinization and PBS immersion were accomplished on the paraffin-embedded tissue sections, and finally antigen retrieval was carried out by high pressure 2 min in antigen repair solution. The prepared cell coverslips were subjected to a 30-min fixation in paraformaldehyde (4%) and subsequently washed using PBS. After goat serum closed, tissue sections and cell coverslips were subjected to an overnight incubation using polyclonal rabbit anti-human STEAP2 antibody (1:100 dilution, PA5-25495, Invitrogen) under a 4°C condition. The specific steps were referred to the instruction of SP staining kit (ZSGB-BIO, China). The paraffin-embedded human prostate carcinoma section (STEAP2 positive) served as the positive control, while the negative control was prepared by substituting PBS for the primary antibody. Every tissue section/cellular coverslip was prepared in triplicates.

The Analysis Of Ihc And Icc

Given the location of STEAP2 on the cellular membrane, the cytoplasmic and membrane brown granules were considered positive. The expression intensity of STEAP2 was evaluated according to a semi-quantitative system of scoring created by Soumaoro et al.[16] The total score of expression was a sum of staining intensity and positive cell proportion scores. Rating of staining intensity was accomplished under 4 grades: total negative scored 0; light yellow slightly higher than background scored 1; brown yellow obviously higher than background scored 2; brown meaning strongly positive scored 3. Furthermore, the positive cells were divided into 5 grades: according to the number of positive cells 0%, 0%-10%, 10%-25%, 25%-75% and ≥ 75%, the score 0, 1, 2, 3 and 4 was recorded in turn. The expression was considered low when the overall score lay between 0–3, whereas high when the score range was 4–7. Each tissue section or cell coverslip was evaluated by two pathologists independently. Any inconsistency was resolved by consulting a third pathologist.

Quantitative Real-time Polymerase Chain Reaction (Qrt-pcr)

After collecting of log-growth cells, sampling of total RNA proceeded by the trizol- chloroform-isopropanol method, followed by reverse transcription to cDNA (complementary DNA) via the RT reagent Kit (TaKaRa Bio, China). Furthermore, preparation of the reaction system was accomplished as per the guidelines of SYBR Primescrip real time PCR kit (TaKaRa), containing 10 μl of Power SYBR Green PCR master mix, 7.2 μl of DNAse/RNAse-free water, 2 μl of cDNA, and each 0.4 μl of upstream and downstream primers. PCR amplification was implemented in accordance with kit instructions for comparing the relative STEAP2 mRNA levels in five cervical carcinoma cells against the β-actin internal reference. The results were analyzed by the convenient $2^{-\Delta\Delta Ct}$ method.[17] Primers design and synthesis were undertaken by TaKaRa,

the specific forward or reverse sequences were shown in Table 1. qRT-PCR experiments were repeated 3 times.

Table 1
The sequence of primer in real time RT-qPCR

Primer name	Sequences
STEAP2	F:5’-CGCTATGGTCCATGTTGCCTA-3’
	R:5’-CCAAGGCTCATTATGCCAAAG-3’
CDH1	F:5’-GGATTGCAAATTCCTGCCATTC-3’
	R:5’-AACGTTGTCCCGGGTGTCA-3’
CDH2	F:5’-CGAATGGATGAAAGACCCATCC-3’
	R:5’-GCCACTGCCTTCATAGTCAAACACT-3’
VIM	F:5’-AACCTGGCCGAGGACATCA-3’
	R:5’-TCAAGGTCAAGACGTGCCAGA-3’
SNAIL	F:5’-GCTCCCTCTTCCTCTCCATACC-3’
	R: 5’-AAGTCCTGTGGGGCTGATGT-3’
SLUG	F: 5’-GAAGCATTTCAACGCCTCCAA-3’
	R: 5’-GTTGTGGTATGACAGGCATGGAGTA-3’
TWIST	F: 5’-CAGCTACGCCTTCTCGGTCT-3’
	R: 5’-CTGTCCATTTTCTCCTTCTCTGG-3’
ZEB2	F: 5’-AAATGCACAGAGTGTGGCAAGG-3’
	R: 5’-CTGCTGATGTGCGAACTGTAGGA-3’
MMP1	F: 5’-CACAAACCCCAAAAGCGTGT-3’
	R: 5’-TCGGCAAATTCGTAAGCAGC-3’
MMP8	F: 5’-TGCTATCACCACACTCCGTG-3’
	R: 5’-ATACCAGTTGGAAGGGATGGC-3’
MMP13	F: 5’-TCCTGGGCCAAATTATGGAG-3’
	R: 5’-GGGTCCTTGGAGTGGTCAAGA-3’
MMP2	F: 5’-CTCATCGCAGATGCCTGGAA-3’
	R: 5’-TTCAGGTAATAGGCACCCTTGAAGA-3’
MMP9	F: 5’-ACGCACGACGTCTTCCAGTA-3’
	R: 5’-CCACCTGGTTCAACTCACTCC-3’

Primer name	Sequences
ACTB	F: 5’-TGGCACCCAGCACAATGAA-3’
	R: 5’-CTAAGTCATAGTCCGCCTAGAAGCA-3’

Western Blotting

Logarithmic growth cells were collected and added with appropriate amount of protein lysate (RIPA:PMSF = 100:1), after centrifugation, extraction of total protein proceeded. The sample amount of protein in each lane was 40μg, SDS-PAGE (10% gel) was performed for isolation of proteins with different molecular weights, and then the electrophoretically isolated proteins were shifted onto PVDF (polyvinylidene difluoride) membranes. Next step was placement of the membranes in a shaker and a 1-h blockage using 5% non-fat milk. After thrice washing in TBST (tris-buffered saline containing Tween 20), an overnight incubation was accomplished using primary antibodies (1:1000 diluents) at 4°C, followed by a further 1-h incubation at ambient temperature using corresponding secondary antibodies on the next day. The enhanced chemiluminescence (ECL) working solution (Solarbio) was used to develop the target protein blot, whose gray value was then examined with the aid of ImageJ program.

Lentiviral Transfection For Steap2 Up-regulation Or Down-regulation

Vector construction and virus packaging for STEAP2 up-/down-regulation were accomplished by the Shanghai Genechem. For construction of the lentiviral over-expression vector of STEAP2, the Ubi-MCS-3FLAG-CBh-gcGFP-IRES-puromycin vector was recombined with STEAP2 (NM_001040665). Meanwhile, the lentiviral knockdown vector of STEAP2 was constructed by recombining the hU6-MCS-CBh-gcGFP-IRES-puromycin vector with the STEAP2 cloning small hairpin RNAs (shRNAs). The cervical cancer cell line Ca Ski was selected for RNA interference (RNAi) to down-regulate the expression of STEAP2, another cancer cell line HT-3 was selected for cDNA transfection to achieve the STEAP2 up-regulation. Negative controls of lentiviral transfections were also designed. Logarithmic phase cells were gathered, seeded onto the 24-well microplate and subjected to a 24-h cultivation in a 37°C incubator including 5% CO_2. According to the pre-test results, the MOI (multiplicity of infection value; quantitative ratio of viruses to cells) was assigned as 50 for RNAi, whereas as 100 for over-expression. To reduce cytotoxicity, complete fresh medium was replenished 12 h later to substitute for the viral mixture. For the transfection efficiency assessment, a fluorescence microscope was utilized to observe the green fluorescence following 72 h of transfection, while for the expression evaluations of STEAP2 mRNA and protein in STEAP2 RNAi, over-expression and negative control groups, the qRT-PCR in conjunction with Western-blot were employed to validate the transfection performance.

Growth Curve Assay

Following digestion and centrifugation, the logarithmic phase cells were quantified, and subsequently each 1×10^4 cells were seeded per well of the 24-well microplate. From the next day, the mean cell counts in 3 wells were regarded as the cell quantity in that day, and the growth curve experiment lasted for one week. Curve plotting was accomplished by taking the cultivation duration as the horizontal axis, and the cell counts derived from daily statistics as the longitudinal axis.

Detection Of Cell Cycle By Flow Cytometry

The logarithmic growth cells were gathered, washed thrice in pre-cooled PBS, and then subjected to overnight fixation at − 20°C in 75% ethanol. A 30-min treatment of the fixed cells proceeded using RNase at 37°C, followed by a 30-min PI (propidium iodide) staining under ambient temperature and dark conditions. Finally, the cellular cycle was assessed with a Muse Cell Analyzer (Merck-Millipore, US).

Cell Plate Cloning Formation Assay

After gathering and PBS washing, each 500 logarithmic growth cells were seeded per well of a 6-well microplate, and subsequently incubated for 10 days, during which cell growth was observed. Ten days later, residual cells and the medium were rinsed in PBS, and then the cells were subjected to a 20-min fixation in inparaformaldehyde (4%) reagent until full coverage, and further subjected to a 10-min staining using hematoxylin dye, followed by removal of the dye solution. Thereafter, the 6-well microplate was continuously washed for about 5 min under the slow water flow of tap water and dried at room temperature. Three repeat wells were established for each group of cells and the experiment was triplicated.

