Inhibition of Ezrin suppresses cell migration and invasion in human nasopharyngeal carcinoma

YUANYUAN TANG[1], XIUZHEN SUN[1], SHEN YU[2], XU BIE[1], JIZHE WANG[1] and LIDAN REN[3]

[1]Department of Otolaryngology Head and Neck Surgery, The Second Affiliated Hospital of Dalian Medical University, Dalian, Liaoning 116027; [2]State Key Laboratory of Structural Analysis for Industrial Equipment, Dalian University of Technology, Dalian, Liaoning 116023; [3]Department of Oncology, The 210th Hospital of PLA, Dalian, Liaoning 116000, P.R. China

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Abstract. Nasopharyngeal carcinoma (NPC) is one of the most severe types of malignant cancer of the head and neck as it is difficult to treat. Ezrin is highly expressed in numerous types of cancer. However, the role of Ezrin in NPC has not been fully investigated and further studies are required in order to uncover its therapeutic potential in the treatment of NPC. The aim of the present study was to investigate the expression of Ezrin in human NPC and to evaluate the effect of knockdown of Ezrin using small interfering (si)-RNA on NPC cell migration and invasion. The expression levels of Ezrin were determined using reverse transcription-quantitative polymerase chain reaction, immunohistochemical staining and western blotting. Following transfection of Ezrin-siRNA into NPC cells, cell invasion and migration were analyzed and the mRNA expression levels of matrix metalloproteinase(MMP)-2 and MMP9 were determined. The results revealed that the expression of Ezrin was markedly increased in human NPC tissue samples compared with normal adjacent nasopharyngeal tissue samples. Ezrin was also highly expressed in the NPC cell lines 6-10B and C6661 when compared with the normal nasopharyngeal cell line NP69. Transfection of NPC cell lines with siRNA targeting Ezrin significantly inhibited NPC cell migration and invasion, and downregulated the mRNA expression level of MMP2; however, no effect was observed on MMP9 mRNA expression. At the same time, knockdown of Ezrin significantly decreased the expression levels of phosphatidylinositol 3-kinase (PI3K) and phosphorylated protein kinase B (Akt), which downregulated the mRNA expression of MMP2. In conclusion, the results revealed that knockdown of Ezrin suppressed NPC migration and invasion by reducing the mRNA expression of MMP2 via the PI3K/Akt signaling pathway. These results highlight the important role of Ezrin in NPC cell migration and invasion. In addition, they indicate that silencing of Ezrin may serve as a potential therapeutic strategy to treat human NPC.

Correspondence to: Professor Xiuzhen Sun, Department of Otolaryngology Head and Neck Surgery, The Second Affiliated Hospital of Dalian Medical University, 467 Zhongshan Road, Dalian, Liaoning 116027, P.R. China
E-mail: sunxiuzhen001@163.com

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Introduction

Nasopharyngeal carcinoma (NPC) is one of the most severe types of head and neck malignant tumor (1). In the United States, the incidence of NPC ranges from 1-2 cases per 100,000 male and 0.4 cases per 100,000 female. The incidence rate of NPC in Asia is very high ranging from 0.6 cases per 100,000 male and 0.5 cases per 100,000 female patients (1). Incidence rates among the Cantonese population of southern China are as high as 25-50 cases per 100,000 and may account for 18% of all cancers in that area. However, the cause of NPC remains unclear (1). In recent years, concurrent radiotherapy and chemotherapy have been used the standard treatment for NPC. However, the recurrence rate of NPC following treatment remains as high as 10-40% (1-3). The combination of chemotherapy and radiation therapy has not improved the 5-year survival rate of patients, as different outcomes have been observed in patients of the same age, sex and clinical stage due to differences in the molecular markers of the tumors (2-4). Therefore, there is an urgent requirement to identify novel molecular markers and reveal their role in the treatment of human NPC.

In 1981, Ezrin was purified from the brush epithelium cells of chicken small intestines (5). The Ezrin gene is located on human chromosome 6q25-6q26 (6). The relative molecular weight of the Ezrin protein is ~81 kDa and consists of 585 amino acids (7). The amino terminus of Ezrin is attached to the cell membrane, while the carboxyl terminal is bound to the actin core. Ezrin is part of the Ezrin-Radixin-Moesin family of proteins, which function as linkers between the plasma membrane and the actin cytoskeleton. As such, they serve key roles in the regulatory networks of numerous cellular processes, including cell survival, proliferation, adhesion and migration (8,9). Although previous studies have revealed that Ezrin is highly expressed in a number of cancer types (10-12),

to the best of our knowledge, the expression and functional roles of Ezrin in NPC have not been fully investigated.

