International Journal of Immunopathology & Pharmacology

Original Research Article

Knockdown of Ezrin inhibited migration and invasion of cervical cancer cells in vitro

International Journal of Immunopathology and Pharmacology
Volume 34: 1–10

DOI: 10.1177/2058738420930899
journals.sagepub.com/home/iji
SAGE

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Abstract

Cervical cancer is the fourth most common malignancy in women. The aim of this study was to investigate the functions of Ezrin in cervical cancer cells. Two cervical cancer cell lines, SiHa and CaSki, were cultured in vitro. Following the knockdown of Ezrin using siRNA, real-time reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis were applied to analyze Ezrin expression at the messenger RNA (mRNA) and protein levels. Subsequently, wound healing assay, transwell assay, and sulforhodamine B (SRB) assay were used to detect the migration, invasion, and viability of cervical cancer cells, respectively. Results revealed that Ezrin siRNA can notably inhibit the migration and invasion of SiHa and CaSki cells ($P < 0.05$). However, knockdown of Ezrin shows no effects on the viability of SiHa and CaSki cells ($P < 0.05$). It is indicated that Ezrin plays a possible role in promoting the migration and invasion of cervical cancer cells and may be a therapeutic target to prevent metastasis of cervical cancer.

Keywords

cervical cancer cell, Ezrin, invasion, migration, viability

Date received: 14 October 2019; accepted: 11 May 2020

Introduction

Cervical cancer is the most common malignant tumor of female reproductive system, which seriously threatens women's health.[1] There are about 529,000 new cases of cervical cancer in the world every year, of which 85% occur in developing countries.[2] China has about 1,315,000 new cases every year, accounting for one-third of the world's new cases of cervical cancer. Direct infiltration and lymph node metastasis are still the main causes of poor prognosis and death of cervical cancer.[3] Although remarkable progresses have been made in diagnosis and treatment of cervical cancer, the mechanism of invasion and metastasis of cervical cancer remains to be further elucidated.

Ezrin is a member of the Ezrin–radixin–moesin (ERM) protein family, and it is also a membrane cytoskeleton connexin that stabilizes the structure and function of the cell membrane region.[4] The main physiological functions of Ezrin protein include participating in the formation of microvilli; maintaining cell morphology; involving in cell movement, adhesion, and cytoskeleton remodeling; and mediating cell signal transduction process.[5] Recently, Ezrin is found to play an important role in tumor invasion and metastasis. A lot of studies have shown that Ezrin is abnormally

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expressed in many tumor tissues and involved in the invasion and metastasis of tumors such as breast cancer,[6] prostate cancer,[7] ovarian cancer,[8] colorectal cancers,[9] thyroid carcinoma,[10] and pancreatic ductal adenocarcinoma.[11]

To present, there are few studies on the role of Ezrin in cervical cancer.[9–15] For instance, aberrant localization of Ezrin has been reported to be involved in cervical cancer.[9] High Ezrin expression was observed in cervical cancer samples, indicating that Ezrin serves as a risk factor for progression of cervical cancer.[12–14] Furthermore, Ezrin can regulate epithelial-mesenchymal transition, and Ezrin down-regulation inhibits cervical cancer progression through the phosphoinositide 3-kinase/Akt pathway.[15] Moreover, the effect and mechanism of Ezrin in the invasion and metastasis of cervical cancer have not been reported. In order to elucidate the function of Ezrin in cervical cancer, siRNA interference technology was used to interfere with Ezrin gene expression and then scratch test, Transwell chamber method, and sulforhodamine B (SRB) method were used to detect the changes of invasion, migration, and viability of cervical cancer cells.

Materials and methods

Cell culture

Human cervical cancer cell lines SiHa and CaSki cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). SiHa and CaSki cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal bovine serum (FBS) at 37°C, 5% CO_2, and saturated humidity. Cells were digested by 0.25% + 0.02% EDTA every 3 days and passaged from 1:3 to 1:4.

siRNA transfection

SiHa and CaSki cells were cultured in 25-mL flat flask, and transfection was required when cells reached 80%–90% fusion. Ezrin siRNA (Forward: 5′-UCCACUA UGUGGAUAAUAA-3′; Reverse: 5′-UUAUUAUCCACAUAGUGGA-3′) and negative control siRNA powder (Forward: 5′-UCCACU AUGUGGAUAAUAA-3′; Reverse: 5′-ACGU GACACGUUCGGAGAA-3′) (Ambion, USA) were added into 100 μL of storage solution, and 10 μL of storage solution was then diluted to 100 μL (10 μm).