Transwell Invasion And Migration Assay

The transwell chamber is divided into two parts: upper and lower by the microporous membrane 8.0 μm in pore dimension, when chemokines act, the upper chamber cells can penetrate into the lower chamber. Counting the cells crossing the microporous membrane can compare the migration ability of different cells. At ambient temperature, BD Matrigel can be polymerized to form a 3D matrix with biological activity, simulating the basal membrane in terms of composition, architecture, functionality and physical properties. In transwell invasion assay, Matrigel was diluted with serum-free cold medium at 1:5 ratio, 50 μl mixture were taken out and covered evenly and carefully on the microporous membrane, and then subjected to a 1-h incubation at 37℃ until thorough solidification of the Matrigel matrix glue. Serum-free medium was utilized to adjust the density of gathered logarithmic cells to 1×10^6 per ml, 200 μl of which was dispensed to the upper chamber. At the same time, the lower chamber was dispensed with 600 μl of 20% serum-containing media as Chemokines. After culturing for 12 h at 37℃ and 5% CO_2, erasing

residual Metrigel and not penetrated upper chamber cells and immersing the chamber for 30 min within paraformaldehyde (4%), the penetrated cells at the bottom of the membrane was fixed and stained by hematoxylin dye. Under microscopy, 5 visual fields were stochastically picked to quantify the cells crossing the membrane and calculate the average value. The experimentation was triplicated. The operational steps of transwell migration assay were fully identical to the invasion experiment in addition to the absence of Matrigel.

Nude Mice Orthotopic Transplantation Tumor Model In Vivo

Female 4–6 weeks of age BALB/C/nu/nu mice (body masses: 18–22 g) were kept in SPF (specific pathogen free) sterile facility and fed sterile food and water on a regular basis. We randomized these mice into Ca Ski, Ca Ski-sh1, Ca Ski-sh2, HT-3 and HT-3-exSTEAP2 5 groups with 5 mice per group. After gathering the logarithmic phase cells and suspending them in sterile saline, subcutaneous administration of 300 μl cell suspension containing 1×10^7 cells proceeded into the mice at back and neck. After injection, the tumor formation and changes were regular observed and the size of the nodule (tumor) was measured and recorded weekly with vernier calipers exploiting a formula: Volume = Length×Width^2×0.5, tumor volume of nude mice per group were calculated. After observing for 8 weeks, mice were all euthanized with CO_2 (carbon dioxide) for dissecting and assessing subcutaneous tumors per group. The approval of all animal experiments were obtained from the Shandong First Medical University's Institutional Animal Care and Use Committee and implemented following the regulatory guidelines.

Statistical analysis

Processing of all the data was accomplished via the SPSS 24.0. The inter-group rates were evaluated for the results of immunohistochemistry through Chi-square or Fisher exact test. Pairwise comparisons of independent samples were accomplished through *t*-test, while the comparisons among multiple groups were made via single-factor ANOVA, and the SNK method was used to make pair comparison between multiple samples. Survival analysis was accomplished by Kaplan-Meier combined with Log Rank procedures. *P* values of < 0.05 were regarded to be significant.

Results

Expression of STEAP2 in normal tissue, cervical intraepithelial neoplasia (CIN) and cervical cancer

The expression levels of STEAP2 in normal tissue, cervical intraepithelial neoplasia (CIN) as well as cervical cancer were determined by immunohistochemical assay. STEAP2 protein expression was very low in normal cervical tissue (Fig. 1A) and CIN (Fig. 1B), as shown in Table 2. However, in most cervical cancer tissues, the expression levels of STEAP2 were high. STEAP2 was slightly over-expressed in stage Ⅰ(Fig. 1C), moderately high-expressed in stage Ⅱ (Fig. 1D) and strongly expressed in stage III and IV of

cervical carcinoma (Fig. 1E). It was found that the strong positive expression of STEAP2 was mostly concentrated in cancer cells and a few interstitial tissues. In addition, the high expression of STEAP2 protein was tightly associated with the low differentiation, advanced clinical stage and positive lymph node metastasis of cervical cancer (Table 3). With the purpose of evaluating the correlation between STEAP2 and the prognosis of patients undergoing cervical cancer, Kaplan-Meier and Log Rank tests were adopted for performing survival analysis. According to the obtained results, patients with low STEAP2 protein expression revealed a notably better prognosis than those suffering from high STEAP2 protein expression (Fig. 1F).

Table 2
Expression of STEAP2 in normal, CIN and cancer tissues

Pattern of tissue	**N**	**STEAP2**	χ^2	*P*
		Low expression High expression		
		n (%) n (%)		
Cervical normal tissues	60	49 (81.7) 11(18.3)	155.022	0.000
CIN	80	62 (77.5) 18 (22.5)		
Carcinoma	248	38 (15.3) 210 (84.7)		

Table 3
The relationship between the expression of STEAP2 and clinicopathological features of cervical carcinoma patients

Pathological features	N	STEAP2	χ^2	*P*
		Low expression High expression		
		n (%) n (%)		
Age			1.199	0.274
≤ 50	104	19 (18.3) 85 (81.7)		
> 50	144	19 (13.2) 125 (86.8)		
TNM clinical stages			13.330	0.000
□ and □ stage	135	31 (23.0) 104 (77.0)		
□ and □ stage	113	7 (6.2) 106 (93.8)		
Histological type			1.878	0.391
Squamous carcinoma	128	17 (13.3) 111 (86.7)		
Adenocarcinoma	95	15 (15.8) 80 (84.2)		
Adenosquamous carcinoma	25	6 (24.0) 19 (76.0)		
Cell differentiation			9.552	0.002
High and Medium	139	30 (21.6) 109 (78.4)		
Low	109	8 (7.3) 101 (92.7)		
Lymphatic metastasis			15.289	0.000
N0	117	29 (24.8) 88 (75.2)		
N+	131	9 (6.9) 122 (93.1)		

Steap2 Expression In 5 Different Cervical Cancer Cell Lines

By western blotting (Fig. 1G), ICC (Fig. 1H) and qRT-PCR (Fig. 1I), STEAP2 mRNA and protein expressions were measured in the 5 cervical cancer cell lines Ca Ski, HeLa, SiHa, C-33A and HT-3. STEAP2 was highly expressed in high-invasive cell line Ca Ski which contained the complete genome of human papillomavirus type 16 (HPV-16, approximately 600 copies per cell) and related sequences of HPV-18, and STEAP2 expression was extremely low in the low-invasive cell lines C-33A and HT-3 which were both negative for human papillomavirus DNA and RNA. STEAP2 expression was both relatively moderate in HeLa cell line with HPV-18 sequence and Siha cell lines with HPV-16 genome (1–2 copies per cell). With the purpose of deeply exploring the function of STEAP2 in the biological characteristics of cervical cancer

cells, we selected STEAP2 high-expressed Ca Ski cell line for RNAi experiment, and STEAP2 low-expressed HT-3 cell line for lentiviral overexpression experiment. In addition, the changes of cell proliferation and invasion ability before and after STEAP2 gene knockdown and overexpression were detected by in vivo and in vitro functional experiments.

Identification The Efficiencies Of Steap2 Over-expressed Or Knockdown Transfection

LV-STEAP2-RNAi down-regulated viruses and LV-STEAP2-overexpressed viruses were used to transfect Ca Ski and HT-3 cells respectively, STEAP2 shRNA1 and shRNA2 transfected Ca Ski cells were referred to as Ca Ski-sh1 and Ca Ski-sh2, meanwhile STEAP2-cDNA transfected HT-3 cells were referred to as HT-3-cDNA. After 72 hours of culture, the fluorescence of cells was observed under a fluorescence microscope, when the proportion of cells with high fluorescence was greater than 80% of the total number of cells, the transfection efficiency was considered to be ideal (Fig. 2AB). After that, the cells were further cultured, proteins were extracted, and the transfection effect was further verified by western blotting and immunocytochemistry. In comparison with the negative control group, the protein expression of STEAP2 was obviously lowered in the Ca Ski-sh1 and Ca Ski-sh2 groups, and significantly increased in the HT-3 cDNA group (Fig. 2C). The results of ICC (Fig. 2D) and qRT-PCR (Fig. 2E) also showed that STEAP2 was successfully down-expressed in Ca Ski-sh1 and Ca Ski-sh2 cells and over-expressed in HT-3-cDNA cells.

Impacts Of Steap2 Knockdown Or Over-expression On Cervical Cancer Cell Proliferation, Cell Cycle And Clonogenicity

The growth curves and cell cycle were performed to identify the impacts of STEAP2 knockdown or over-expression on cervical cancer cell growth and proliferation. STEAP2 knockdown markedly hindered the growth of cervical cancer cells, meanwhile the cells transfected with STEAP2 cDNA grew faster than the negative control cells (Fig. 3A). Cell cycle results showed that after STEAP2 knockdown, numerous cells stayed in the G1 phase (the gap time before DNA replication), leading to a sharp decrease in the number of cells in the S phase (DNA replication), in comparison with the negative control. On the contrary, the over-expression of STEAP2 significantly reduced the proportion of cells in the G1 phase and enhanced the number of cells entering the S phase (Fig. 3B). The data consistently showed that STEAP2 knockdown inhibited the growth and proliferation of cervical cancer cells via blocking the transition of cells from G1 phase to S phase, while over-expression of STEAP2 promoted cell growth and proliferation by enhancing the proportion of cells entering S phase. The cell plate cloning formation assays were carried out in order to decide the clonogenesis potential of cells after down-expression and over-expression of STEAP2. According to the results, the average number of colonies formed in the STEAP2 shRNA1 and shRNA2 transfected groups was obviously lower when compared with that in the negative

control group, while the average number of colonies formed in the STEAP2 cDNA transfected group was notably higher than that in the negative control group (Fig. 4C). In one word, STEAP2 may be an important positive regulator in stimulating the proliferation and clonogenicity of cervical cancer cells.

