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Chloride channel-3 is required for efficient tumour cell migration and invasion in human cervical squamous cell carcinoma

Yutao Guan [a], Yi Luan [b], Yong Xie [a], Hui Zhou [c], Weifeng Li [a], Xiaolu Zhang [a], Ximing Shen [d], Yangping Chen [a], Lijiang Xu [a], Zhongqiu Lin [c,**], Gang Wang [a,*]

[a] Department of Obstetrics and Gynecology, The First People's Hospital of Foshan, Foshan, China
[b] Department of Laboratory Medicine, Sun Yat-Sen Memorial Hospital of Sun Yat-Sen University, Guangzhou, China
[c] Department of Obstetrics and Gynecology, Sun Yat-Sen Memorial Hospital of Sun Yat-Sen University, Guangzhou, China
[d] Department of Pathology, Sun Yat-Sen Memorial Hospital of Sun Yat-Sen University, Guangzhou, China

HIGHLIGHTS

- ClC-3 is increased expression in cervical squamous cell carcinoma.
- ClC-3 silencing inhibited cervical squamous carcinoma cell migration and invasion.
- ClC-3 facilitated cervical squamous carcinoma cell migration and invasion via PI3K/Akt/mTOR signaling pathway.
- ClC-3 is a positive regulator of MMP-9 expression via PI3K/Akt/mTOR signaling pathway.

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ABSTRACT

Objective. Chloride channel-3 (ClC-3) plays significant roles in various physiological and physiopathological activities, including cell migration and invasion ability. The purpose of this study was to evaluate whether ClC-3 influences the migration and invasion of cervical squamous cell carcinoma cells and its possible mechanisms.

Methods. Paraffin-embedded cervical tissues, including normal cervical tissues, cervical squamous cell carcinoma (SCC) and homologous paracancerous tissues, were collected. The cervical squamous cell carcinoma and matched paracarcinoma fresh tissues specimens were collected from 49 patients with SCC, and the normal cervical tissues were collected from 45 non-cervical squamous cell carcinoma patients. The human cervical squamous carcinoma cell line SiHa was cultured. ClC-3 expression was assessed by real-time RT-PCR, immunohistochemistry and Western blot, and the expression of phospho-PI3K/Akt/mTOR and matrix metalloproteinase-9 (MMP-9) was detected by Western blot. Small interfering RNA (siRNA) technology was used to knockdown ClC-3 expression. SiHa cell migration and invasion ability were measured using Transwell assays with or without Matrigel-coated membranes.

Results. ClC-3 mRNA and protein expression in SCC tissues from cervical squamous cell carcinoma patients was significantly upregulated, and no significant difference was noted between the matched paracarcinoma fresh tissue from the same patients and non-cervical cancer patients. SiHa cell migration and invasion and phospho-PI3K/Akt/mTOR and MMP-9 expression were attenuated by knocking down ClC-3 expression using ClC-3 siRNA.

Conclusions. ClC-3 participates in the processes of SCC cell migration and invasion and regulates MMP-9 expression via the PI3K/Akt/mTOR signaling pathway.

1. Introduction

Cervical carcinoma is the second most frequent gynaecological malignancy, causing thousands of deaths annually in women worldwide. The vast majority of cases are caused by high-risk human papillomaviruses (HPV) infection. Although cervical cytology screening has facilitated early detection and treatment of cervical carcinoma, its

* Correspondence to: G. Wang, Department of Obstetrics and Gynecology, The First People's Hospital of Foshan, Foshan 528000, China.
** Correspondence to: Z. Lin, Department of Obstetrics and Gynecology, Sun Yat-Sen Memorial Hospital of Sun Yat-Sen University, Guangzhou 510120, China.
E-mail addresses: lin-zhongqiu@163.com (Z. Lin), wallace1971@163.com (G. Wang).

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incidence rate has increased yearly, and trend of younger onset is noted. Despite the close aetiological relationship between HPV and cervical carcinoma, persistent HPV infection is not sufficient to lead to the progression of human cervical carcinoma, and its specific pathogenesis has not been fully elucidated. Therefore, it is hypothesized that other cofactors may be related to the pathogenesis of cervical carcinoma. The identification of new factors associated with the progression of cervical carcinoma is particularly significant for the treatment and improvement of prognosis.

One such cofactor is chloride channel-3 (ClC-3), a member of the superfamily of voltage-gated chloride ion channels that participates in various cellular processes, including physiological and pathophysiological processes. In addition, ClC-3 plays a key role in regulating tumour cell proliferation, migration and invasion. Recently, increasing studies have found that ClC-3 is closely related to the malignant behaviour of cancer cells. In human osteosarcoma (OS) cells, ClC-3 is not only critical in cell proliferation but also an important factor for cell migration [1]. Studies have reported that ClC-3 is involved in promoting migration and invasion of glioma cells by mediating MMP-3 and MMP-9 expression [2]. Additionally, ClC-3 is involved in the regulation of various signal pathways, such as the TGF-beta/Smad signaling pathway [3], nuclear factor-kappaB pathway [4] and PI3K/Akt/mTOR pathway [5]. A previous study demonstrated that ClC-3 expression levels are increased in cervical cancer lesions and closely related to the progression and prognosis of women with cervical cancer. However, the exact mechanism of ClC-3 in cervical squamous cell carcinoma remains unclear.

The PI3K/AKT/mTOR signaling pathway is involved in regulating cell proliferation, apoptosis [6], migration and invasion [7]. Dysfunction of PI3K/AKT/mTOR signaling pathway is closely related to cancer development and therapeutic resistance. Downstream effectors of PI3K/AKT/mTOR include members of the matrix metalloproteinase (MMP) family, which participate in extracellular matrix and basement membrane degradation. MMPs are often activated in multiple cancers, which represents a significant event in cancer progression. Previous studies demonstrated an activated PI3K/Akt/mTOR signaling pathway significantly upregulates MMP-9 expression.