Cells at concentration of 1×10^6 cells/mL were incubated in six-well plates using RPMI-1640 medium containing 10% FBS for 24 h. Ezrin siRNA (30 nM) or negative control siRNA was transfected into each hole of six-well plates using siPORT™ NeoFX™ (Ambion). Cells treated with transfection reagent without siRNA were used as the blank control group, and cells transfected with negative control siRNA were used as the negative control group.

Real-time reverse transcription-polymerase chain reaction

Reverse transcription-polymerase chain reaction (RT-PCR) was used to detect messenger RNA (mRNA) expression of Ezrin after 48 h of transfection. Briefly, 2×10^5 cells were inoculated into six-well plates containing 2.5 mL of cell culture medium. After 24 h of culture, cells were transfected with Ezrin siRNA and negative control. Forty-eight hours later, RNA was extracted by phenol and chloroform. According to the instructions of miScript Reverse Transcription Kits (Takara, Japan), 1 g RNA was retrieved into cDNA. Primers for Ezrin (Biosune Biotechnology Shanghai Co., China) were as follows: 5′-GGAGCAGCTG ATTGAATTACGG-3′ (Forward), 5′-AGAGAG AGTAGCAGATG-3′ (Reverse). The relative expression of Ezrin was calculated by $2^{(-\Delta\Delta Ct)}$ method.[16] Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as the internal control.

Western blot

Protein expression of Ezrin was determined by Western blot after 48 h of transfection. The cells were lysed using lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% NP-40 (Sigma; St. Louis, MO, USA), 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mg/mL aprotinin, 1 mg/mL leu-peptin, and 1 mg/mL pepstatin. The protein concentrations were determined using the Bradford assay. Sixty micrograms of protein was separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA) for immunoblotting. The blots were blocked with 5%

non-fat dry milk in phosphate-buffered saline Tween-20 buffer (Sigma). The blots were then washed and incubated with primary Ezrin and GAPDH antibodies (Santa Cruz, CA, USA) at a 1:1000 dilution overnight at 4°C. The blots were washed with phosphate buffer solution containing tween 20 (PBST) and then incubated for 30 min with secondary antibody conjugated with horseradish peroxidase (1:10,000 dilution, Shanghai Youning Technology Co., Ltd, China). Immunoreactive protein signals were visualized by an enhanced chemiluminescence kit (ECL, Thermo, USA).

Scratch test

Cells were inoculated on the six-well plates. After cells grew to 100% abundance, the six-well plates were taken out and scratched on them with 100 μL aseptic gunhead. After the scratched cells were washed gently by phosphate-buffered saline (PBS), the six-well plates were placed in the medium containing 15% FBS and taken out 24 h later. The wound healing of cells was observed under a microscope. Five visual fields were selected randomly and migration distances were counted to show the migration ability of cells.

Transwell assay

Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was thawed in refrigerator at 4°C and then diluted by 1:5 in serum-free Opti-MEM medium (Sigma) at the next day. Fifty microliters of matrigel was added to the upper chamber of Transwell chamber which was put in a static position at 37°C for 1 h until coagulation. Cells were digested by trypsinase-EDTA at 24 h after siRNA transfection and resuspended in serum-free Opti-MEM medium at concentration of 1×10^6 cell/mL. One hundred microliters of cell suspension was inoculated in Transwell chamber (1×10^5 cells/each chamber) with 600 μL RPMI-1640 medium containing 10% FBS. After cells were incubated at 37°C for 24 h, Tweezers were taken out of Transwell chamber and washed with PBS for twice. Cells on the upper side of the filter membrane were gently wiped off with cotton swab, washed with PBS for twice, and stained with hematoxylin for 15 min. Six visual fields (10 × 20-fold) were photographed under the microscope for each sample.