Impacts Of Steap2 Knockdown Or Over-expression On Cervical Cancer Cell Migration And Invasion

The transwell chamber was adopted for evaluating the impact of STEAP2 on the migration and invasion capacity of cervical cancer cells, via the comparison of the number of cells that migrated or invaded the microporous membrane before and after the STEAP2 knockdown or over-expression. The results of cell migration assay (Fig. 3D) and invasion assay (Fig. 3E) showed that the number of cells transfected with STEAP2 shRNA1 and shRNA2 migrating through the PVPF membrane or invading via the Matrigel was less than that of the negative control cells, STEAP2 knockdown significantly inhibit cervical cancer cell migration and invasion. When STEAP2 was over-expressed, the average number of invaded or migrated cells transfected with STEAP2 cDNA was apparently higher when compared with that of negative control cells, cervical cancer cell migration and invasion capacities were remarkably enhanced. In vitro studies, STEAP2 knockdown or over-expression could make a noticeable impact on the migration and invasion of cervical cancer cells. Meanwhile, further in vivo experiments would confirm our conclusions.

Subcutaneous Tumor Transplantation Experiment In Nude Mice

The impacts of STEAP2 on tumor growth in vivo was further investigated by establishing the xenograft tumor model in nude mice. STEAP2 shRNA1 and shRNA2 transfected cells, STEAP2 cDNA transfected cells and consistent negative control cells were subcutaneously seeded into 5 nude mice, separately. The growth and the size of the tumor during the 8-week follow-up were shown in Fig. 4A and Fig. 4B. In vivo, tumor growth and size were delayed in the STEAP2 shRNA1 and shRNA2 transfection groups, STEAP2 knockdown inhibited tumor formation and growth. On the contrary, the average tumor size of subcutaneously inoculated with STEAP2 cDNA transfected cells was much larger when compared with that of the control group, STEAP2 over-expression promoted tumor growth in vivo.

Impacts of STEAP2 knockdown or over-expression on the key Epithelial to mesenchymal transition (EMT) hallmarks and matrix metalloproteinases (MMPs)

EMT refers to a vital biological process for malignant tumor cells of epithelial origin in order to grasp the capability of migrating and invading. Via EMT, epithelial cells lose cell polarity, connection to the basement membrane together with other epithelial phenotypes, which can subsequently acquire

interstitial phenotypes like higher migration and invasion abilities and the capacity to degrade extracellular matrix.[18] We have confirmed that STEAP2 could play a critical function in the migration and invasion ability of cervical cancer cells. Therefore, it could be considered that STEAP2 knockdown or over-expression could affect the EMT process. We obtained consistent results from Western blotting (Fig. 4C) and real time RT-qPCR (Fig. 4D) that STEAP2 knockdown could obviously enhance the expression of epithelial marker E-cadherin, and lowered that of mesenchymal markers N-cadherin and Vimentin, as well as transcription factors Snail, Slug, Twist and Zeb2, inhibiting E-cadherin transcription. In contrast, in STEAP2 cDNA-transfected HT-3 cells, STEAP2 over-expression remarkably lowered the expression of epithelial marker E-cadherin and added the expression of N-cadherin, Vimentin, Snail, Slug, Twist and Zeb2. The MMPs family, named because they require metal ions such as Ca^{2+} and Zn^{2+} as cofactors, have been isolated and identified as 26 members, which were classified into 6 classes according to the substrate and fragment homology.[19] In our research, collagenases (MMP-1, MMP-8 and MMP-13) and gelatinases (MMP-2 and MMP-9) were identified by adopting Western blotting and real time RT-qPCR in STEAP2 down-expression or over-expression lentiviral transfection system. According to Fig. 5A and 5B, at the both protein and mRNA levels, the similar results were concluded that STEAP2 knockdown weakened the expression of MMP-1 and MMP-13, while exerted no impact on the expression of MMP-2, MMP-9 and MMP-8, simultaneously, STEAP2 over-expression remarkably enhanced the expression of MMP-1 and MMP-13 while did not alter the expression of MMP-2, MMP-9 and MMP-8. Briefyly, the STEAP2 functionality gain or loss could influence the EMT process and the expression of some MMPs. STEAP2 over-expression promoted EMT, increased the expression of MMP1 and MMP13, promoted the degradation of extracellular matrix and cell invasion and metastasis; STEAP2 knockdown inhibited EMT, decreased the expression of MMP1 and MMP13, inhibited extracellular matrix degradation and the invasion and metastasis of cervical cancer cells.

Impacts Of Steap2 Knockdown Or Over-expression On The Pi3k/akt Signaling Pathway

The PI3K/AKT/mTOR axis is found to generate a vital function in tumor EMT, and the functional activation of the PI3K/AKT leads to the expression of downstream mesenchymal proteins and the inhibition of E-cadherin.20 The current work explored whether the PI3K/AKT/mTOR signaling pathway was hindered or activated in STEAP2 down-expression or over-expression lentiviral transfection system. According to the obtained results, STEAP2 knockdown could obviously restrain the phosphorylation of PI3K, AKT and mTOR in Ca Ski enhanced the phosphorylation of PI3K, AKT and mTOR in HT-3 cells tranfected with STEAP2 cDNA (Fig. 5C). In a word, STEAP2 knockdown could inactivate the PI3K/AKT/mTOR signaling pathway but STEAP2 over-expression activated it. Furthermore, the PI3K inhibitor LY294002 and AKT inhibitor MK2206 were supplemented in the culture medium of HT-3 cells transfected with STEAP2 cDNA for 48h to investigate weather these inhibitors could hamper the enhancement of cancer cell migration and invasion ability as well as the activation of the PI3K/AKT/mTOR signaling pathway, which was caused by STEAP2 over-expression. As shown in Fig. 6A,

PI3K inhibitor LY294002 and AKT inhibitor MK2206 could significantly weaken the migration and invasion ability of HT-3 cDNA cells. As the concentration of the drug increased, this inhibition was further enhanced (Fig. 6B). The PI3K inhibitor LY294002 could significantly decrease the phosphorylation levels of PI3K, and its downstream effector proteins AKT and mTOR, and further restrained the expression of mesenchymal proteins and transcription factors, leading to enhanced expression of E-cadherin. Meanwhile, the AKT inhibitor MK2206 could remarkably inhibit the phosphorylation of AKT and mTOR while exerted no impact on PI3K phosphorylation, and resulted in decreased mesenchymal protein expressions and increased expression of epithelial protein (Fig. 6C). In conclusion, STEAP2 over-expression could promote the EMT process of cervical cancer cells through the activation of the PI3K/AKT/mTOR signaling pathway.

Discussion

In our present research, the STEAP2 up-regulation in cancerous cervical tissues and cells was identified, and its high level was found linked positively to the high histological grade, advanced clinical stage, positive metastasis to lymph nodes, as well as poor outcome of patients. Through down-expression and up-expression of STEAP2 after lentivirus transfection and cell function experiments in vitro and in vivo, STEAP2 was found to play an oncogene role in the development of cervical cancer by promoting the EMT event and initiating the PI3K/Akt axis.

According to our findings, the high STEAP2 level in cervical carcinomas were linked tightly to the clinically malignant phenotype and poor patient outcome. Besides, knockdown of STEAP2 inhibited the multiplicative and invasive capacities of cervical carcinoma cells, while its up-regulation facilitated the multiplication and infiltration of these cells. Kim K et al. had reported that when STEAP1 and STEAP2 were co-expressed, heterotrimer could be formed,[21] so their ability to form heterooligomers might explain the tendency to co-express in some cancers. In prostate cancer, the levels of STEAP1 and STEAP2 were both high,[22] and down-regulation of these genes could repress the cellular multiplication and infiltration.[14, 23] In the case of breast carcinoma, however, the STEAP1 and STEAP2 in tumor tissues were both down-regulated, the outcome of patients highly expressing STEAP1/STEAP2 was favorable,[24] and their enhanced expressions significantly repressed the migratory and invasive capacities of breast carcinoma cells.[25, 26] Over-expression of STEAP1 and STEAP2 had also been noted jointly in a few other types of human carcinomas, including pancreatic, colonic, bladder, ovary and Ewing's sarcoma.[9, 10] Influenced by tumor microenvironment, their carcinogenic or anticancer function may be determined by the type of different cancers, and there were obvious tissue specificity.[27] Comprehensive analysis shows that, just like STEAP1, STEAP2 has great role and clinical significance in the human carcinomas as well. Deeper probing into the STEAP2's effects on cancer pathophysiology and the co-expression mechanism of STEAP1 and STEAP2 are expected to bring about better diagnosis and management of human carcinomas.