In the present study, the expression of Ezrin was assessed using immunohistochemical (IHC) staining, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis with human NPC tissue samples, adjacent normal nasopharyngeal tissue samples and the human NPC cell lines 6-10B and C6661. Subsequently, small interfering RNAs (siRNAs) targeting Ezrin were transfected into NPC cells. Transwell assays were performed to investigate the effect of Ezrin silencing on NPC cell migration and invasion. The mRNA expression levels of matrix metalloproteinase (MMP)2 and MMP9 were also investigated and the molecular mechanism of Ezrin in human NPC cell migration and invasion was further examined.

Materials and methods

Tissues, cell lines, cell culture and inhibitor treatment. The NPC cell line C6661, the normal human nasopharyngeal epithelial cell line NP69, and HT29 cells were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The human NPC 6-10B cell line was obtained from the Sun Yat-Sen University Cancer Centre (State Key Laboratory of Oncology in South China, Guangzhou, China). RPMI-1640 cell culture medium and fetal calf serum (FCS) were purchased from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Cells were cultured in RPMI-1640 medium supplemented with 10% FCS at 37˚C in a humidified incubator under 5% CO_2. Confluent cells (70-80%) were passaged using 0.25% (w/v) trypsin solution in 0.02% (w/v) EDTA.

For analysis of the role of the phosphatidylinositol 3-kinase (PI3K) pathway, 1 h after passage of cells, PI3K inhibitor (LY294002; 20 μM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added to the culture medium and the cells were cultured at 37˚C for a further 24 h. The mRNA expression of MMP2 was detected by RT-qPCR.

Due to the limitation of funds, 20 human NPC tissue samples and 20 respective adjacent normal nasopharyngeal epithelial tissue samples (1 cm apart) were collected from patients at the Department of Otolaryngology Head and Neck Surgery of the Second Affiliated Hospital of Dalian Medical University (Dalian, China) and written informed consent was obtained from all participants. A total of 20 patients were recruited between January 2016 and January 2017 (12 males and 8 females; age range, 41-78 years, mean age 51 years). Patients had confirmed NPC diagnoses and had not previously undergone radiation or chemotherapy prior to cancer tissue resection. During routine surgery that was performed at the Second Affiliated Hospital of Dalian Medical University, cancer tissues and adjacent normal nasopharyngeal epithelial tissues were collected. The present study was approved by the Ethical Committee of Dalian Medical University (Dalian, China).

IHC staining. IHC staining of Ezrin was performed using an anti-Ezrin antibody. Briefly, tissue sections (4-μm-thick) embedded in paraffin were sequentially deparaffinized and rehydrated through a descending alcohol series (100, 95, 90, 75, 50%), and washed with xylene. Then antigens were retrieved at 95˚C using 10 mM citrate buffer (pH 6.0; Merck KGaA). Freshly prepared 3% H_2O_2 was used to quench the endogenous peroxidase activity. Non-specific staining was blocked with 10% normal serum (Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 1 h. Subsequently, sections were incubated with anti-Ezrin primary antibodies (1:300; cat no. NBP2-16396; Novus Biologicals, LLC, Littleton, CO, USA) at 37˚C for 2 h, which was followed by incubation with goat anti-rabbit IgG biotinylated antibodies (1:1,000; cat no. BAF008; R&D Systems, Inc., Minneapolis, MN, USA) at room temperature for 30 min. Following this, sections were washed with PBS and then incubated with horseradish peroxidase-conjugated streptavidin (1:2,000; cat no, N100; Thermo Fisher Scientific, Inc.) for 5 min at room temperature. Then, 3,3'-diaminobenzidine substrate (Gene Tech Co., Ltd., Hong Kong, China) was used to develop the immunostaining for 10 min at room temperature. Finally, sections were counterstained with hematoxylin (Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 3 min. Sections were incubated with PBS as a negative control. The cells were examined under an inverted-TS100 microscope (magnification, x200; Nikon Corporation, Tokyo, Japan).

RT-qPCR. RNA from cells was obtained using a RNeasy Mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol (13). RT was performed as follows: A mixture of 2 μg total RNA and 1 μl Oligo (dT) was incubated at 70˚C for 5 min, then rapidly chilled on ice and subsequently added to a reaction mixture containing 5 μl M-MLV RT 5X Buffer (Promega Corporation, Madison, WI, USA), 0.5 μl deoxyribonucleotide triphosphate (25 μM; Promega Corporation), 0.7 μl RNA inhibitors (Promega Corporation) and 1 μl M-MLV reverse transcriptase (200 U/μl; Promega Corporation). The reaction volume was made up to 25 μl with RNA-free water and subsequently incubated at 42˚C for 1 h, followed by 70˚C for 10 min. qPCR was performed on an Applied Biosystems 7900HT thermal cycler instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Briefly, qPCR was performed in a 20 μl reaction volume including 2X Mix SYBR Green I (10 μl; Promega Corporation), primer (0.25 μl, 10 pmol/l), template DNA (1 μl) and sterile water. The thermocycling conditions were as follows: Initial denaturation at 95˚C for 2 min, followed by 50 cycles of 95˚C for 15 sec and 60˚C for 45 sec. Fold changes of target gene expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (14). The following primer sequences were used: Human Ezrin forward, 5'-CCAACAGCAGACACG CATCT-3' and reverse, 5'-GGTGAGCTGCTCCCTGTCTT-3'; human MMP2 forward, 5'-CTCGGTAGGGACATGCTAAGT AGAG-3' and reverse, 5'-CCTCTGGAGGTTCGACGTGA-3'; human MMP9 forward, 5'-TCATGAGGAAGAGCTCTG AGT-3' and reverse, 5'-TCATGAGGAAGAGCTCTGAGT-3'; human PI3K forward, 5'-AGATAACTGAGAAAATGAAAG CTCACTCT-3' and reverse, 5'-TGTTCATGGATTGTGCAA TTCC-3'; and human GAPDH forward, 5'-GAAGGTGAA GGTCGGAGTC-3' and reverse, 5'-GAAGATGGTGATGGG ATTTC-3'. The relative mRNA expression levels of Ezrin, PI3K, MMP2 and MMP9 were normalized to the respective endogenous GAPDH mRNA expression.