SRB staining

Cell viability was detected by SRB staining (230162, Sigma). Briefly, cells were inoculated into 96-well plates at concentration of 5×10^3 per hole with the 100 μL final volume, and the incubation was closed after 48 h of transfection. Cells were treated with 50 μL of precooled 30% trichloroacetic acid (TCA) and fixed at 4°C for 1 h. Then, cells were washed with ddH_2O for five times, dried in a fume cabinet, and stained with 100 μL of 0.4% SRB dye solution (prepared with 0.1% glacial acetic acid) at room temperature for 30 min. Cells were washed using 0.1% glacial acetic acid for five times and then dried in a hood. One hundred microliters of Tris (10 mmol/L) was put into each hole of plates and oscillated for 10 min. Finally, the absorbance was detected by the microplate reader (Olympus, Tokyo, Japan) at 570 nm wavelength.

Statistical analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) software (Windows version 16.0; SPSS, Chicago, IL, USA). Data are expressed as mean ± standard deviation (SD). Mean difference between the two groups was determined by t-test; $P < 0.05$ represents a significant difference.

Results

Transfection efficiency of Ezrin siRNA

The mRNA and protein expression of Ezrin in both SiHa and CaSki cells was observed following Ezrin siRNA transfection. It was suggested that, compared with the negative control group, mRNA expression in Ezrin siRNA-treated SiHa cells (Figure 1(a)) and CaSki cells (Figure 1(b)) was down-regulated by 79% and 81%, respectively ($P < 0.01$). Furthermore, the protein expression level of Ezrin in SiHa cells (Figure 1(c)) and CaSki cells (Figure 1(d)) was decreased significantly compared with the negative control group ($P < 0.01$). However, there was no significant difference in Ezrin expression between negative control group and blank control group ($P > 0.05$) (Figure 1). The expression of comprehensive mRNA and protein of Ezrin was decreased significantly, indicating that the transfection efficiency of Ezrin was excellent, and it could be used in the functional tests.

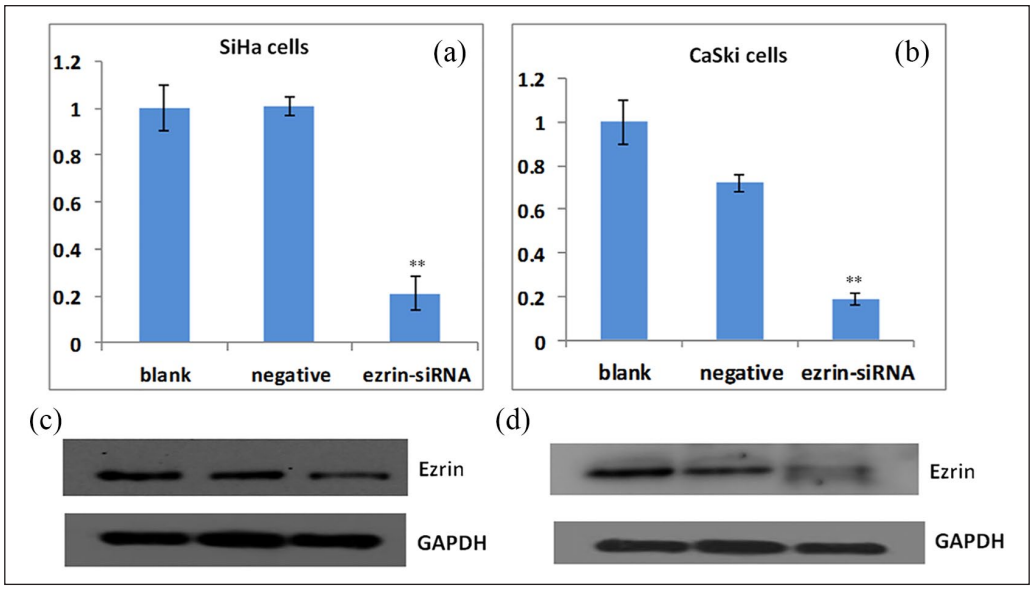

Figure 1. Transfection efficiency of Ezrin siRNA in SiHa and CaSki cells. Analysis of mRNA expression of Ezrin by RT-PCR in (a) SiHa cells and (b) CaSki cells. Analysis of Ezrin protein expression by Western blot in (c) SiHa cells and (d) CaSki cells.
**$P < 0.01$ compared with control.