Over the last two decades, the role of EMT event has been proven crucial in carcinoma progression. As demonstrated by ever-growing researches, EMT program is activated during malignant progression, and the mesenchymal traits triggered by it enable fulfillment of multiple invasion-metastasis cascade steps for the carcinoma cells.[28, 29] In our study, up-regulation of STEAP2 could inhibit the epithelial marker (E-cadherin) expression and elevate the mesenchymal marker (N-cadherin and vimentin) expressions, which caused the cell-to-cell junctions between adjacent epithelial cells to become looser, allowing cancer cells to dissociate and thus to spread. Snail, Slug, Twist and Zeb2 are all key transcription factors that mediate EMT by blocking the transcription of E-cadherin.[31] In the case of down-regulated STEAP2, the expression levels of these transcription factors were all decreased, leading to the weakening of their negative regulations and the increase of E-cadherin expression, thus inhibiting the EMT process. Excessive STEAP2 expression led to increased expression of these transcription factors, as well as enhanced negative regulation, so that E-cadherin expression was reduced and EMT was activated. It was suggested that STEAP2 could promote the migratory and invasive capacities of cervical carcinoma cells by impacting the transcriptional factors and triggering the EMT. At present, the STEAP family is identified to be located at the intercellular junctions, which is considered an ion transporter/channel involved in the iron/protein transport and the intercellular communication, eventually impacting the carcinoma cell invasion and multiplication.[7, 8] However, the association of STEAP members with the EMT has scarcely been reported. In breast cancer, both STEAP1 and STEAP2 acted as tumor suppressor genes to suppress tumor progression and played inhibitory roles in their relationship with EMT.[25, 26] On the contrary, in gastric cancer[32], lung adenocarcinoma[33] and ovarian carcinoma[34], STEAP1 was found to be an oncogene that induced EMT to facilitate the carcinoma cell migration and infiltration. But, in these carcinomas, no literature on STEAP2's effect on the tumor evolution has been retrieved. Although members of the STEAP family are involved in the tumorigenesis and evolution of diverse tumors, the precise mechanism is still elusive, which requires further exploration. More data will help formulate more optimal regimens for the management of cervical carcinomas and improvement of patient prognosis.

MMPs play a pivotal role in facilitating the cervical carcinoma progression,[35] among them, MMP1 promoted cervical tumor growth and lymphatic metastasis,[36] MMP13 expression was apparently increased in cancerous cervical tissues, and its elevated level in cervical carcinoma cells led to EMT and induced tumor metastasis.[37] In our experimental data, STEAP2 knockdown inhibited the infiltration of cervical carcinoma cells and down-regulated the MMP-1 and MMP-13, while STEAP2 over-expression remarkably promoted such cellular infiltration and up-regulated the MMP-1 and MMP-13. Therefore, MMP1 and MMP13 were implicated in the mechanism whereby STEAP2 facilitates the infiltration of carcinoma cells. Apart from that, MMP13 was detected as a downstream target of STEAP2 for driving prostate cancer cell invasion.[14] MMP2 and MMP9 are over-expressed in most tumors and are considered to be markers of cancer aggressiveness.[38] However, at both protein and mRNA levels, knockout or overexpression of STEAP2 had no effect on their expression in cervical cancer cells. But for STEAP1, it could promote ovarian carcinoma cell infiltration by up-regulating MMP2 and MMP9 and enhancing their activities.[34] Co-expression of STEAP1 and STEAP2 is found in many tumors and seem to play a similar

effect on tumor evolution, although the particular mechanisms may be different, and a reasonable explanation for this phenomenon needs further in-depth study.

The effect exerted by PI3K/Akt/mTOR axis on the malignant cell growth, survival and proliferation is crucial, and abnormal PI3K-reliant signaling is usually noted in diverse types of tumors, including cervical carcinoma.[39] In the present work, implication of PI3K/Akt/mTOR axis was also noted in the EMT regulation of cervical carcinoma cells by STEAP2. Knockdown of the STEAP2 weakened the PI3K, Akt and mTOR phosphorylation and hampered EMT, while, over-expression of STEAP2 enhanced the PI3K, Akt and mTOR phosphorylation and elicited the EMT. Further, LY294002 (a PI3K inhibitor) and MK2206 (an AKT inhibitor) could significantly reduce the phosphorylation level of their downstream effector proteins, further inhibiting EMT. In a word, in cervical cancer, STEAP2 could facilitate EMT through the PI3K/ Akt/mTOR axis initiation. In the case of breast carcinomas, STEAP2 suppressed cancer cell invasion by repressing the EMT event and hampering the PI3K/AKT axis initiation.[26] For STEAP1, its up-regulation could facilitate the multiplication, migration and infiltration of gastric carcinoma cells via the AKT/FoxO1 axis initiation and EMT.[40] The relationship between STEAP1 or STEAP2 and EMT process also appears to be related to the tumor microenvironment, with both promoting and inhibiting effects. So far, the studies of the STEAP family members at the molecular and cellular levels are still in their infancy, and a profound future direction of STEAP family studies is the identification of downstream effector molecules and more possible signaling pathways.

In conclusion, the expression of STEAP2 was elevated in cancerous cervical tissues, and its high level was linked positively to the malignant phenotype of cervical cancer and the poor patient outcome. Besides, STEAP2 facilitated the cervical carcinoma cell multiplication and invasion, and initiated the PI3K/Akt axis to promote EMT process. We believe that STEAP2 provides a new therapeutic target for inhibiting the cervical carcinoma infiltration and metastasis, which is profoundly meaningful for improving the prognosis of patients.

Abbreviations

six-transmembrane epithelial antigen of prostate 2
STEAP2
epithelial interstitial transformation
EMT
matrix metalloproteinases
MMPs
six-transmembrane protein of the prostate 1
STAMP1
cervical intraepithelial neoplasia
CIN
International Federation of Gynecology and Obstetrics
FIGO

minimum Eagle's medium
MEM
fetal bovine serum
FBS
immunohistochemistry
IHC
immunocytochemistry
ICC
streptavidin-biotin-peroxidase
SP
Quantitative Real-time polymerase chain reaction
qRT-PCR
complementary DNA
cDNA
polyvinylidene difluoride
PVDF
tris-buffered saline containing Tween 20
TBST
enhanced chemiluminescence
ECL
small hairpin RNA
shRNA
RNA interference
RNAi
multiplicity of infection
MOI
propidium iodide
PI
specific pathogen free
SPF
carbon dioxide
CO_2

Declarations

Acknowledgments

Not applicable.

Authors' contributions

The manuscript has been read and approved by all the authors. FW took charge of the experimental conception and design, MW NL took charge of the experimental implementation, NL CY FW took charge of the data analysis, and MW FW took charge of the manuscript writing.

Funding

The present work was supported under grant No. 2020M682219 by the Postdoctoral Science Foundation of China and under grant No. ZR2020MH066 by the Natural Science Foundation of Shandong Province. The funders had no role in the design of research, acquisition/analysis of data, publication decision, or manuscript formulation.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The present work was approved by the Shandong First Medical University's Institutional Medical Ethics Committee. Methodological procedures were all implemented following the relevant guidelines and regulations. The patients enrolled in this study have signed informed consents in a written form. Murine experimental protocols were all in line with the PRC State Council Regulations for the Administration of Affairs Concerning Experimental Animals.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Cohen PA, Jhingran A, Oaknin A, Denny L. Cervical cancer. Lancet. 2019;393:169–182.
2. Dickinson JA. Age of initiation of cervical cancer screening. JAMA. 2019;321:611–612.
3. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68:394–424.
4. Liontos M, Kyriazoglou A, Dimitriadis I, Dimopoulos MA, Bamias A. Systemic therapy in cervical cancer: 30 years in review. Crit Rev Oncol Hematol. 2019;137:9–17.
5. Ayen A, Jimenez Martinez Y, Boulaiz H. Targeted gene delivery therapies for cervical cancer. Cancers. 2020;12:5.

