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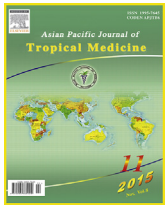

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Expression of MAPK1 in cervical cancer and effect of *MAPK1* gene silencing on epithelial-mesenchymal transition, invasion and metastasis

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ABSTRACT

Objective: To discuss the expression of mitogen-activated protein kinase 1 (MAPK1) in the cervical cancer and effect of *MAPK1* gene silencing on epithelial-mesenchymal transition and invasion and metastasis.

Methods: Immunohistochemistry, western blot and RT-PCR method were employed to detect the expression of MAPK1 protein and mRNA in cervical cancer tissue and adjacent normal tissue. The constructed siRNA-MAPK1 was transferred into human cervical cancer HeLa cells using Lipofectamine™2000. MTT method was used to detect the cell vitality, transwell method to detect the cell invasion, and western blot to detect the expression of matrix metalloproteinases (MMP)-2, MMP-9, tissue inhibitor of metalloproteinase (TIMP)-1, TIMP-2, zinc finger transcription factor (Snail), epithelial-mesenchymal transition related protein (EMT) E-cadherin and vimentin in cells.

Results: The expression of MAPK1 protein and mRNA in the cervical cancer tissue was significantly higher than the one in the adjacent normal tissue ($P < 0.01$); after transfecting the siRNA-MAPK1 into the human cervical cancer HeLa cells through liposome, compared with the control group, its cell vitality was significantly decreased ($P < 0.01$), cell invasion was significantly decreased ($P < 0.01$); expressed of MMP-2, MMP-9, Snail and vimentin was significantly decreased ($P < 0.01$), and expression of TIMP-1, TIMP-2 and E-cadherin was significantly increased ($P < 0.01$).

Conclusions: Because of the high expression of MAPK1 in the cervical cancer tissue, the interference in the expression of MAPK1 can significantly inhibit the invasion and metastasis of cervical cancer HeLa cells, which is related to the interference in the expression of MMPs/TIMP and Snail-mediated generation of EMT.

1. Introduction

The cervical cancer is the common gynecologic malignant tumor and its morbidity ranks the second place and the mortality the first place among malignant tumors in women. Besides, with the development of cytology screening technology, it's found that the onset of cervical cancer tended to be in young women, with the gradually decreased incidence in the terminal stage, but the significantly increased incidence in the early stage [1,2]. Accordingly, based on the means of surgery, molecular biological diagnosis and gene therapy, the possibility to cure the cervical cancer has been greatly increased in the clinical practice. However, the invasion and metastasis and local recurrence of cervical cancer are still the major reasons for the treatment failures of most patients. It is the difficulty that should be settled urgently in the laboratory research, clinical diagnosis and treatment. In consequence, to confirm the pathogenesis of cervical cancer and seek the new efficient markers of molecular biological diagnosis and gene therapy for the cervical cancer, it will be capable to maximally inhibit

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the invasion and metastasis of tumors and thus prevent and cure the cervical cancer to the greatest extent.

The mitogen-activated protein kinase (MAPK) is the protein kinase that consists of serine-threonine kinases. It is highly conserved in the process of biological evolution. Without the assistance of signal transduction system of second messenger, it is capable to independently transducer the extracellular signals into cells, in order to regulate the expression of related genes and join in the physiological processes of mitosis, proliferation, inflammation, invasion and metastasis of cells. According to previous researches, many enzymes of MAPK pathway had the abnormal expression in the female reproductive tract cancers, breast cancer, ovarian cancer and cervical cancer [3,4]. Besides, many chemotherapeutic drugs through such pathway have been widely in the clinical practice. Most of scholars believed that MAPK signaling pathway was the therapeutic target of tumors. Researches mainly focused on the proliferation and apoptosis of tumor cells [5], but there were limited researches on the invasion and metastasis of tumor cells. MAPK1 is the subfamily of MAPK pathway, which has the overexpression in the follicular lymphoma [6]. It has also been proved that the interference in the expression of MAPK1 in HeLa cells can significantly inhibit the proliferation of cancer cells and induce the cell apoptosis [7,8]. Some research also found that the activation of MAPK1 signaling pathway could significantly promote the generation of epithelial-mesenchymal transition (EMT) in cervical cancer cells [9]. It indicated that MAPK1 had the abnormal expression in the cervical cancer and it's closely related to the development of cervical cancer, but its specific mechanism of action has been unknown. Accordingly, in this study, the immunohistochemistry, western blot and RT-PCR method were employed to detect the expression of MAPK1 protein and mRNA in the cervical cancer tissue and adjacent normal tissue. Besides, according to the interference in the expression of MAPK1 in HeLa cells of cervical cancer, it is to discuss the effect and specific effect of *MAPK1* gene silencing on the invasion and metastasis of HeLa cells and EMT in vitro.