Western blot analysis. Radioimmunoprecipitation assay buffer (Sigma-Aldrich; Merck KGaA) and protease inhibitor (Sigma-Aldrich; Merck KGaA) were used to lyse cells. Following the collection of protein, a BCA Pierce assay (Thermo Fisher Scientific, Inc.) was used to quantify the protein concentration. A total of 50 μg protein from each sample was denatured and resolved by 10% SDS-PAGE (EMD Millipore, Billerica, MA, USA) and then electro-blotted to a polyvinylidene fluoride (PVDF) membrane (EMD Millipore). The PVDF membrane was incubated with 5% non-fat milk for 1 h at room temperature, and then incubated with specific monoclonal antibodies against Ezrin (1:500; cat no. NBP2-16396; Novus Biologicals, LLC), PI3K (1:500; cat no. 4292; Cell Signaling Technology, Inc., Danvers, MA, USA), phosphorylated (p)-Akt (1:500; cat no. 4060S; Cell Signaling Technology, Inc.) , Akt (1:500; cat no. 4691s; Cell Signaling Technology, Inc.) and β-actin (1:1,000; cat no. NB600-503; Novus Biologicals, LLC) at 4˚C overnight. After washing with TBST three times, the PVDF membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (1:3,000; cat no. NB710-57836; Novus Biologicals, LLC) for 1.5 h at room temperature. Finally, signals were developed by enhanced chemiluminescence (Pierce; Thermo Fisher Scientific, Inc.). The optical density of each protein band was quantified using a scanning densitometer and Quantity One software (version 4.4.1; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each lane of protein band density was normalized against the corresponding β-actin densities.

Knockdown of Ezrin and PI3K in 6-10B and C6661 cells by RNA interference. The expression of Ezrin and PI3K in 6-10B, C6661 cells was silenced using specific siRNAs targeting Ezrin (cat no. AM16708; Thermo Fisher Scientific, Inc.) and PI3K (cat no. 143975; Thermo Fisher Scientific, Inc.). NPC cells transfected with scrambled-siRNA (siNC; cat no. 4390846; Thermo Fisher Scientific, Inc.) were used as the control. The Ezrin siRNA sequences were: Sense, 5'-GCUCAAAGAUAA UGCUAUGTT-3', and antisense, 5'-CAUAGCAUUAUCUUU GAGCTT-3'. The PI3K siRNA group sequences were: Sense, 5'-GCCAGUACCUCAUGGAUUAGAAGdAdT-3', and anti-sense, 5'-AUCUUCUAAUCCAUGAGGUACUGGCUU-3'. The scrambled siRNA sequences were: Sense, 5'-UUCUCC GAACGUGUCACGUdTdT-3', and antisense, 5'-ACGUGA CACGUUCGGAGAAdTdT-3'. Briefly, 6-10B and C6661 cells ($1x10^5$ cells/ml) were re-suspended in cell culture medium. Transfection complexes were prepared by mixing RNAiMAX Lipofectamine™ transfection agent (Ambion; Thermo Fisher Scientific, Inc.) and siRNA (20 nM) in cell culture medium. Then, 6-10B and C6661 cells and the transfection complexes were mixed and incubated for 24 h at 37˚C in 6-well plates ($2x10^5$ cells/well; Nalge Nunc International, Penfield, NY, USA). Cells were collected at 48 h post-transfection for RNA analysis and at 72 h for protein analysis. The knockdown of Ezrin was confirmed by RT-qPCR and western blot analysis.