6. Hubert RS, Vivanco I, Chen E, Rastegar S, Leong K, Mitchell SC, Madraswala R, Zhou Y, Kuo J, Raitano AB, Jakobovits A, Saffran DC, Afar DE. STEAP: a prostate-specific cell-surface antigen highly expressed in human prostate tumors. Proc Natl Acad Sci U S A. 1999; 96:14523–8.
7. Grunewald TG, Bach H, Cossarizza A, Matsumoto I. The STEAP protein family: versatile oxidoreductases and targets for cancer immunotherapy with overlapping and distinct cellular functions. Biol Cell. 2012;104:641–57.
8. Gomes IM, Maia CJ, Santos CR. STEAP proteins: from structure to applications in cancer therapy. Mol Cancer Res. 2012;10:573–87.
9. Sikkeland J, Sheng X, Jin Y, Saatcioglu F. STAMPing at the crossroads of normal physiology and disease states. Mol Cell Endocrinol. 2016;425:26–36.
10. Lindstad T, Jin Y, Wang L, Qu S, Saatcioglu F. STAMPs at the crossroads of cancer and nutrition. Nutr Cancer. 2010;62(7):891–5.
11. Porkka KP, Helenius MA, Visakorpi T. Cloning and characterization of a novel six-transmembrane protein STEAP2, expressed in normal and malignant prostate. Lab Invest. 2002;82:1573–82.
12. Korkmaz KS, Elbi C, Korkmaz CG, Loda M, Hager GL, Saatcioglu F. Molecular cloning and characterization of STAMP1, a highly prostate-specific six transmembrane protein that is overexpressed in prostate cancer. J Biol Chem. 2002;277:36689–96.
13. Whiteland H, Spencer-Harty S, Morgan C, Kynaston H, Thomas DH, Bose P, Fenn N, Lewis P, Jenkins S, Doak SH. A role for STEAP2 in prostate cancer progression. Clin Exp Metastasis. 2014;31:909–20.
14. Burnell SEA, Spencer-Harty S, Howarth S, Bodger O, Kynaston H, Morgan C, Doak SH. STEAP2 Knockdown Reduces the Invasive Potential of Prostate Cancer Cells. Sci Rep. 2018;8:6252.
15. Barroca-Ferreira J, Pais JP, Santos MM, Goncalves AM, Gomes IM, Sousa I, Rocha SM, Passarinha LA, Maia CJ. Targeting STEAP1 Protein in Human Cancer: Current Trends and Future Challenges. Curr Cancer Drug Targets. 2018;18:222–230.
16. Soumaoro LT, Uetake H, Higuchi T, Takagi Y, Enomoto M, Sugihara K. Cyclooxygenase-2 expression: a significant prognostic indicator for patients with colorectal cancer. Clin Cancer Res. 2004;10:8465–71.
17. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402–8.
18. Dongre A, Weinberg RA. New insights into the mechanisms of epithelial-mesenchymal transition and implications for cancer. Nat Rev Mol Cell Biol. 2019;20(2):69–84.
19. Carey P, Low E, Harper E, Stack MS. Metalloproteinases in Ovarian Cancer. Int J Mol Sci. 2021;22(7):3403.
20. Pearlman RL, Montes de Oca MK, Pal HC, Afaq F. Potential therapeutic targets of epithelial-mesenchymal transition in melanoma. Cancer Lett. 2017;391:125–140.
21. Kim K, Mitra S, Wu G, Berka V, Song J, Yu Y, Poget S, Wang DN, Tsai AL, Zhou M. Six-Transmembrane Epithelial Antigen of Prostate 1 (STEAP1) Has a Single b Heme and Is Capable of Reducing Metal Ion

Complexes and Oxygen. Biochemistry. 2016;55(48):6673–6684.

22. Burnell SEA, Spencer-Harty S, Howarth S, Bodger O, Kynaston H, Morgan C, Doak SH. Utilisation of the STEAP protein family in a diagnostic setting may provide a more comprehensive prognosis of prostate cancer. PLoS One. 2019;14(8):e0220456.
23. Gomes IM, Rocha SM, Gaspar C, Alvelos MI, Santos CR, Socorro S, Maia CJ. Knockdown of STEAP1 inhibits cell growth and induces apoptosis in LNCaP prostate cancer cells counteracting the effect of androgens. Med Oncol. 2018;35(3):40.
24. Wu HT, Chen WJ, Xu Y, Shen JX, Chen WT, Liu J.The Tumor Suppressive Roles and Prognostic Values of STEAP Family Members in Breast Cancer. Biomed Res Int. 2020;2020:9578484.
25. Xie J, Yang Y, Sun J, Jiao Z, Zhang H, Chen J. STEAP1 Inhibits Breast Cancer Metastasis and Is Associated With Epithelial-Mesenchymal Transition Procession. Clin Breast Cancer. 2019;19:e195-e207.
26. Yang Q, Ji G, Li J. STEAP2 is down-regulated in breast cancer tissue and suppresses PI3K/AKT signaling and breast cancer cell invasion in vitro and in vivo. Cancer Biol Ther. 2020;21:278–291.
27. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. Nat Med. 2013;19:1423–37.
28. Zhang Y, Weinberg RA. Epithelial-to-mesenchymal transition in cancer: complexity and opportunities. Front Med. 2018;12(4):361–373.
29. Mittal V. Epithelial Mesenchymal Transition in Tumor Metastasis. Annu Rev Pathol. 2018;13:395–412.
30. Kaszak I, Witkowska-Piłaszewicz O, Niewiadomska Z, Dworecka-Kaszak B, Ngosa Toka F, Jurka P. Role of Cadherins in Cancer-A Review. Int J Mol Sci. 2020;21(20):7624.
31. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol. 2014;15(3):178–96.
32. Zhang Z, Hou WB, Zhang C, Tan YE, Zhang DD, An W, Pan SW, Wu WD, Chen QC, Xu HM. A research of STEAP1 regulated gastric cancer cell proliferation, migration and invasion in vitro and in vivos. J Cell Mol Med. 2020;24(24):14217–14230.
33. Huo SF, Shang WL, Yu M, Ren XP, Wen HX, Chai CY, Sun L, Hui K, Liu LH, Wei SH, Wang XX, Wang Y, Tian YX. STEAP1 facilitates metastasis and epithelial-mesenchymal transition of lung adenocarcinoma via the JAK2/STAT3 signaling pathway. Biosci Rep. 2020;40(6):BSR20193169.
34. Jiao Z, Huang L, Sun J, Xie J, Wang T, Yin X, Zhang H, Chen J. Six-transmembrane epithelial antigen of the prostate 1 expression promotes ovarian cancer metastasis by aiding progression of epithelial-to-mesenchymal transition. Histochem Cell Biol. 2020;154(2):215–230.
35. Barillari G, Monini P, Sgadari C, Ensoli B. The Impact of Human Papilloma Viruses, Matrix Metallo-Proteinases and HIV Protease Inhibitors on the Onset and Progression of Uterine Cervix Epithelial Tumors: A Review of Preclinical and Clinical Studies. Int J Mol Sci. 2018;19(5):1418.

36. Tian R, Li X, Gao Y, Li Y, Yang P, Wang K. Identification and validation of the role of matrix metalloproteinase-1 in cervical cancer. Int J Oncol. 2018;52(4):1198–1208.
37. Li RM, Nai MM, Duan SJ, Li SX, Yin BN, An F, Zhai YQ, Liu J, Chu YR, Yu Y, Song WY. Down-expression of GOLM1 enhances the chemo-sensitivity of cervical cancer to methotrexate through modulation of the MMP13/EMT axis. Am J Cancer Res. 2018;8(6):964–980.
38. Henriet P, Emonard H. Matrix metalloproteinase-2: Not (just) a "hero" of the past. Biochimie. 2019;166:223–232.
39. de Melo AC, Paulino E, Garces ÁH. A Review of mTOR Pathway Inhibitors in Gynecologic Cancer. Oxid Med Cell Longev. 2017;2017:4809751.
40. Zhang Z, Hou WB, Zhang C, Tan YE, Zhang DD, An W, Pan SW, Wu WD, Chen QC, Xu HM. A research of STEAP1 regulated gastric cancer cell proliferation, migration and invasion in vitro and in vivos. J Cell Mol Med. 2020;24(24):14217–14230.