Effects of Ezrin on migration of cervical cancer cells

SiHa cells and CaSki cells were scratched in blank group (Figures 2(a) and 3(a)), negative group (Figures 2(b) and 3(b)), and Ezrin siRNA group (Figures 2(c) and 3(c)) before transfection. After 48 h of transfection, changes in migration ability of cervical cancer cells were evaluated. It was found that after 48 h of Ezrin siRNA transfection, SiHa cells migrated from both sides of the wound to the middle, but the ability of cell migration was significantly inhibited. The distance of wound migration in the blank group was 5.20 times (Figure 2(d)) and in the negative group 3.25 times (Figure 2(e)) to that in Ezrin siRNA group (Figure 2(f)), and the difference was statistically significant ($P < 0.05$). Furthermore, compared to blank group (Figure 3(d)) and negative group (Figure 3(e)), migration ability of CaSki in Ezrin siRNA group (Figure 3(f)) was also inhibited significantly ($P < 0.05$).

Effects of Ezrin on invasion of cervical cancer cells

As shown in Figure 4, the invasive ability of SiHa cells in Ezrin siRNA group (Figure 4(c)) was significantly lower than that in the blank control group (Figure 4(a)) and the negative control group (Figure 4(b)). In addition, it was also found that, compared to the negative control (Figure 5(b)), Ezrin siRNA obviously inhibited invasion of CaSki cells (Figure 5(c)). The number of cells passing through in the Ezrin siRNA group was significantly less than that in the blank control group ($P < 0.01$), but there was no significant difference between the negative control group and the blank group (Figure 5(a)) ($P > 0.05$).

Effects of Ezrin on viability of cervical cancer cells

The viability of SiHa cells (Figure 6(a)) and CaSki cells (Figure 6(b)) transfected with Ezrin siRNA for 48 h was detected by SRB assay. Compared with the blank group and negative control group, the growth of SiHa cells and CaSki cells transfected with Ezrin-siRNA did not changed significantly at all time points, indicating that knockdown of Ezrin had no effect on the viability of cervical cancer cells ($P > 0.05$).

Discussion

A large number of studies have shown that Ezrin promotes tumor metastasis, but it is not clear how Ezrin exerts its functions in tumor cells.[17–19] Khanna et al.[20] first studied the role of Ezrin in osteosarcoma cells and found that Ezrin is involved