However, the clinical implication and biological effects of ClC-3 and the PI3K/Akt/mTOR signaling pathway in cervical squamous cell carcinoma have not been elucidated to date. The purpose of this research was to evaluate whether ClC-3 influences migration and invasion of cervical squamous cell carcinoma cells via the PI3K/Akt/mTOR signaling pathway.

2. Materials and methods

2.1. Ethical authorization

This research conforms to and acts in accordance with the Enhancing the QUAlity and Transparency of Health Research guidelines (http://www.equatornetwork.org/). All fresh human tissue specimens were collected by informed consent on the basis of the request of the Foshan First People's Hospital scientific research ethics committee from December 2017 to August 2018.

2.2. Reagents and antibodies

LY294002 (M1925) and Everolimus (RAD001) were purchased from AbMole BioScience (Houston, USA). Reagents were formulated and stored until use in accordance with the manufacturer's protocol. Most antibodies were purchased from Abcam (Cambridge, UK), including primary antibodies against ClC-3 (ab28736), PI3K (ab32089), p-PI3K (ab207485), Akt (ab126433), p-Akt (ab126580), mTOR (ab2732), p-mTOR (ab109268) and MMP-9 (ab137867) as well as horse radish peroxidase (HRP)-conjugated goat secondary antibodies against rabbit and mouse.

2.3. Human tissue collection

Paraffin-embedded cervical tissue specimens, including normal cervical tissues (n = 35), SCC (n = 40) and the corresponding paracancerous tissue (PC, n = 40), were collected for immunohistochemistry assay (IHC) from the files of the Pathology Department of The Sun Yat-Sen Memorial Hospital of Sun Yat-Sen University in 2017.

Chinese-Han fresh cervical tissue specimens, including cervical tissue (N, n = 45) from patients who accepted laparoscopic surgery for myoma of the uterus as well as cervical squamous cell carcinoma (SCC, n = 49) and relevant paracancerous normal cervical tissues (PC, n = 49) from patients who accepted laparoscopic surgery for cervical cancer as confirmed by pathological examination, were collected from the Department of Gynecology and Obstetrics (The First People's Hospital of Foshan, Sun Yat-Sen University) from December 2017 to August 2018 for protein and mRNA assessment. The relevant paracancerous normal cervical tissues were acquired 3 cm from the margin of cervical carcinoma tissues. According to the classification of International Federation of Gynaecology and Obstetrics (FIGO) in 2009, carcinoma patients were classified as stage IB to IIA. All of the patients did not undergo neoadjuvant chemotherapy or radiotherapy before radical operation.

2.4. Cell culture

SiHa human cervical squamous carcinoma cells were purchased from Guangzhou Jennio Biotech Co., Ltd. and cultured using DMEM medium (GIBCO) containing 10% foetal bovine serum (HyClone, USA) and double antibiotics (100 Units/ml penicillin and 100 μg/ml streptomycin) (GIBCO). SiHa cells were incubated in a humid atmosphere at 37 °C with 5% CO_2.

2.5. ClC-3 was silenced in SiHa cells

Small interfering RNA (siRNA) and negative control siRNA (NC) for ClC-3 were synthesized by Gene Pharmacy Technology (GenePharma Co., Ltd., Shanghai, China) and transfected into SiHa cells following previous experimental methods [8]. The primer sequences were as follows: ClC-3 siRNA sense 5′-CAAUGGAUUUCCUGUCAUATT-3′ and ClC-3 siRNA antisense 5′-UAUGACAGGAAAUC CAUUGTA-3′; negative control siRNA sense 5′-CAAUGGAUUUCCUGUCAUATT-3′ and negative control siRNA antisense 5′-UAUGACAGG AAAUCCAUUGTA-3′. Western blotting confirmed successful gene silencing.

2.6. Immunohistochemistry assay

Routine deparaffinization and rehydration were performed for the paraffin-embedded cervical tissue samples. Antigen retrieval was accomplished in 10 mM sodium citrate buffer, pH 6.0, for 10 min at 92–98 °C; the samples were then treated with 0.3% H_2O_2 for 15 min. Normal goat serum incubation was performed for 20 min to eliminate nonspecific binding, and after washing with PBS, the samples were incubated in the mouse anti-ClC-3 antibody at 4 °C overnight. The sections were washed with PBS and incubated in biotinylated secondary antibody for 60 min (1:800 dilution, Beyotime Biotechnology Inc.). The sections were then processed in ABC solution for 30 min at 37 °C prior to treatment with DAB (3,3′-diaminobenzidine) for 5 min. Counterstaining was performed with Harris haematoxylin. Positive staining was visualized as brown staining in the cell membrane or the cytoplasm.

The slides were imaged in an inverted microscope. For each slide, images of five random fields were acquired; using the Image-Pro Plus 6.0 image analysis software (Media Cybernetics, Rockville, MD) the mean immunostaining density was measured as described before [8].

2.7. MTT assay

About 3×10^3 SiHa cells transfected with siRNA-ClC3 or siRNA-NC were seeded in each well of 96-well plates and cultured for 48 h. Each well was treated with 20 μl of MTT reagent (5 mg/ml) and incubated for 4 h at 37 °C in 5% CO_2. After removing medium, 100 μl DMSO was added to each well and the crystals were dissolved in the dark for 15 min. The 490 nm absorbance was measured with a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Each chemical was tested in three independent experiments. Growth rate (%) = $(OD_{siRNA} - OD_{Solution}) / (OD_{Control} - OD_{Solution}) \times 100\%$.

2.8. Quantitative real-time PCR

ClC-3 mRNA levels in fresh cervical tissues and cultured cells were detected with real-time RT-PCR (Bio-Rad, USA) using the SYBR Green fluorescence signal test kit according to the manufacturer's operation protocol. Real-time RT-PCR quantified the levels of mRNA encoding and was performed in triplicate for each sample. The cycling conditions were described previously [8]. The sequences of the primer pairs for human ClC-3 are as follows: forward primer 5′-CCAGCTACAATGGATT TCCT-3′ and reverse primer 5′-AAAGGGCTCATGTCAAGAAT-3′. The human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as internal reference and amplified with the following primers: 5′-GCTGAGTACGTCGTGGAGTC-3′ (forward) and 5′-GATGGC ATGGACTGTGGTCA-3′ (reverse). The $2^{-\Delta\Delta Ct}$ method was employed to assess relative gene levels (folds), and all the tests were performed at least thrice.