Figures

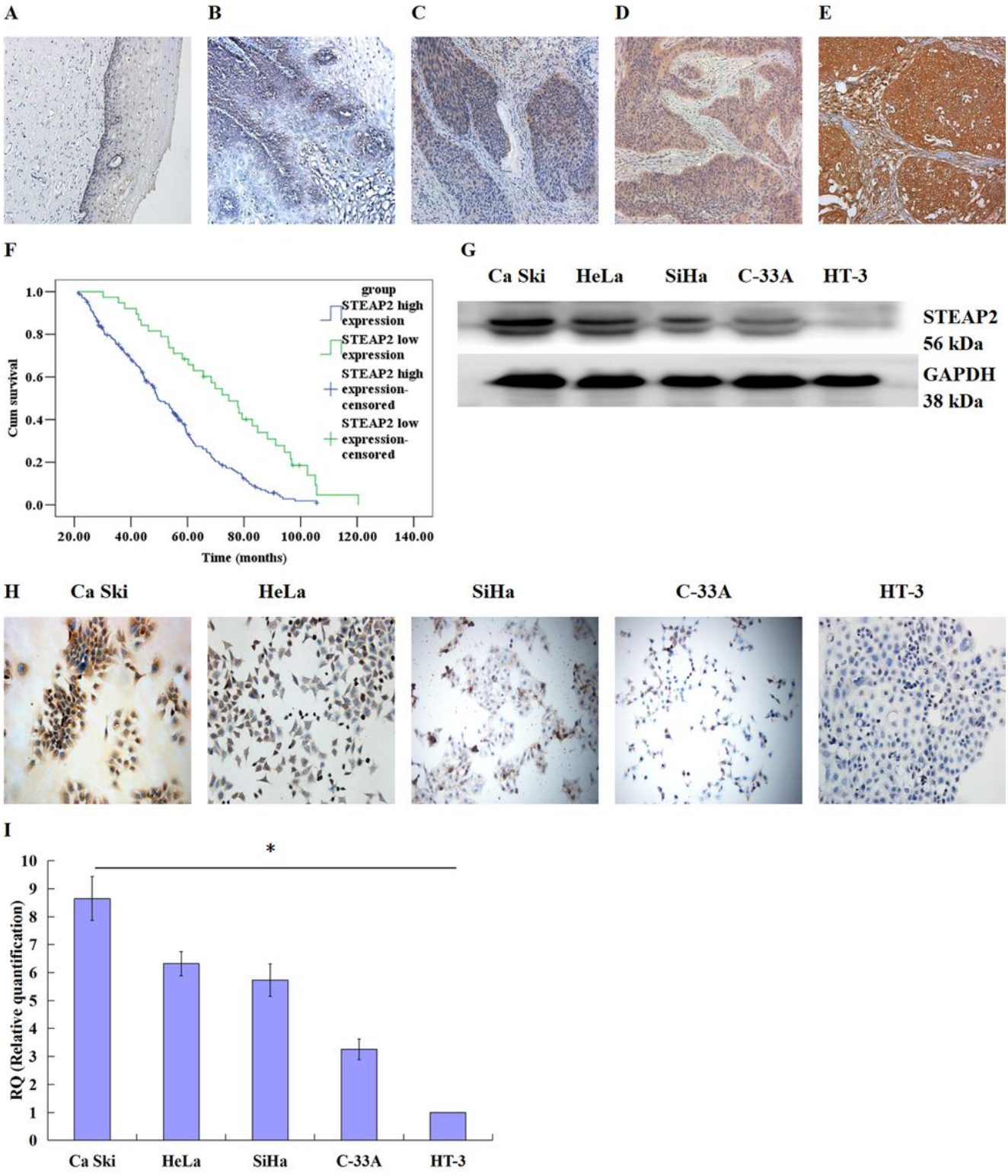

Figure 1

STEAP2 levels in healthy, CIN and cancerous tissues, as well as in five cervical carcinoma (Ca Ski, HeLa, SiHa, C-33A and HT-3) cells. STEAP2 expressions of (A) healthy human cervical tissue, (B) CIN (cervical intraepithelial neoplasia), (C) stage I CC (cervical carcinoma), (D) stage II CC and (E) stages III and IV CCs were measured by IHC. (F) By Kaplan-Meier plotter analysis, the outcome of CC patients highly expressing STEAP2 (blue line) was worse compared to those lowly expressing STEAP2 (green line). The expressions

of STEAP2 in 5 cervical cancer cell lines were evaluated by (G) western-blot (cropped blot), (H) ICC staining, as well as (I) qRT-PCR under ×200 magnification (*$P < 0.05$).

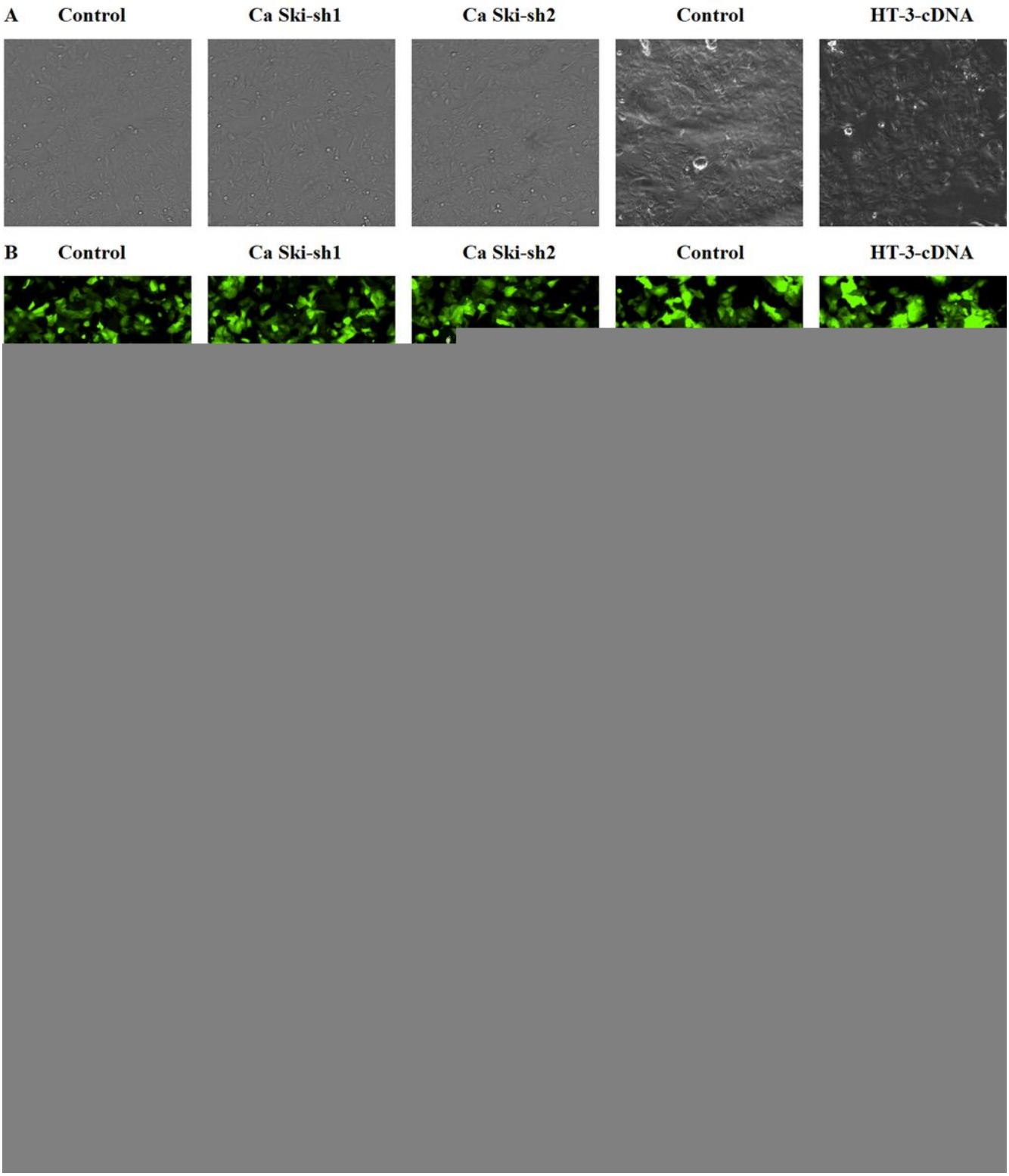

Figure 2

Lentiviral transfaction efficiency validation for the knockdown/over-expression of STEAP2. (A) Phase contrast micrographs and (B) GFP fluorescence micrographs revealed valid lentiviral transfaction, when

STEAP2 was up- or down-regulated, the fluorescent cell count > 80% indicated good transfection efficiency. The expression of STEAP2 in STEAP2 shRNA1 and shRNA2 transfected Ca Ski cells, STEAP2 cDNA transfected HT-3 cells and negative control cells were evaluated by (C) western-blot (cropped blot), (D) ICC staining, as well as (E) qRT-PCR under ×200 magnification (*P < 0.05).