In vitro cell migration and invasion assay. Cell migration was measured using the BD Falcon HTS multi-well insert system (pore size, 8.0 μm) (15). The lower chamber of the wells was filled with 500 μl RPMI-1640 cell culture medium containing 10% FCS. After siRNA transfection, 6-10B and C6661 cells suspended in serum-free cell culture medium were added into the upper chamber of the insert at a density of $1.5x10^5$ cells/ml and incubated for 6 h at 37˚C. Cotton swabs were used to gently scrape off the non-migrated cells. The migrated cells were fixed with 4% formaldehyde for 20 min at room temperature, and then stained with 0.1% crystal violet for 30 min at room temperature. Milli-Q water was used to wash away the excess stain and the filters were dried overnight. Migrated cells were counted in five random fields and images were obtained using a light microscope and a camera (magnification, x200; Nikon Digital Camera Dxm 1200F; Nikon Corporation).

The cell invasion assay was performed using BioCoatMatrigel invasion chambers (BD Biosciences, Franklin Lakes, NJ, USA) (16). A total of 50 μg of reduced serum Matrigel (BD Biosciences) was added to the upper chamber of the inserts to coat the Transwell membrane. Following transfection with siRNA, 6-10B and C6661 cells were plated into the upper chambers at a density of $1x10^6$ cells/ml. RPMI-1640 cell culture medium with 10% FCS was added to the lower chamber and served as a chemo-attractant. Following 48 h of incubation at 37˚C, the membrane facing the lower chamber was gently taken off and mounted on a glass slide. The slide was then fixed with 4% formaldehyde for 20 min at room temperature and stained with 0.1% crystal violet at room temperature for 30 min. Invaded cells were counted in five random fields and images were obtained using a light microscope and a camera (magnification, x200; Nikon Digital Camera Dxm 1200F; Nikon Corporation).

Statistical analysis. The data are presented as the mean ± standard deviation of at least three independent experiments performed in triplicate. The difference between two groups was analyzed using a two-tailed Student's t-test and one-way analysis of variance followed by Tukey's post hoc test was used to assess significant differences among >2 groups. SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of Ezrin in human NPC tissue samples and cell lines. IHC was used to detect the protein expression of Ezrin in human NPC tissue samples and the respective adjacent normal nasopharyngeal epithelial tissue samples. The results indicated that the protein expression of Ezrin was higher in NPC tissue samples (Fig. 1A, right-hand panel) compared with the respective adjacent normal nasopharyngeal epithelial tissue samples (Fig. 1A, left-hand panel). The immunostaining of Ezrin was widely localized in the cytoplasm and membrane of carcinoma cells.

The mRNA expression level of Ezrin was determined in the human NPC cell lines 6-10B and C6661, as well as the normal nasopharyngeal epithelial cell line NP69, using RT-qPCR. Significantly higher levels of Ezrin mRNA were observed in the NPC cell lines compared with the normal human nasopharyngeal epithelial NP69 cells (Fig. 1B). Correspondingly, the protein levels of Ezrin were significantly higher in the NPC cell lines 6-10B and C6661 when compared with the normal NP69 cells (Fig. 1C).