2.9. Western blotting

Western blotting analysis is based on previous experimental methods [8]. Protein samples separated by SDS-PAGE were transferred to polyvinylidene difluoride (PVDF) membrane and incubated with the primary antibodies against ClC-3, PI3K, pPI3K, Akt, pAkt, mTOR, pmTOR and MMP-9, separately and detected by enhanced chemiluminescence (Amersham, GE Lifesciences). The proteins were measured by using the ECL system (CWBIOTECH, China) with the ChemiDoc XRS system (Bio-Rad, Philadelphia, USA). Using the Image-Pro Plus 6.0 image analysis software (Media Cybernetics, Rockville, MD) measured the density.

2.10. Cell migration and invasion

For the migration assays, SiHa cells were seeded to 24-well Transwell chambers at a density of 4×10^5 per well with 200 μl FBS-free medium in the upper chambers (8 μm, BD, Bedford, MA, USA). For the invasion assays, in addition to precoating with 24 μg/μl Matrigel (R&D Systems, USA) on Transwell membranes, the remainder of the steps were analogous to that of the cell migration tests, and 4×10^5 cells were plated in the above compartment. Then, 500 μl culture medium was used to fill the bottom wells, and 30% foetal bovine serum was added to induce cell migration and invasion. The cells on the upper surface of the filter are wiped off with a cotton swab after 24 h of culture. The cells on the submembrane surface were fixed with 4% ethanol polyformaldehyde for 20 min, stained with 0.1% crystal violet for 2 min, and photographed with a CCD camera under a microscope. Five fields were selected at random, and the number of cells were counted in each field.

2.11. Statistical analysis

All data are represented by the mean ± standard deviation (SD). Student's *t*-test was used for statistical comparison. Statistical analysis was performed using the SPSS 16.0 software (SPSS Inc., Chicago, USA). *P*-values < 0.05 were considered to indicate significant differences.

3. Results

3.1. Increased ClC-3 mRNA expression in cervical squamous cell carcinoma tissues

ClC-3 mRNA expression levels were detected in normal fresh tissues of the non- cervical cancer patients (normal) and cervical squamous cell carcinoma and matched paracarcinoma tissues from cervical squamous cell carcinoma patients. As presented in Fig. 1A, ClC-3 mRNA expression was significantly increased in the cervical squamous cell carcinoma specimens ($n = 49$, $P < 0.05$) compared with that in the matched paracarcinoma tissues specimens from the same patients ($n = 49$) and the control group ($n = 45$). In the 49 cases of cervical squamous cell carcinoma, 77.6% cervical squamous cell carcinoma specimens (38 cases out of 49 patients) exhibited increased ClC-3 expression (2.5-fold difference compared with paracarcinoma, $P < 0.05$), and 22.4% cervical squamous cell carcinoma specimens (11/49) did not exhibit increased ClC-3 mRNA expression. Compared with the control normal group (0.5 ± 0.12), no significant differences in ClC-3 mRNA expression levels were noted in paracarcinoma samples from cervical squamous cell carcinoma patients ($n = 49$, 0.7 ± 0.017) (Fig. 1A, $P < 0.05$).

3.2. Increased ClC-3 protein expression in cervical squamous cell carcinoma tissues

ClC-3 protein expression was tested by immunohistochemistry and Western blot. As presented in Fig. 1B, C, ClC-3 protein expression was increased in cervical squamous cell carcinoma specimens from cervical squamous cell carcinoma patients ($n = 49$, $P < 0.01$, Fig. 1B, C) compared with corresponding paracancerous tissue from the same patients ($n = 49$) and the control normal group ($n = 45$). ClC-3 protein expression was significantly increased in 38 out of 49 patients.

For further verification, ClC-3 protein expression in fresh cervical squamous cell carcinoma tissues was detected by Western blot (Fig. 1D, E). ClC-3 proteins in the cervical cancer tissue samples were remarkably overexpressed in 38 patients (out of 49 patients). Conversely, ClC-3 protein expression was low both in the paracarcinoma tissue and the control normal cervical tissues. These data indicated that ClC-3 protein expression is upregulated in cervical squamous cell carcinoma tissues.

3.3. ClC-3 silencing inhibited SiHa cell migration and invasion

The number of SiHa cell that migrated and invaded across the polycarbonate Transwell filters to reach the submembrane of the filter membrane was significantly reduced in cells transfected with ClC-3 siRNA compared with the control group or si-NC group ($P < 0.01$, Fig. 2). Compared with the control group, negative control siRNA had no significant effect on cell migration and invasion ($P > 0.05$). These data provide evidence that ClC-3 is a key protein that regulates human cervical squamous carcinoma cell migration and invasion.

3.4. ClC-3 silencing inhibited phospho-PI3K/Akt/mTOR in a cervical squamous carcinoma cell line

We assessed the relationship between ClC-3 expression and phosphorylation of the PI3K/Akt/mTOR signaling pathway using siRNA technology in the human cervical squamous carcinoma cell line SiHa. Using ClC-3 knockdown, the level of phospho-PI3K decreased by 2.4-fold (Fig. 3B), and phospho-AKT and mTOR phosphorylation were reduced by 2.5-fold compared with control (Fig. 3C, D). Phospho-PI3K/Akt/mTOR was not significantly changed in the negative control siRNA compared with the control normal group. Thus, ClC-3 clearly regulates the PI3K/AKT/mTOR signaling pathway.