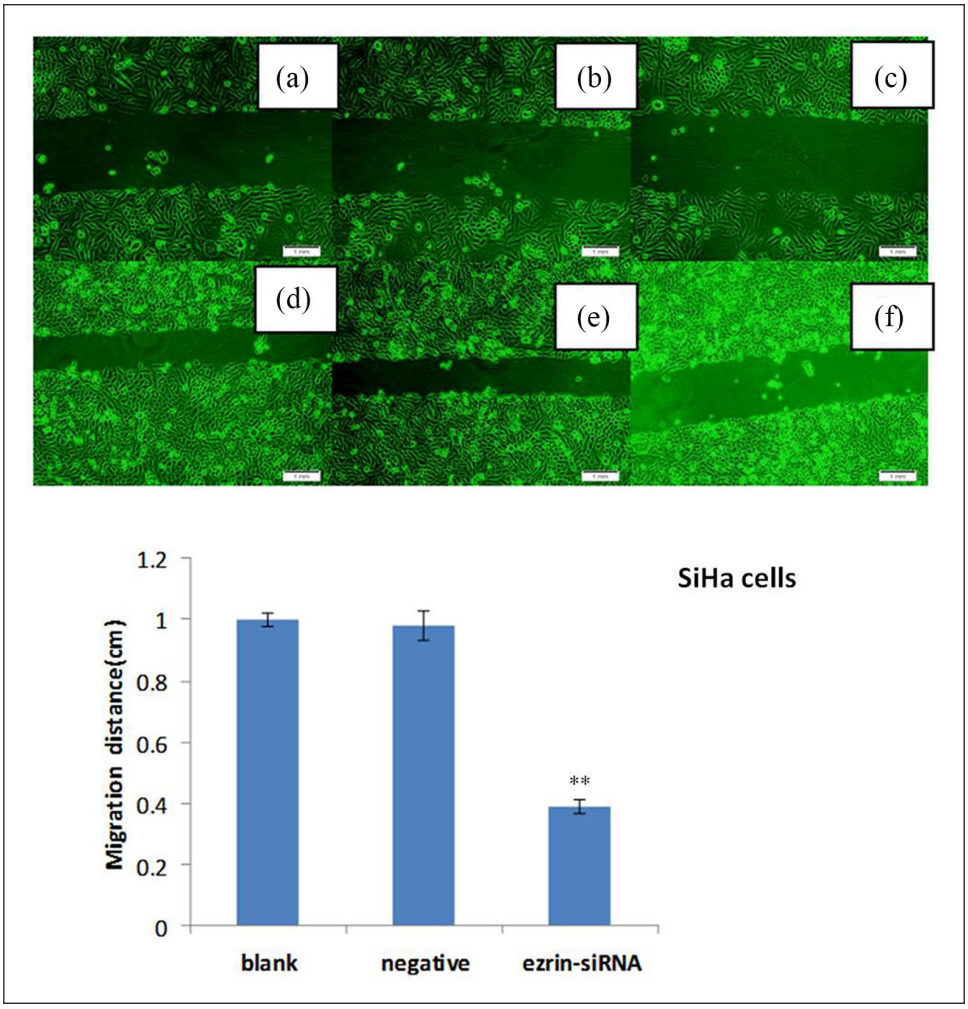

Figure 2. Effects of Ezrin on migration of SiHa cells. SiHa cells were scratched in (a) blank group, (b) negative group, and (c) Ezrin siRNA group before the transfection. Following 48 h of transfection, changes of migration ability in (d) blank group, (e) negative group, and (f) Ezrin siRNA group were evaluated.
**$P < 0.01$ compared with control.

in regulating cell invasion, migration, and adhesion. Furthermore, there is a significant difference in the expression of Ezrin between the two models of osteosarcoma with different invasiveness. In the study of osteosarcoma and rhabdomyosarcoma, Khanna et al.[21,22] screened Ezrin as a highly metastasis-related gene-by-gene chip. Transfection of Ezrin gene into low metastatic potential cell lines can lead to lung metastasis. Interestingly, in high metastatic potential cell lines, Ezrin expression inhibited by RNA interference and antisense RNA significantly reduces lung metastasis.

The same results also appeared in research of hepatocellular carcinoma. Zhang et al.[23] found that the expression of Ezrin is increased, which is significantly related to the proliferation, invasion, and migration of hepatocellular carcinoma cells. When the expression of Ezrin protein is specifically down-regulated, the invasion ability of hepatocellular carcinoma cell lines with high metastatic potential is also decreased significantly. Ilmonen et al.[24] found that the immunoreactivity of Ezrin is related to the thickness and invasiveness of melanoma. When the thickness of melanoma is increased, the higher immunoreactivity of Ezrin and the stronger invasiveness of tumor cells are found together. In another study, Ezrin was reported to promote invasion and metastasis of pancreatic cancer cells. The expression of Ezrin in Mia ez22-B cell line is 3.8 times higher than that in Miapcb6 cell line. It is proved that high expression of Ezrin can significantly increase the membrane processes