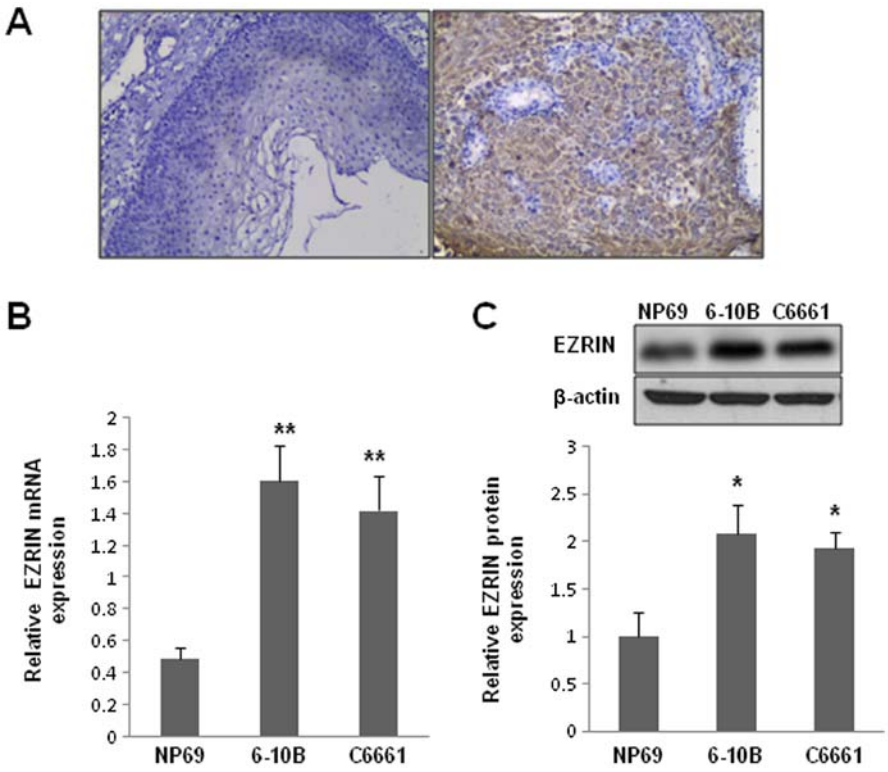

Figure 1. Expression of Ezrin in human NPC tissue samples and cell lines. (A) The expression and localization of Ezrin in (right panel) human NPC tissue samples and (left panel) adjacent normal tissue samples were determined by immunohistochemical staining with an anti-Ezrin antibody. The expression of Ezrin was higher in NPC tissue samples compared with non-cancerous tissue samples. Magnification, x200. (B) The mRNA expression level of Ezrin in the human NPC cell lines 6-10B and C6661, as well as the normal human nasopharyngeal epithelial cell line NP69, was detected by reverse transcription-quantitative polymerase chain reaction assay. (C) The protein expression of Ezrin in the cell lines was evaluated by western blotting Relative protein levels of Ezrin were measured by quantifying the grey density of the protein bands. $^{*}P<0.05$, $^{**}P<0.01$ vs. NP69 (n=3). NPC, nasopharyngeal carcinoma.

Silencing of Ezrin at the gene and protein level by siRNA targeting Ezrin. Ezrin-siRNA or siNC were transfected into the human NPC cell lines 6-10B and C6661. 6-10B and C6661 cells transfected with siNC were used as the control. The mRNA and protein expression levels of Ezrin were determined by RT-qPCR and western blotting, respectively. The mRNA and protein expression levels of Ezrin in 6-10B and C6661 cells transfected with Ezrin-siRNA were significantly reduced following transfection compared with that observed in 6-10B and C6661 cells transfected with siNC (Fig. 2A and B).

Knockdown of Ezrin suppresses 6-10B and C6661 cell migration and invasion, and downregulates the mRNA expression of MMP2, but not MMP9. Cell migration and invasion were assessed following knockdown of Ezrin in 6-10B and C6661 cell lines using a Transwell assay. The results revealed that there was a significant decrease in cell migration following silencing of Ezrin in 6-10B and C6661 cells when compared with cells transfected with siNC (Fig. 3A). Similarly, the ability of cells to invade was significantly reduced in 6-10B and C6661 cells transfected with Ezrin-siRNA when compared with the control cells (Fig. 3B). In summary, the results indicate that knockdown of Ezrin inhibited NPC cell migration and invasion.

The mRNA expression levels of MMP2 and MMP9 were then examined by RT-qPCR. In 6-10B and C6661 cells transfected with Ezrin-siRNA, the expression of MMP2 was significantly downregulated when compared with that of cells transfected with siNC (Fig. 3C). However, no significant difference was observed for the mRNA level of MMP9 following knockdown of Ezrin (Fig. 3D). The results revealed that silencing of Ezrin led to a downregulation of MMP2 mRNA expression.

Knockdown of Ezrin inhibits mRNA expression of MMP2 via the PI3K/Akt signaling pathway. To further investigate the mechanisms and role of Ezrin in NPC cell migration and invasion, the present study investigated the effects of Ezrin-siRNA transfection on the PI3K/Akt signaling pathway. The expression levels of PI3K and p-Akt were detected by western blotting. The results demonstrated that knockdown of Ezrin could significantly decrease the expression levels of PI3K and p-Akt (Fig. 4A). This suggests that knockdown of Ezrin inhibited the PI3K/Akt signaling pathway. Therefore, the aforementioned results indicate that Ezrin may affect the mRNA expression of MMP2 via the PI3K/Akt signaling pathway. To validate the results, siRNA targeting PI3K and the specific inhibitor LY294002 were used to suppress the PI3K/Akt pathway. Following transfection with PI3K siRNA, the downregulation of PI3K was verified by RT-qPCR and western blotting (Fig. 4B and C). Subsequently, the mRNA expression of MMP2 was assessed following PI3K-knockdown. It was revealed that silencing of PI3K significantly decreased the mRNA expression of MMP2 (Fig. 4D). Inhibition of the PI3K/Akt pathway by LY294002 treatment also significantly reduced the mRNA level of MMP2 (Fig. 4E). In summary, these results indicate that knockdown of Ezrin reduced the mRNA expression of MMP2 via the PI3K/Akt signaling pathway.