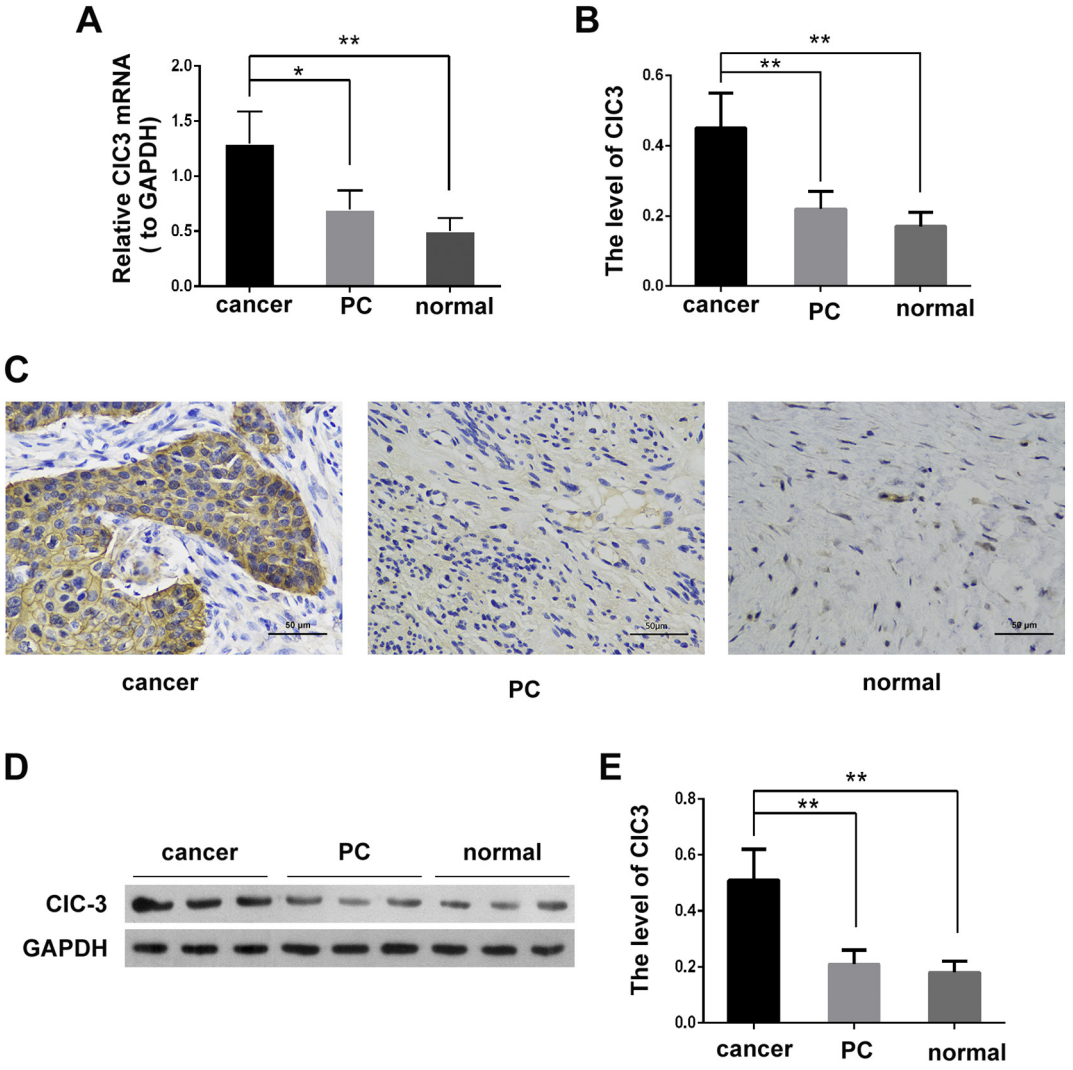

Fig. 1. Increased ClC-3 mRNA and protein expression in cervical cancer tissues from cervical cancer patients. (A) ClC3 mRNA expression levels detected by quantitative real time RT-PCR in the cervical cancer and matched paracarcinoma (PC) tissues from 49 patients with cervical cancer and in control cervical tissues (normal) from 45 non-cervical cancer patients. (B) Densitometric analysis of ClC3 protein expression in different tissues by immunohistochemical staining (35–40 patients). (C) Images of different tissues exhibiting ClC-3 immunohistochemical staining. Scale bars, 50 μm. (D) Representative Western blotting of ClC3 protein and GAPDH from different tissues (45–49 patients). (E) Densitometric analysis of ClC3 protein levels in different tissues detected by Western blot. Data in A, B and E were mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

3.5. ClC-3 facilitates cervical squamous carcinoma cell migration and invasion via the PI3K/Akt/mTOR signaling pathway

LY249002, a PI3K inhibitor, and Everolimus, a mTOR inhibitor, were employed to illuminate the effects of ClC-3 on cervical squamous carcinoma cell migration and invasion through the PI3K/AKT/mTOR pathway. SiHa cell migration and invasion decreased by 74% and 72%, respectively, after treatment with LY249002 (10 μM), and SiHa cell migration and invasion were reduced by 74% and 71%, respectively, after treatment with Everolimus (20 nM) (Fig. 4).

3.6. ClC-3 is a positive regulator of MMP-9 expression via the PI3K/Akt/mTOR signaling pathway

To elucidate the molecular mechanism of ClC-3 in the activation of cervical squamous carcinoma cell SiHa migration and invasion, we assessed the levels of MMP-9, a key member matrix metalloproteinases (MMPs) family that is often increased in cancer cells. We found that MMP-9 protein was decreased in cells wherein ClC-3 was downregulated with siRNA, and similar changes were noted with both LY249002 and Everolimus treatment in SiHa cells. Compared with the control group, negative control siRNA had no significant effect on MMP-9 expression, indicating that MMP-9 expression regulated by ClC-3 depends on the PI3K/Akt/mTOR signaling pathway (Fig. 5). These data collectively indicate that MMP-9 is regulated by ClC-3 in cervical squamous carcinoma cell through the PI3K/Akt/mTOR signaling pathway.

4. Discussion

Although ClC-3 plays an important role in cancer progression as confirmed by numerous studies, the role of ClC-3 in the development and progression of cervical squamous carcinoma has not been clarified. By studying a cervical squamous carcinoma cell line with a hyperactive PI3K/AKT/mTOR signaling pathway that results from high expression of the ClC-3 tumour promoter, we demonstrated that ClC-3 is a main positive regulator of cell migration and invasion, which is a hallmark of carcinoma development. The rate-limiting metalloproteinase MMP-9 is a primary downstream effector of mTOR signaling and is crucial for oncogenic PI3K/AKT/mTOR-mediated tumourigenesis.