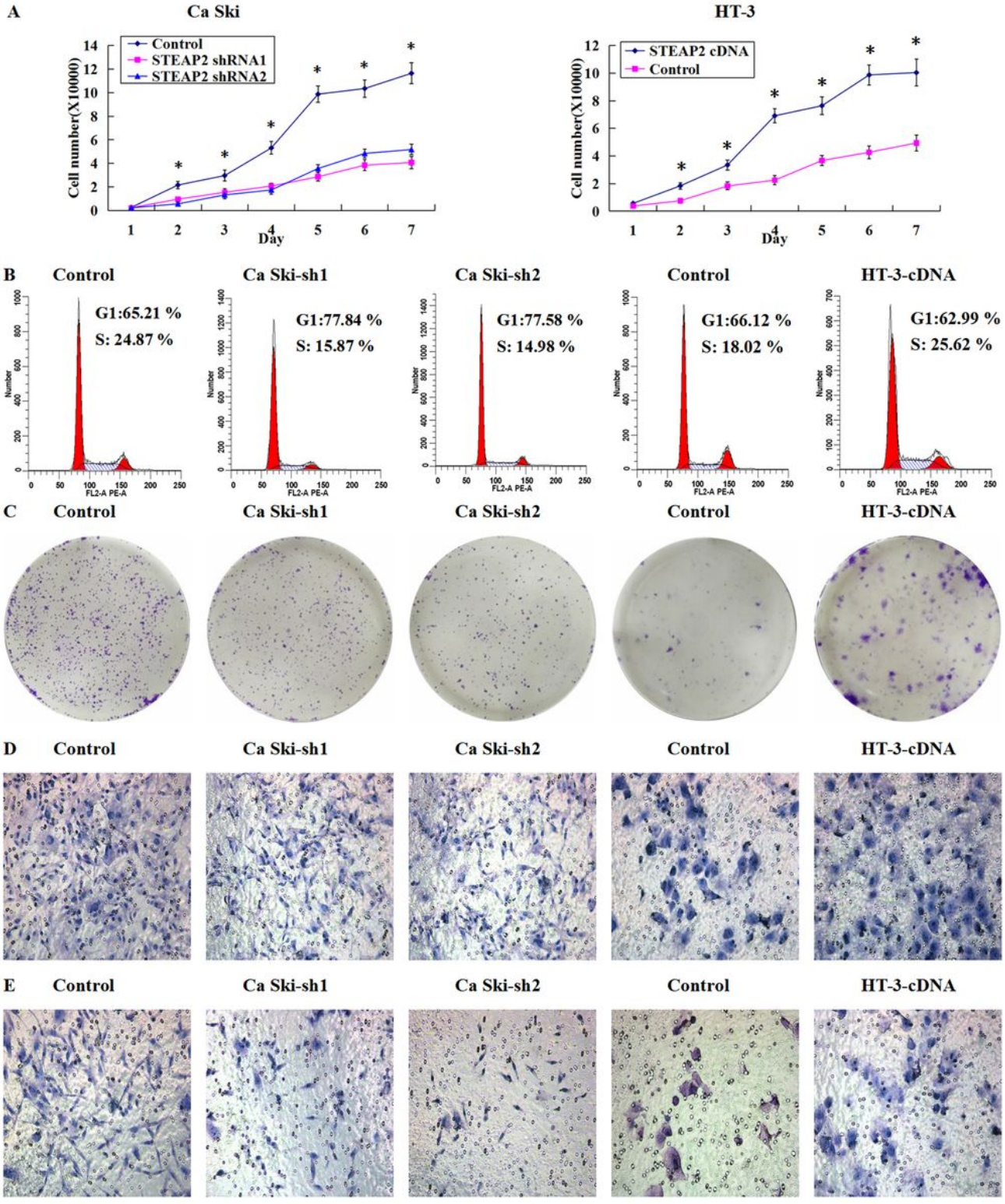

Figure 3

Effects of STEAP2 knockdown/over-expression on the CC cell multiplicative and invasive capacities. The multiplicative and invasive capacities of CC cells were evaluated by growth curve method, flow cytometry cell cycle assay, plate cloning formation test and transwell invasion and migration assay. (A) STEAP2 knockdown significantly hampered the proliferation capacity of Ca Ski cells, while STEAP2 over-expression remarkably improved the proliferation capacity of HT-3 cells. (B) STEAP2 knockdown inhibited cells from entering the DNA replication phase, leading to elevated G1 cell proportion and declined S cell proportion, while the effect of STEAP2 over-expression was just the opposite. (C) Prominently less colonies were formed in the Ca Ski shRNA1 and shRNA2 groups compared to the negative control, meanwhile the number of clones formed in HT-3 cDNA group was markedly increased, compared to negative control group. (D) Migration assay micrographs in the STEAP2 shRNA1- and shRNA2-transfected Ca Ski cells, STEAP2 cDNA transfected HT-3 cells and negative control cells. (E) Invasion assay micrographs in the STEAP2 shRNA1- and shRNA2-transfected Ca Ski cells, STEAP2 cDNA transfected HT-3 cells and negative control cells. (Magnification×200). *$P < 0.05$.

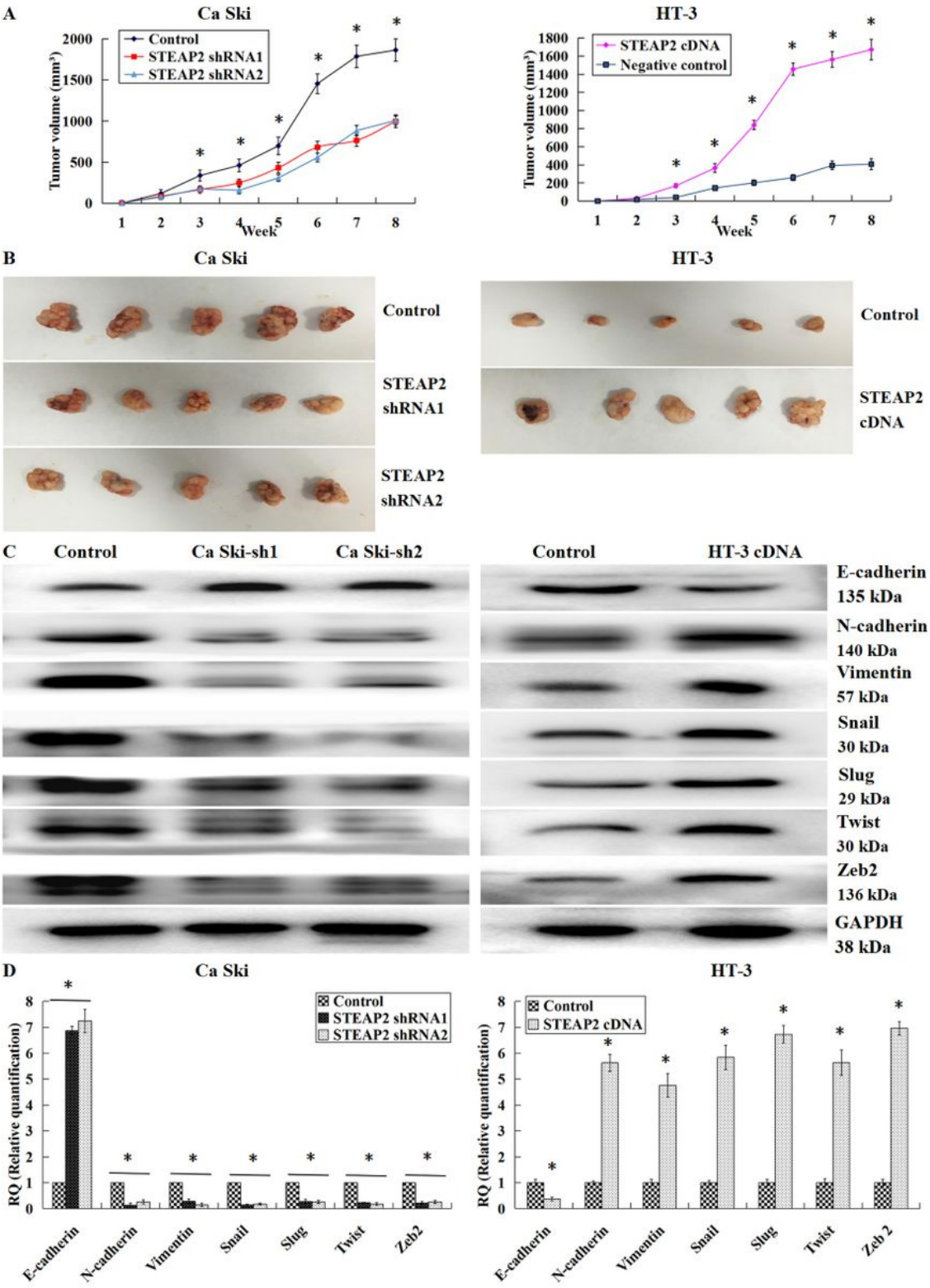

Figure 4

Effects of STEAP2 knockdown or over-expression on tumor growth in vivo and the process of EMT. (A) A persistent 8-week observation concerning the growth of tumors formed by the Ca Ski cells transfected with STEAP2 shRNA1 and shRNA2, the STEAP2 cDNA transfected HT-3 cells and negative control cells. Tumor growth in the Ca Ski shRNA1 and shRNA2 group was slower in contrast to the negative control, while that in the HT-3 cells transfected with STEAP2 cDNA was faster in vivo than those of negative

control cells. (B) Xenograft tumor micrographs following subcutaneous challenge with the STEAP2 shRNA1- and shRNA2-transfected Ca Ski cells, STEAP2 cDNA transfected HT-3 cells and negative control cells. By (C) western blotting (cropped blot) and (D) qRT-PCR, the hallmarks of EMT (E-cadherin, N-cadherin, Vimentin, Slug, Snail, Twist and Zeb2) were determined in STEAP2 shRNA1 and shRNA2 transfected Ca Ski cells, STEAP2 cDNA transfected HT-3 cells and negative control cells. STEAP2 knockdown increased the E-cadherin (epithelial hallmark) level, while decreased the levels of N-cadherin and Vimentin (mesenchymal hallmarks), as well as Slug Snail, Twist and Zeb2 (transcriptional factors). Contrastively, the excessive STEAP2 expression down-regulated the E-cadherin, while up-regulated the rest investigated hallmarks (*P<0.05).