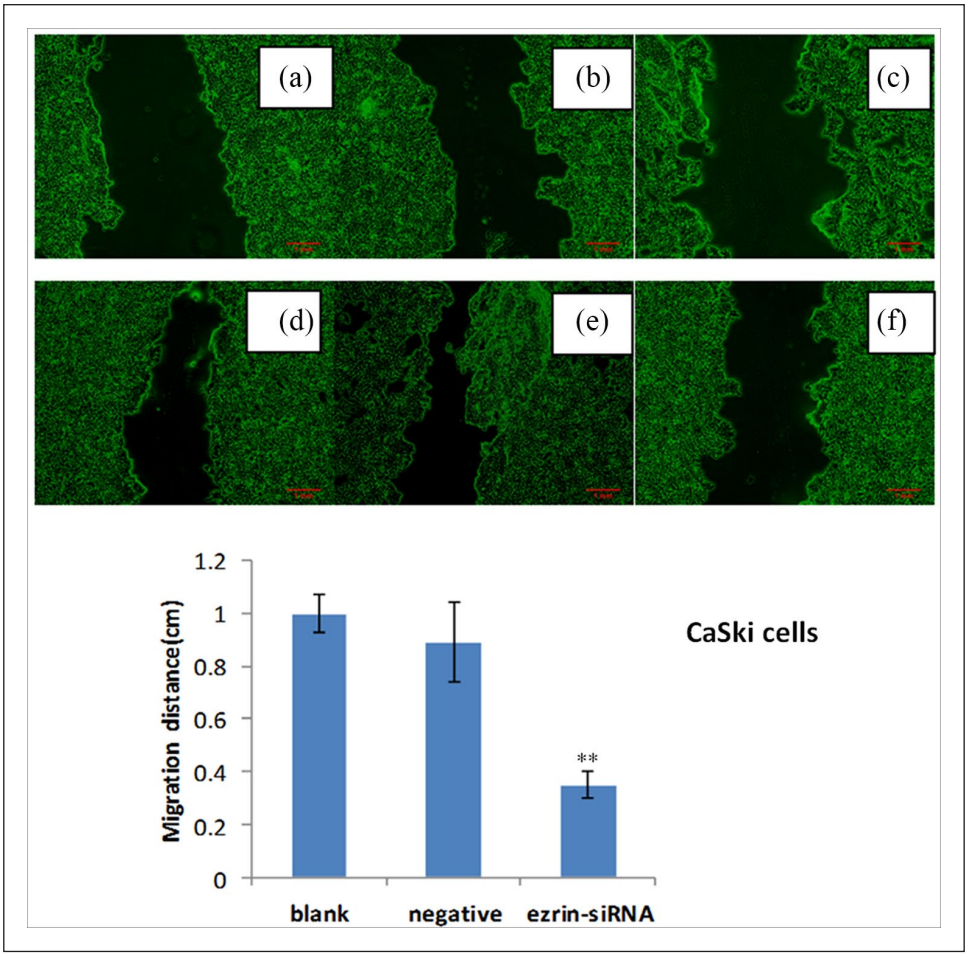

Figure 3. Effects of Ezrin on migration of CaSki cells. CaSki cells were scratched in (a) blank group, (b) negative group, and (c) Ezrin siRNA group before the transfection. Following 48 h of transfection, changes of migration ability in (d) blank group, (e) negative group, and (f) Ezrin siRNA group were evaluated.
$^{**}P < 0.01$ compared with control.

on the cell edge, cell movement, and invasion.[25] In a study investigating the functions of Ezrin in human metastatic melanoma, it was found that Ezrin and related molecules participate in the formation of phagocytic vacuoles, vacuole fusion, enhanced migration ability, and promote melanoma metastasis.[26] In vitro cell culture studies showed that the loss of Ezrin is related to the increase of motility and invasiveness of tumors and can affect the adhesion molecules on the cell surface and promote the invasion and metastasis of tumors.[27,28] In this study, it was suggested that interfering with Ezrin expression can effectively inhibit the migration and invasion of SiHa cells and CaSki cells, which is consistent with the role of Ezrin in various tumors reported in the literature.

Ezrin has been well studied in the metastasis of osteosarcoma and may be a key molecule to inhibit the metastasis of osteosarcoma. The differential expression of Ezrin between metastatic and non-metastatic osteosarcoma tissues was investigated, and the expression of Ezrin in metastatic lung tissues was found to be four times higher than that in non-metastatic lung tissues.[29] Detection of Ezrin protein expression in 32 patients with osteosarcoma showed that the high expression of Ezrin is negatively correlated with lung metastasis time and total survival time.[30] The expression rate of Ezrin protein in primary and metastatic lesions of osteosarcoma is 62% and 84.5%, respectively. The average expression of Ezrin protein in pulmonary metastases is significantly higher than that in primary lesions of osteosarcoma.[31]