In cervical cancer [9], under the hypotonic conditions of cervical cancer cell lines and primary culture of carcinoma cells in situ, the volume-sensitive chloride ion channels were activated, but these channels were

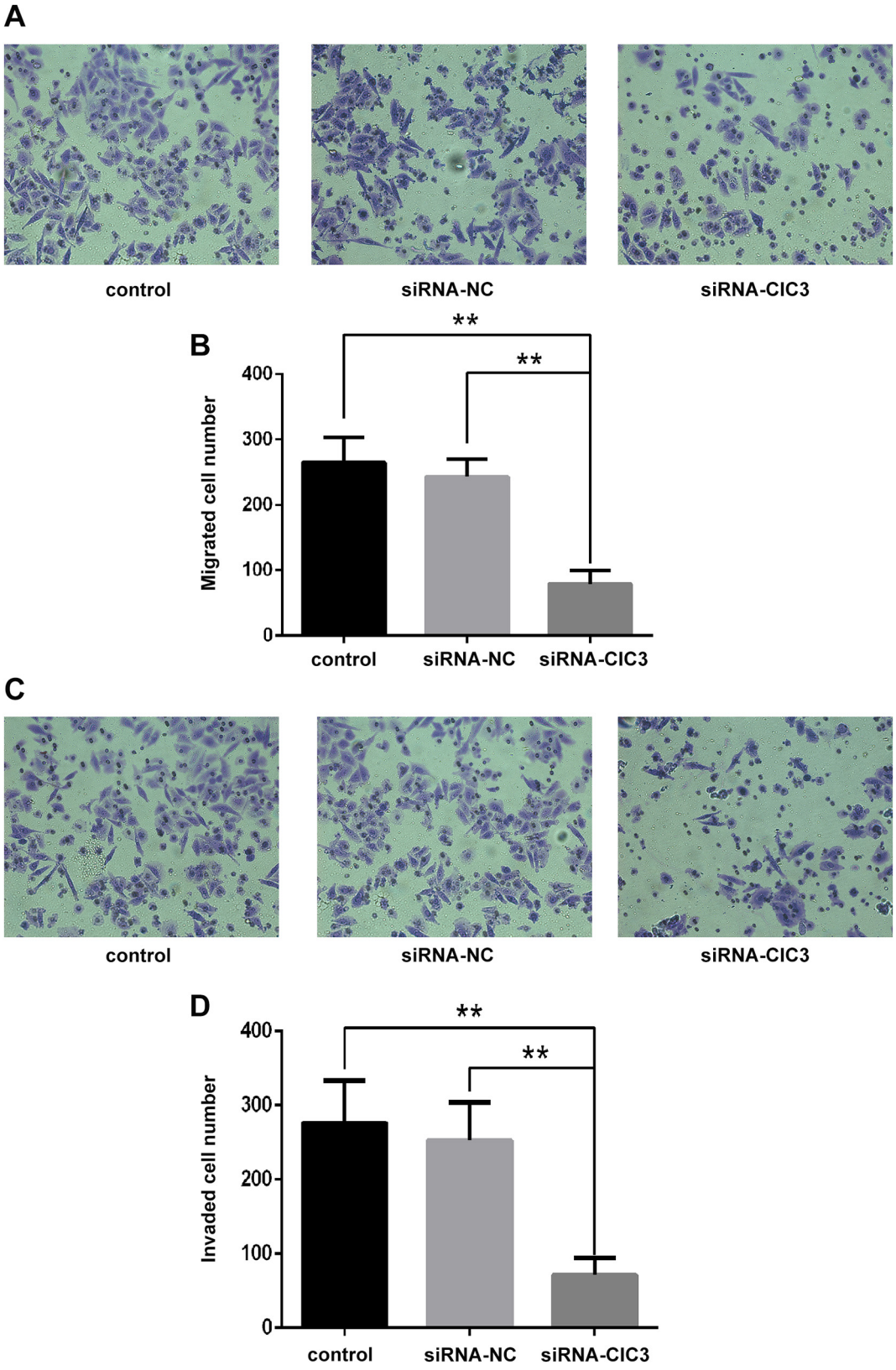

Fig. 2. Inhibition of SiHa cervical cell migration and invasion via knockdown of ClC-3 expression with ClC-3 siRNA. Cervical cells were incubated in control medium (control, no additives) or transfected with 20 μM ClC-3 siRNA (siRNA-ClC3) or negative control siRNA (siRNA-NC) in the presence of the transfection agent lipofectamine (5 μl/ml) for 48 h before detection of migration and invasion ability by Transwell assay. (A) Images depicting migrated SiHa cells on the lower surface of the Transwell membranes in different groups. Scale bar, 100 μm. (B) Number of migrated SiHa cells in five random fields as assessed via microscopy in different groups. (C) Images depicting invaded SiHa cells on the lower surface of the Transwell membranes in different groups. (D) Number of invaded SiHa cells in five random fields as assessed via microscopy in different groups (mean ± SD, n = 6). $^{**}P < 0.01$.