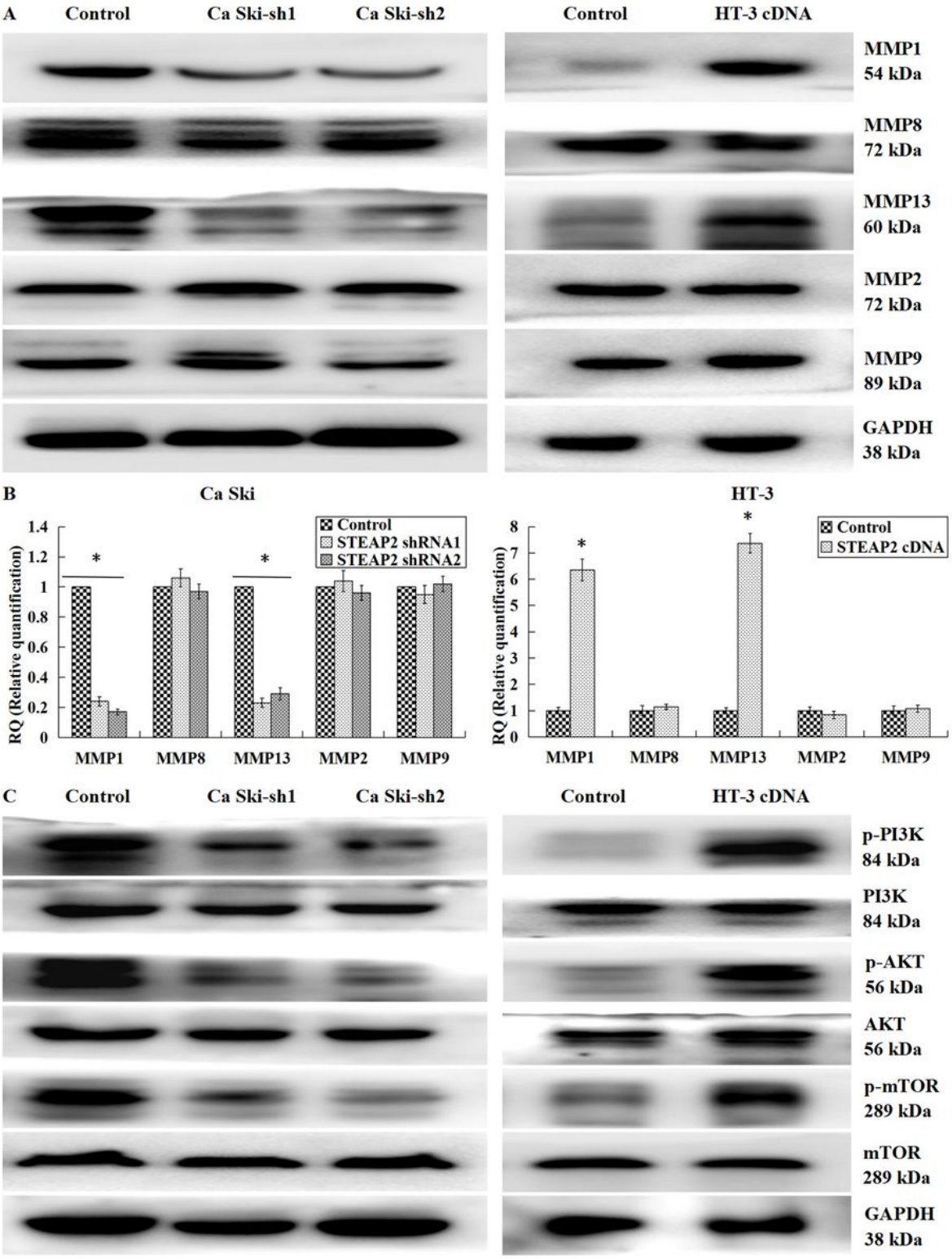

Figure 5

Effects of STEAP2 knockdown or over-expression on the MMP expressions and the PI3K/Akt/mTOR axis. Through (A) western-blott (cropped blot) combined with (B) qRT-PCR, the expressions of MMP-1, MMP-2, MMP-8, MMP-9 and MMP-13 were measured in STEAP2 shRNA1 and shRNA2 transfected Ca Ski cells, STEAP2 cDNA transfected HT-3 cells and negative control cells. STEAP2 knockdown weakened the MMP-1 and MMP-13 expressions, while failing to impact the MMP-2, MMP-8 or MMP-9 level. Contrastively,

STEAP2 over-expression enhanced the MMP-1 and MMP-13 expressions, while failing to alter the MMP-2, MMP-8 or MMP-9 level. (C) In STEAP2 shRNA1 and shRNA2 transfected Ca Ski cells, STEAP2 knockdown significantly reduced the PI3K, AKT and mTOR phosphorylation activities, while in the HT-3 cells transfected with STEAP2 cDNA, STEAP2 over-expression remarkably enhanced the PI3K, AKT and mTOR phosphorylation (*P<0.05).

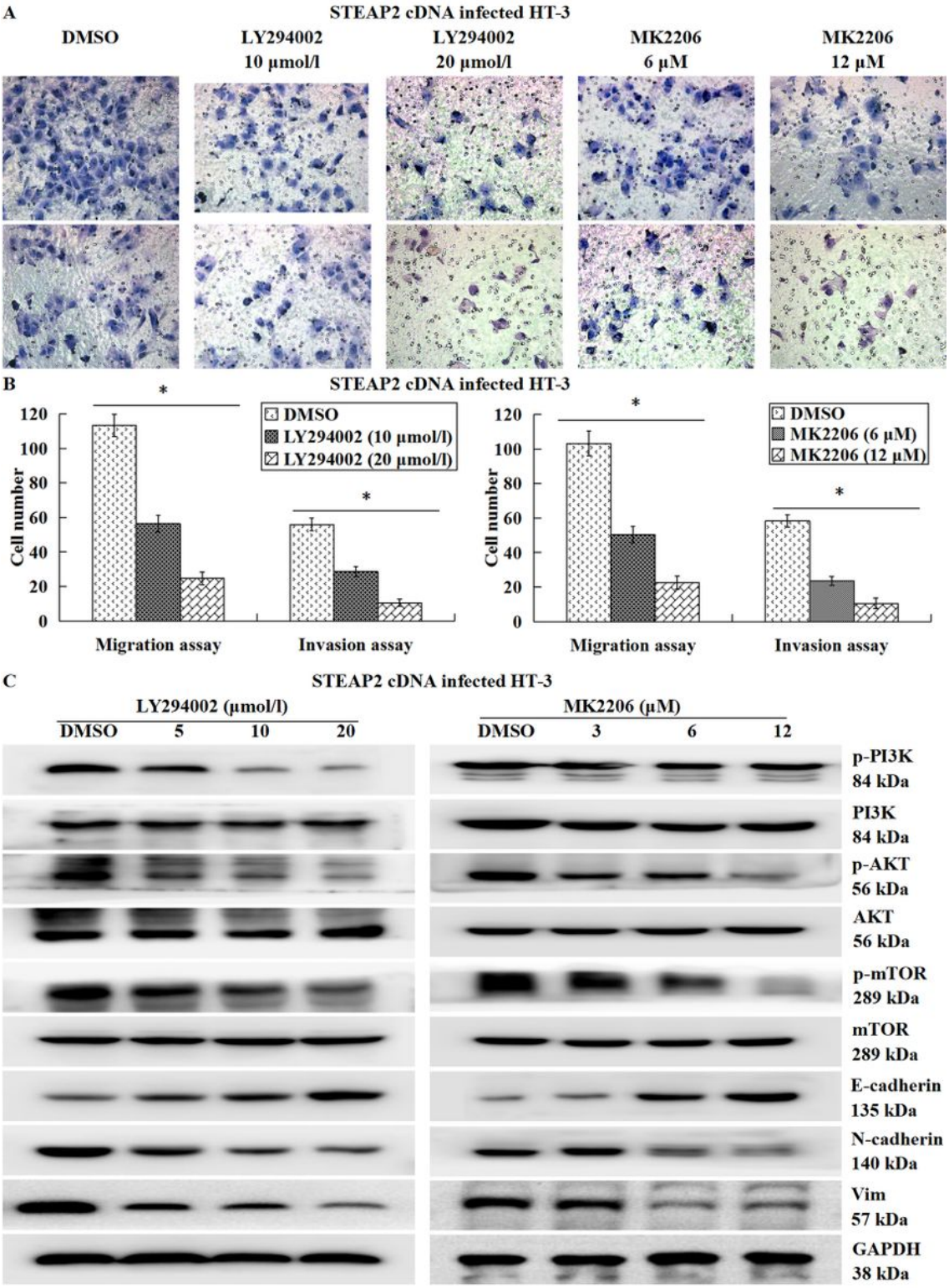

Figure 6

Effects of PI3K inhibitor LY294002 and AKT inhibitor MK2206 on the CC cell invasive and migratory capacities and the EMT event. (A) LY294002 and MK2206 could weaken the invasive and migratory capacities of HT-3 cDNA cells pronouncedly, which was caused by STEAP2 up-regulation. (B) As the concentration of the drug increased, this inhibition was further enhanced. (C) The PI3K inhibitor LY294002, could significantly hinder PI3K phosphorylation and EMT process, both of which were activated by STEAP2 over-expression. In the same way, the AKT inhibitor MK2206 could remarkably reduce the AKT and mTOR phosphorylation levels of, while failing to impact the PI3K phosphorylation, which also hampered the process of EMT.