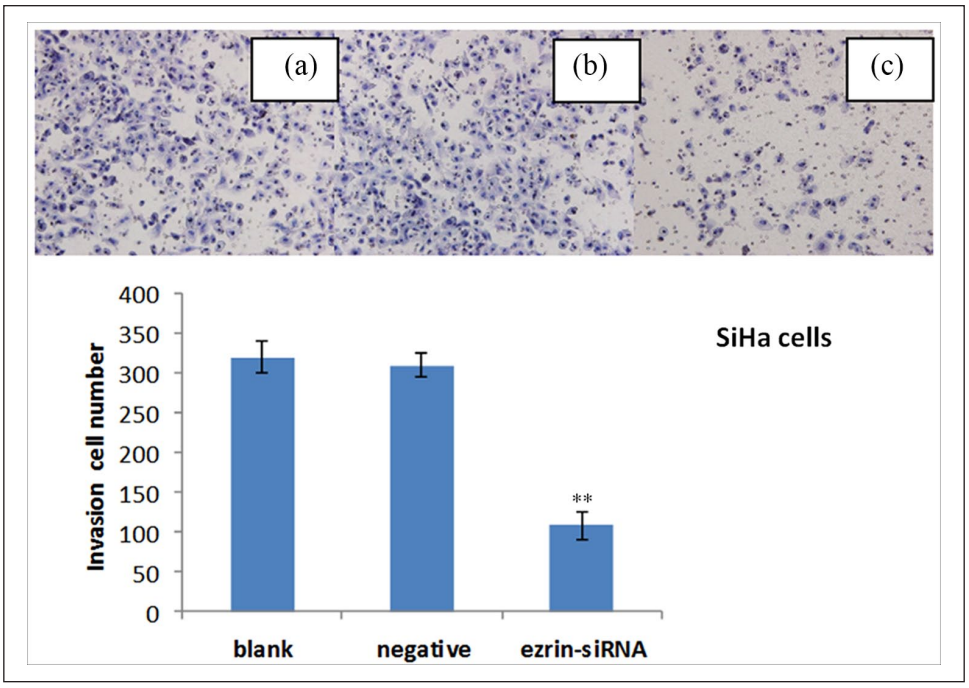

Figure 4. Effects of Ezrin siRNA on invasion of SiHa cells (×400). Invasion of SiHa cells was measured by transwell assay in (a) blank, (b) negative, and (c) Ezrin siRNA groups.
**$P < 0.01$ compared with control.

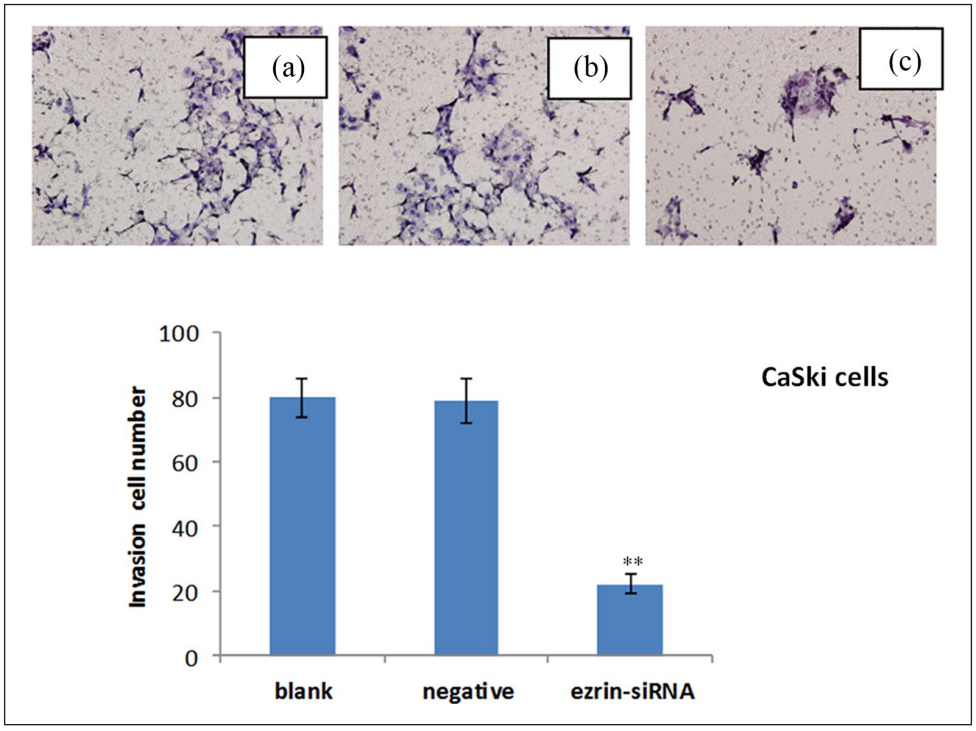

Figure 5. The effects of Ezrin siRNA on invasion of CaSki cells (×400). Invasion of CaSki cells was measured by transwell assay in (a) blank, (b) negative, and (c) Ezrin siRNA groups.
**$P < 0.01$ compared with control.