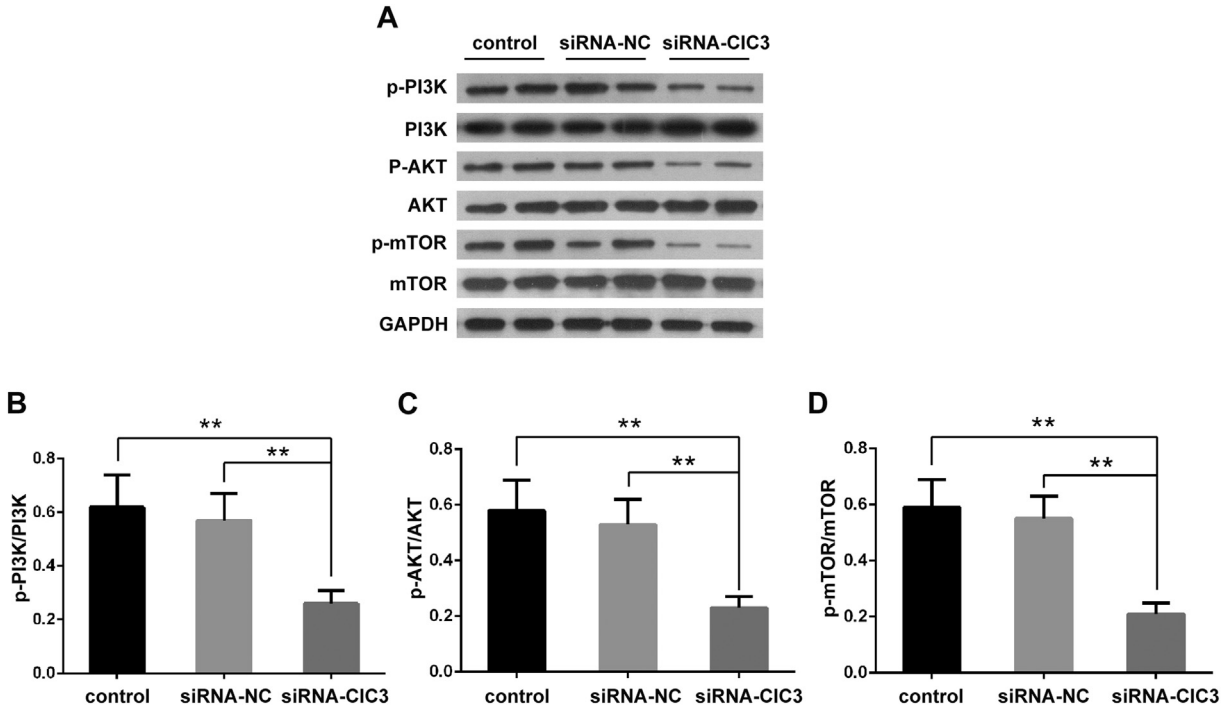

Fig. 3. ClC-3 mediates the PI3K/AKT/mTOR pathway in SiHa cells. (A) Western blotting analysis PI3K, p-PI3K (Tyr458), AKT, p-AKT (Ser473), mTOR, and p-mTOR (Ser2448) expression in SiHa cells. GAPDH was used as the loading control. (B–D) The intensities of the bands were quantified by densitometry analyses and normalized based on the amount of PI3K, AKT, or mTOR. Data are presented as the mean ± SD of three independent experiments; $^{**}P < 0.01$ compared with the control group.

not activated in normal cervix and human papillomavirus-immortalized cells. Moreover, the expression of volume-sensitive chloride currents was not associated with positivity for human papillomavirus infection. Several studies demonstrated that ClC-3 plays a role in regulating many cellular functions, including cell volume, proliferation, migration and invasion. ClC-3 is overexpressed in the plasma membrane of human glioma cells, and its activity facilitates glioma cells invasion into normal brain via facilitating dynamic regulation of cell volume [2]. Peng et al. [3] demonstrated that high expression of ClC-3 protein promotes the proliferation, migration and invasion of gastric cancer cells. In addition, high ClC-3 expression is related to adverse clinical pathology indicators and predicted poor prognosis. Nevertheless, silencing ClC-3 protein expression can suppress ovarian cancer cell migration and invasion [10].

Tumour cell migration and invasion are committed steps in tumour metastasis, and the geometrical morphology and volume of tumour cells must change during this process. Chloride ion channels may play a vital role in cell migration and invasion given their ability to coordinate ion and water movement through the plasma membrane. Interestingly, ClC-3 is involved in this process. Li et al. [11] demonstrated that ClC-3 increased endometrial cancer cell migration by promoting Cl^- ion transport through the endometrial cancer Cl^- channel and causing tumour cell volume changes. Moreover, it is believed that Ca^{2+}-activated K^+ channels bound to ClC-3 Cl^- channels to release K^+ and Cl^- ions together with obligatory water, causing glioma cells to rapidly shrink. This shrinkage facilitated cell entry into the narrow twisting extracellular brain space and promoted cell invasion and distant metastasis formation [12], suggesting that ClC-3 may accelerate cell migration and invasion via adjusting cell volume. In the present study, we demonstrated ClC-3 expression was closely related to cervical squamous carcinoma cell migration and invasion. On the other hand, knockout of ClC-3 expression can significantly restrain cervical squamous carcinoma cell migration and invasion. These data suggested ClC-3 might serve as an oncogene in cervical squamous carcinoma and exhibit an association with the progression of cervical squamous carcinoma.

ClC-3 is not only a chloride ion channel but also a regulatory protein involved in the regulation of multiple signaling pathways. The PI3K/AKT/mTOR signaling pathway plays a crucial role in regulating cell growth, migration, invasion, and distant metastasis [13,14]. Although abnormal PI3K/AKT/mTOR activation often occurs in both benign and malignant sicknesses, the mechanism of cervical cancer development modulated by the PI3K/AKT/mTOR signaling pathway remains unclear. Previous studies indicated that inhibition of PI3K/Akt/mTOR signaling pathway activation suppresses cancer cell growth, migration and invasion and inhibit cancer progression [15–17]. Moreover, studies have demonstrated that the PI3K/Akt/mTOR pathway is often dysregulated in cervical carcinoma and may be a latent therapeutic target for this malignant tumour [18], and suppression the PI3K/Akt/mTOR cascade can significantly attenuate cervical cancer cell proliferation and invasion [6]. Moreover, studies indicated that ClC-3 is involved in modulating the PI3K/AKT/mTOR signaling pathway. Fujimoto et al. [19] found that intracellular Cl^- regulation by ANO1/ClC-3 is involved in the transcription of human epidermal growth factor receptor 2 (HER2) and mediates the PI3K/AKT/mTOR signaling pathway in breast carcinoma cells. In our research, we found that inhibiting ClC-3 expression significantly decreased the expression of a phosphorylated PI3K/AKT/mTOR signaling pathway. Moreover, inhibiting the PI3K/AKT/mTOR pathway could partially suppress cervical squamous carcinoma cell migration and invasion. These results indicated that the canonical PI3K/AKT/mTOR signaling pathway participates in ClC-3-induced cervical squamous carcinoma cell migration and invasion.