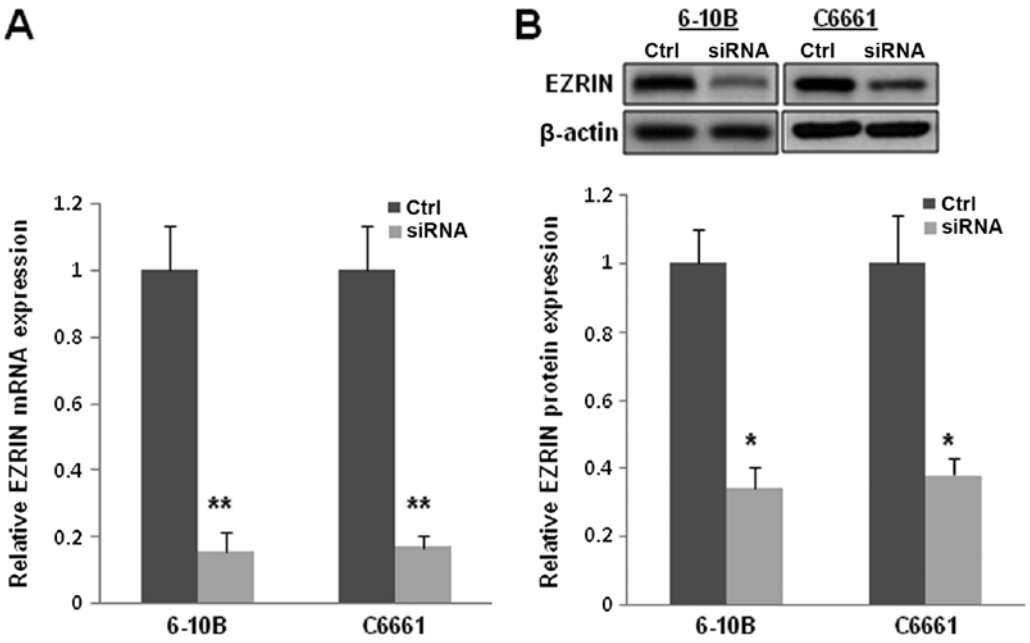

Figure 2. Silencing of Ezrin in the 6-10B and C6661 cell lines was achieved by transfection with siRNA targeting Ezrin and confirmed by reverse transcription-quantitative polymerase chain reaction and western blotting. (A) Transfection of Ezrin-siRNA into 6-10B and C6661 cells resulted in a significant decrease in the mRNA expression level of Ezrin compared with those transfected with the negative control. (B) The protein expression of Ezrin was significantly reduced following transfection with Ezrin-siRNA in 6-10B and C6661 cells as determined by western blotting. β-actin served as the protein loading control. *P<0.05, **P<0.01 vs. Ctrl (n=3). Ctrl, control; siRNA, small interfering RNA.