At present, the upstream regulation mechanism of Ezrin is seldomly studied. It has been suggested that the protein kinase C (PKC) pathway dynamically regulates the expression of Ezrin. PKC

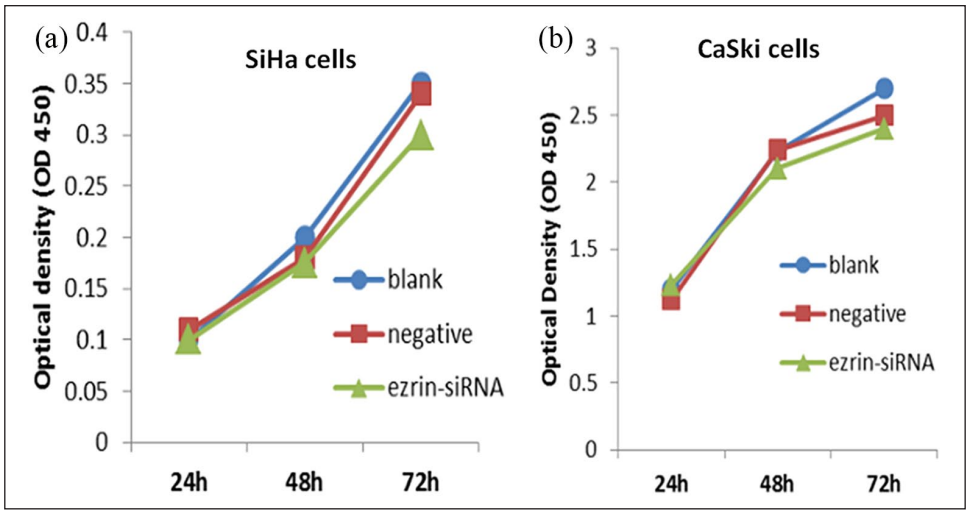

Figure 6. Effects of Ezrin siRNA on viability of SiHa and CaSki cells. The viability of (a) SiHa cells and (b) CaSki cells was determined by SRB assay.

pathway can phosphorylate 567 threonine at the carboxyl end of Ezrin protein and then activate Ezrin protein and mediate the metastasis of tumors. Inhibition of PKC expression decreases the metastasis of tumors. Interestingly, interfering with the expression of Ezrin also has the same effect as inhibiting the expression of PKC.[31] Another study has demonstrated that Ezrin and beta 4 integrin can promote the metastasis of osteosarcoma. In osteosarcoma cell lines with high metastatic potential, silencing or interfering with the beta-4 integrin gene significantly reduces the metastasis of osteosarcoma and inhibits the occurrence of lung metastasis.[32,33] However, the detailed mechanism of Ezrin in the metastasis of tumors still needs more researches.

There are still several limitations in this study. First, we did not take in vivo investigations to prove the results in vitro. Second, how overexpressed Ezrin exerts its function in SiHa and CaSki cells remains unknown. Third, the target of Ezrin in regulating the functions of cervical cancer cells is not studied. Fourth, current studies have not fully revealed the relationship between the Ezrin expression level and location and the occurrence and development of cervical cancer, the relationship between Ezrin expression and human papillomavirus (HPV), and whether Ezrin is involved in the progress of cervical precancerous lesions. So, it needs to further investigate the participation of Ezrin in specific signaling pathways of cancer metastasis and to find effective intervention targets for comprehensively studying the metastasis process of cervical cancer.

Conclusion

In summary, it is proved that knockdown of Ezrin inhibits invasion and migration of SiHa and CaSki cell lines. However, it shows no effect on viability of SiHa and CaSki cell lines. It is indicated that Ezrin is involved in the regulation of cervical cancer, which maybe a crucial target for cervical cancer treatment.

Author contributions

M.X. and W.T. are responsible for the guarantor of integrity of the entire study, study concepts and design, definition of intellectual content, literature research, clinical studies, experimental studies, data acquisition and analysis, statistical analysis, manuscript preparation, and editing and review. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

Informed consent

For this type of study, informed consent is not required.

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