Tumour cells undergoing invasion and distant metastasis required degradation of the extracellular matrix. Interestingly, in our study, ClC-3 expression is closely related to MMP-9 expression. Up-regulation of ClC-3 expression is correlated with cervical squamous carcinoma cell migration and invasion probably due to increased MMP-9 expression through the activated PI3K/AKT/mTOR signaling pathway. MMP-9 is a member of matrix metalloproteinase (MMP) family proteins, which degrade the extracellular matrix and play a vital role in migration and invasion of malignant tumours. In vitro studies demonstrate

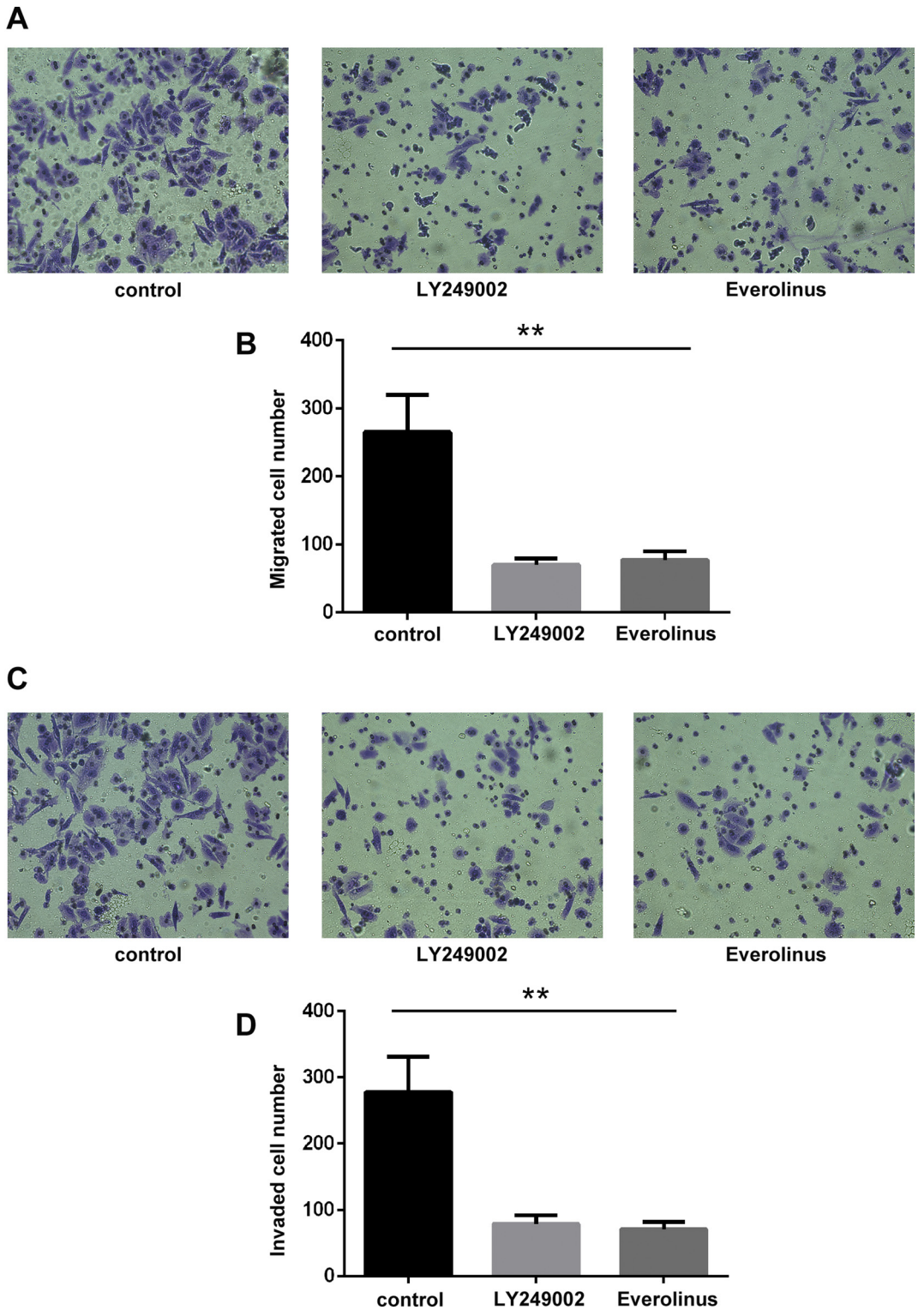

Fig. 4. Inhibition of SiHa cell migration and invasion by PI3K and mTOR inhibitors. SiHa cells were incubated in control medium (control, treated with DMSO) or treated with LY249002 (10 μM, PI3K inhibitor) or Everolimus (20 nM, mTOR inhibitor) for 48 h before detection of migration and invasion ability by Transwell assay. (A) Images depicting migrated SiHa cells on the lower surface of the Transwell membranes in different groups. (B) Number of migrated SiHa cell in five random fields as assessed by microscopy in different groups. (C) Images depicting invaded SiHa cells on the lower surface of the Transwell membranes in different groups. (D) Number of invaded SiHa cells in five random fields as assessed by microscopy in different groups (mean ± SD, n = 6). $^{**}P < 0.01$.

that silencing ClC-3 with SiClC-3 significantly reduces MMP-9 expression and decreases ovarian cancer cell [10] and glioma cell invasion and migration [2], but the underlying mechanism remains unclear. Fujimoto et al. [19] revealed that ClC-3 participates in the regulation of intracellular Cl^- and HER2-positive breast carcinoma cell transcription through the PI3K/AKT/mTOR signaling pathway. Furthermore, the activated PI3K/Akt/mTOR signaling pathway significant up-regulates the expression of MMP-9 [20], and mTOR is an activator of MMP-9 [21,22]. mTOR is the upstream regulator of hypoxia-inducible factor (HIF)-1 alpha [23]. HIF-1 alpha is a transcriptional factor which can