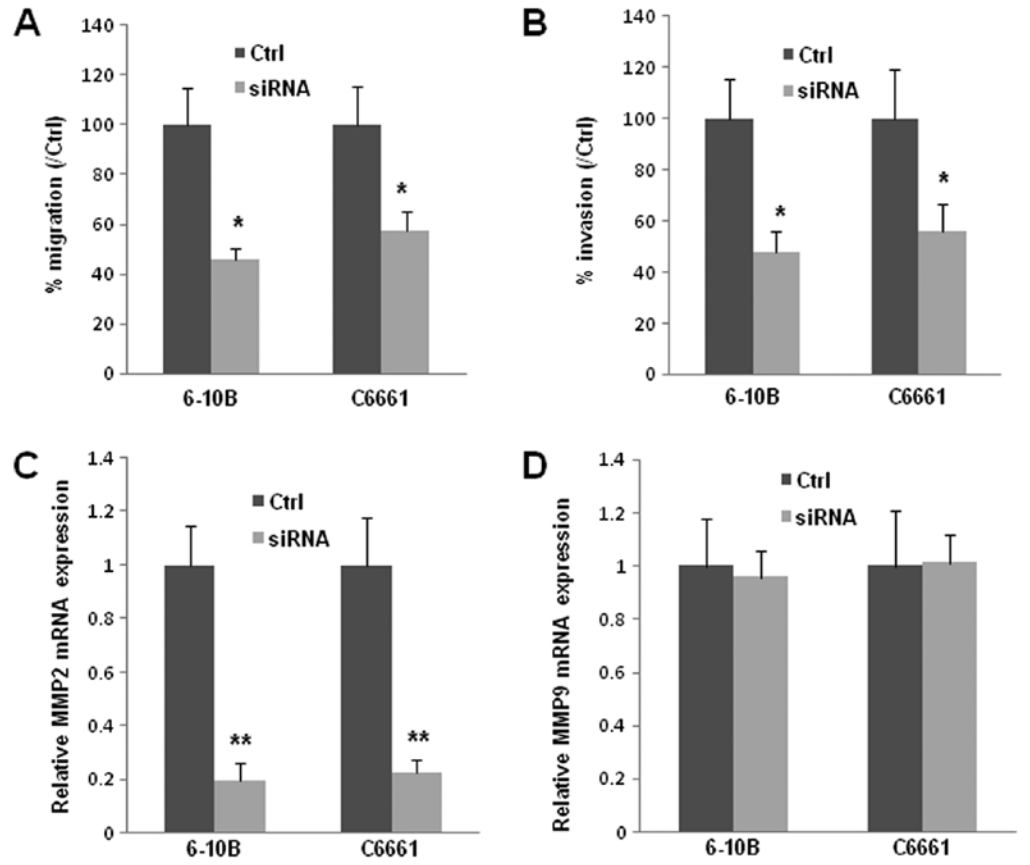

Figure 3. Knockdown of Ezrin inhibits the migration and invasion ability of 6-10B and C6661 cells and decreases the mRNA level of MMP2. Using Transwell and Matrigel assays, cell migration and invasion were observed after silencing of Ezrin in 6-10B and C6661 cells. The migrating and invading cells were fixed with cold methanol and then stained with crystal violet. The percentage of (A) cell migration and (B) cell invasion was calculated after knockdown of Ezrin and compared with Ctrl cells that were transfected with scrambled-siRNA. Cell migration and invasion were significantly reduced in cells transfected with Ezrin-siRNA when compared with the cells transfected with scrambled-siRNA. The mRNA expression level of (C) MMP2 and (D) MMP9 following silencing of Ezrin was assessed using reverse transcription-quantitative polymerase chain reaction. *P<0.05, **P<0.01 vs. Ctrl (n=3). Ctrl, control; siRNA, small interfering RNA; MMP, matrix metalloproteinase.

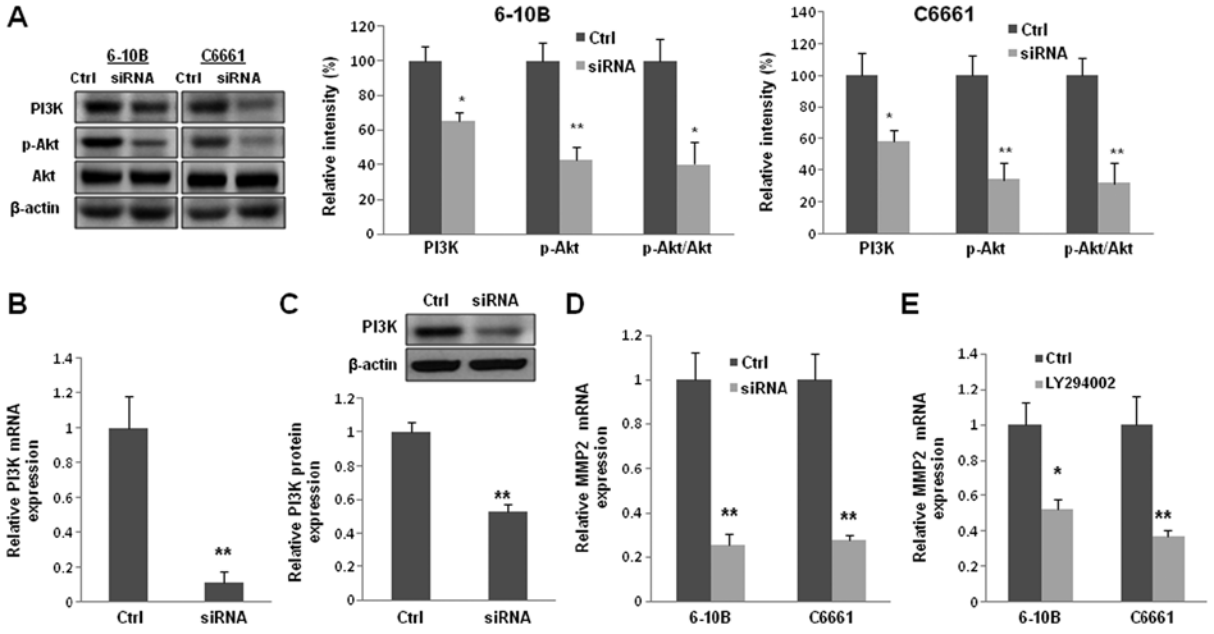

Figure 4. Silencing of Ezrin expression decreases the mRNA level of MMP2 via the PI3K/Akt signaling pathway. 6-10B and C6661 cells were transfected with Ezrin-siRNA (siRNA group) and scramble-siRNA (Ctrl group). (A) The protein expression levels of PI3K and p-Akt were evaluated by western blotting and the relative protein levels of PI3K and p-Akt were then measured by quantifying the grey density of the protein bands. (B) To validate the results, knockdown of PI3K was performed and the silencing efficiency was confirmed using reverse transcription-quantitative polymerase chain reaction. (C) The protein expression of PI3K was also downregulated following transfection with PI3K siRNA. (D) The mRNA expression of MMP2 was revealed to be significantly reduced following silencing of PI3K in 6-10B and C6661 cells. (E) The mRNA expression of MMP2 was significantly decreased following treatment with LY294002. *P<0.05, **P<0.01 vs. Ctrl (n=3). p-, phosphorylated; Akt, protein kinase B; Ctrl, control; siRNA, small interfering RNA; PI3K, phosphatidylinositol 3-kinase; MMP, matrix metalloproteinase.