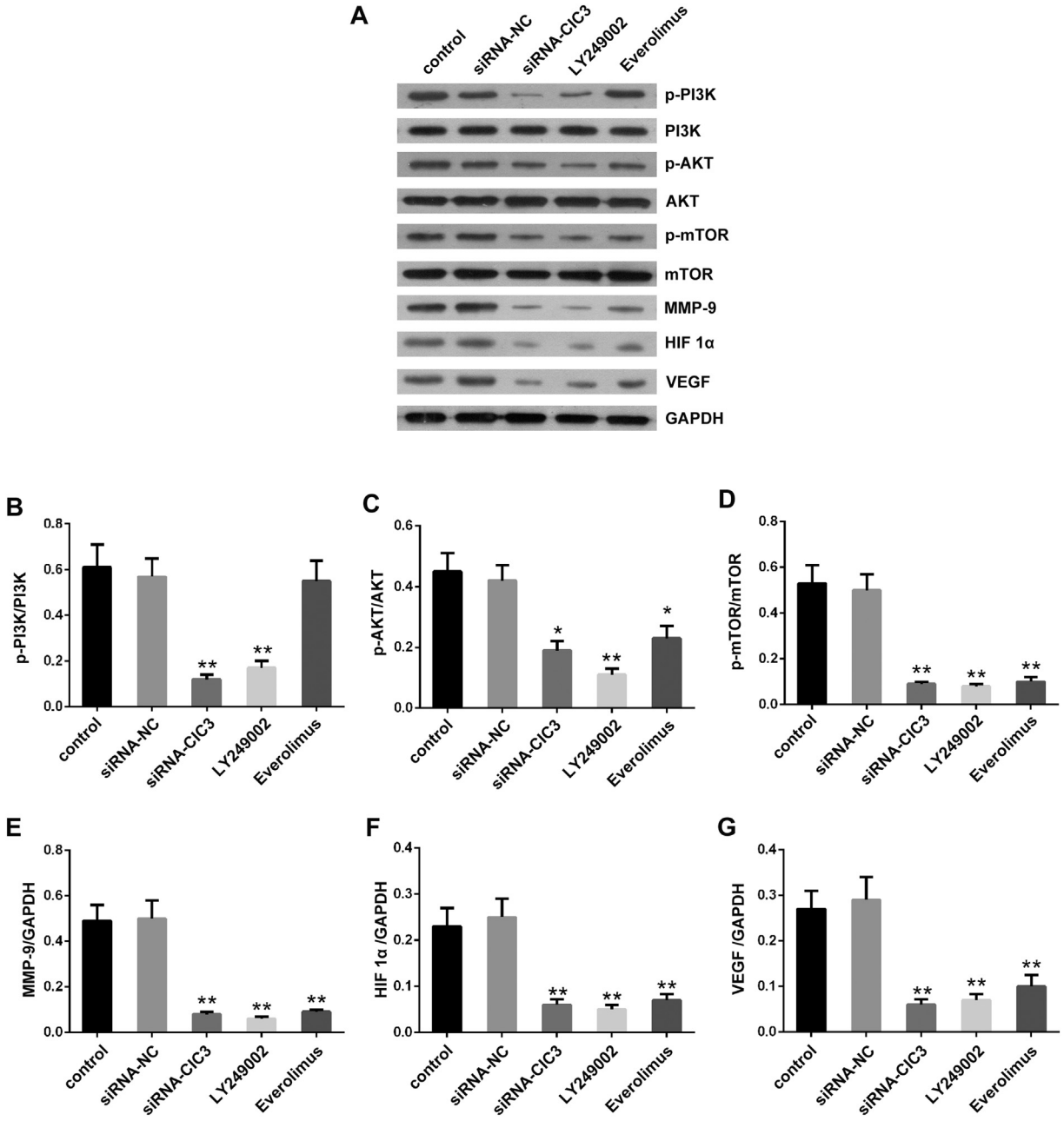

Fig. 5. ClC-3 is a positive regulator of MMP-9 expression via the PI3K/Akt/mTOR signaling pathway. (A) Western blotting analysis of PI3K, p-PI3K (Tyr458/Tyr199), AKT, p-AKT (Ser473), mTOR, p-mTOR (Ser2448), HIF 1 alpha, VEGF and MMP-9 expression in Siha cells treated with DMSO, siRNA-NC, siRNA-ClC3, LY249002 (10 mM), or Everolimus (10 mM), respectively. GAPDH was used as the loading control. (B–G) The intensities of the bands were quantified by densitometric analyses and normalized by the amount of PI3K, AKT, mTOR, HIF 1 alpha, VEGF or MMP-9 respectively. Data are presented as the mean ± SD of three independent experiments; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group.

active many genes including vascular endothelial growth factor (VEGF) and MMP-9 [24].

In summary, our study confirmed that ClC-3 activated PI3K/AKT/mTOR signaling pathway. Subsequently, through this signaling pathway, ClC-3 simulates MMP-9 expression and enhances cervical squamous carcinoma cell migration and invasion. This newly identified ClC-3/PI3K/AKT/mTOR/MMP-9 cascade is essential for cervical squamous carcinoma progression. We hypothesize that ClC-3 may be necessary to activate PI3K/AKT/mTOR signaling in the process of multistep tumourigenesis contributes to the progression of numerous human carcinomas. This research provides us with a mechanistic understanding of ClC-3 as a novel remedial target for tumour development, and ClC-3 may thus be targeted in cervical squamous carcinoma therapy.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Author contributions

Yu-Tao Guan, Zhong-Qiu Lin and Gang Wang designed the research. Hui Zhou, Weifeng Li, Xiao-Lu Zhang and Yi Luan performed experiments and analyzed data. Yang-Ping Chen and Li-Jiang Xu contributed

to sample collection. Xi-Ming Shen performed pathology detection. All authors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygyno.2019.03.006.

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