Discussion

Invasion and metastasis are the main biological characteristics of tumors, which closely reflect the prognosis of patients. The main clinical and pathological feature of NPC is invasive growth (17). The vast majority of NPC cases develop into metastatic NPC, which is the main cause of mortality among patients with NPC (17,18). Invasion and metastasis are complex processes, which involve a myriad of factors and proteins (18). Therefore, it is important to identify the key molecules and elucidate the details of the molecular mechanisms involved in NPC cell invasion and metastasis in order to inhibit the development of metastatic NPC.

A number of studies have reported an aberrant expression of Ezrin in numerous type of tumor cell and the upregulation of Ezrin has been demonstrated to be closely associated with tumor cell invasion (19-22). As a membrane-type cytoskeletal junction protein, Ezrin is involved in a variety of cell activities, including cell survival, cell adhesion, cell motility, signal transduction and tumor formation (8-10). There have also been an increasing number of studies that have investigated the functional roles of Ezrin in biological processes and the development and progression of tumors (23,24). For example, Chen *et al* (25) reported that the phosphorylation status of Ezrin was closely associated with liver cancer invasion. In addition, Ou-Yang *et al* (26) demonstrated that knockout of the Ezrin gene could suppress the migration and invasion of the gastric cancer cell line SGC-7901. It has also been reported that Ezrin is highly expressed in metastatic NPC cells compared with non-metastatic NPC cells (27). Additionally, Epstein-Barr virus latent membrane protein 1-induced upregulation of the expression of Ezrin subsequently can promote the cell metastasis of the NPC cell line CNE1 (28). The results of the present study revealed that Ezrin was highly expressed in human NPC tissue samples compared with the adjacent normal nasopharyngeal tissue samples. Similarly, the expression of Ezrin was significantly higher in the NPC cell lines 6-10B and C6661 compared with the normal nasopharyngeal cell line NP69. Silencing of Ezrin in 6-10B and C6661 cells significantly inhibited cell migration and invasion. These results highlight the important role of Ezrin in human NPC cell migration and invasion.

To date, the mechanisms involved in the invasion and metastasis of NPC remains unclear. It has been reported that an imbalance between MMPs and tissue inhibitors of metalloproteinases serves an important role in invasion and metastasis (21,29). Membrane-type MMPs directly or indirectly degrade various components of the extracellular matrix (ECM), which results in the breakdown of connective tissue barriers, including collagens, fibronectin, laminins, heparan sulfate proteoglycans and vitronectin (29,30). MMP2 and MMP9 are members of the MMP protein family, and have the ability to cleave collagen and other types of ECM proteins. pro-MMP-2 is converted into active MMP-2 that forms a dimer with MMP-9, which consequently exerts its function in matrix degradation (30,31). A previous study has also revealed that MMPs are essential contributors to the metastatic process of cancer cells (31). The results of the present study revealed that knockdown of Ezrin significantly downregulated the mRNA expression of MMP2, but no effect on MMP9 mRNA expression was observed. These results indicate that knockdown of Ezrin suppresses NPC

cell migration and invasion via a downregulation of MMP2. A number of signaling pathways are involved in tumor metastasis. Several studies have reported that focal adhesion kinase/PI3K/Akt/mammalian target of rapamycin signaling is involved in cell proliferation, differentiation, survival and tumor metastasis, and the signaling pathway is also involved in the regulation of MMP2 and MMP9 (32-34). It has also been reported that the PI3K/Akt signaling pathway could activate the downstream transcription factors, activator protein-1, Sp1 and nuclear factor-kB, which are reported to stimulate the expression of MMPs at the transcriptional level (35). Furthermore, certain studies have reported that inhibition of PI3K and Akt can result in a downregulation of MMP2/MMP9 expression and activity in cancer cells (33,34,36). In the present study, to investigate the role of the PI3K/Akt signaling pathway in Ezrin-regulated MMP2 expression, migration and invasion in NPC cells, the expression levels of PI3K and p-Akt were detected following Ezrin knockdown. In addition, siRNA targeting PI3K and the PI3K/Akt inhibitor LY294002 were used to observe the mRNA levels of MMP2 following inhibition of PI3K. The results revealed that knockdown of Ezrin reduced the mRNA expression of MMP2 via the PI3K/Akt signaling pathway. However, the downstream mechanism involved following the downregulation of MMP2 remains to be elucidated.

In conclusion, the results of the present study indicate that knockdown of Ezrin suppresses NPC cell migration and invasion by inducing the downregulation of MMP2 via the PI3K/Akt signaling pathway. The results highlight the important role of Ezrin in NPC cell migration and invasion. They also suggest that inhibition of Ezrin may serve as a potential therapeutic strategy to treat human NPC.

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Availability of data and materials

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

Authors' contributions

YT and XS conceived and designed the present study. YT, SY, XB and JW performed the experiments. YT, SY and LR analyzed the data. XS and LR wrote the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethical Committee of Dalian Medical University (Dalian, China). Samples were taken from the Department of Otolaryngology Head and Neck Surgery of the Second Affiliated Hospital of Dalian Medical University (Dalian, China) once written informed consent was obtained from all patients recruited.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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