2. Materials and methods

2.1. Samples

A total of 20 samples of cervical cancer tissue and 20 samples of adjacent normal tissue that were surgically removed by surgical excision in this hospital from June 2014 to June 2015 were collected. All patients had not received the chemotherapy before the operation, but they were diagnosed by the pathological and imaging examination.

2.2. Reagents and instruments

The human cervical cancer HeLa cells was purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Registered No.: TCHu187); siRNA Tim-3 and control were synthesized by Shanghai GenePharma Co, Ltd.; the rabbit anti metalloproteinases (MMP)-2, MMP-9, tissue inhibitor of metalloproteinase (TIMP)-1, TIMP-2, Snail, E-cadherin, vimentin monoclonal antibody were purchased from Epitmics; methyl thiazolyl tetrazolium from Gibco; fetal bovine serum, DMEM culture medium from Hyclone; transwell chamber from Corning, mini vertical electrophoresis tanks and mini Trans-blot electrophoresis tank from Beijing Liuyi Instrument Factory; and ChemiDoc™ XRS gel imaging system from Bio-Rad.

2.3. Detection of expression of MAPK1 in cervical cancer tissue by RT-PCR

The total RNA was extracted by referring to the instruction manual of trizol kit (Invitrogen) and the extraction was performed in the condition without RNAase. Primers were as follows: *MAPK1* upstream primer: TGGATTCCCTGGTTCTCTC-TAAAG, downstream primer: GGGTCTGTTTTCCGAG-GATGA; reference *GADPH* upstream primer: AAAAACC-TGCCAAATATGATGACA, downstream primer: AGCTTGA-CAAAGTGGTCGTTGA. RNA was reversely transcripted into cDNA through one-stop RT-PCR kit and then PCR amplification was performed. A total of 5 μL amplification product was used for the further detection using 2% agarose gel. Primers were added into 25 μL PCR reaction system respectively, with the reaction conditions of degeneration at 94 °C for 45 s, renaturation at 59 °C for 45 s and extension at 72 °C for 60 s, with 35 cycles. The UV spectrophotometer was used to detect the electrophoretic bands and take the photos.

2.4. Detection of expression of MAPK1 protein in cervical cancer tissue by immunohistochemical assay

Samples were deparaffinized with dimethylbenzene, dehydrated with anhydrous, 95% and 80% ethanol, washed with the running water, repaired with the antigen retrieval, closed with the horse serum, closed with primary antibody, closed with secondary antibody, immersed in the hematoxylin, immersed in the hydrochloric acid alcohol, washed with the running water until it appeared to be antiblue, dehydrated with the gradient ethanol, transparented with dimethylbenzene, dried and then mounted with the neutral resin, and the microscopic examination. The positive staining of MAPK1 appeared to be light yellow and brown yellow, which was located in the cytoplasm.

2.5. Detection of vitality of cervical cancer HeLa cells by MTT

The human cervical cancer HeLa cells were seeded onto 96-well plate. When the confluency of cells reached to 70%, it was transfected with siRNA-MAPK1 (synthesized by Ambion) and NC using Lipofectamine™2000 respectively. 48 h after the transfection, 20 μL 5 mg/mL MTT was added for the continuous culture of 4 h and then the culture medium was sucked out. Afterwards, 150 μL DMSO was added in each well and then it was shaken to fully dissolve the crystals. CD value was measured at 560 nm of analyzer of enzyme-linked immunosorbent assay.

2.6. Cell invasion assay

Matrigel basement membrane was evenly paved on the micromembrane (5 μm) of Transwell chamber to be prepared as the gel for further use. The human cervical cancer HeLa cells were seeded onto 6-well plate. When the confluency of cells reached to 70%, it was to transfect siRNA-MAPK1 and NC using Lipofectamine™2000 respectively. Afterwards, cells were digested and added in the upper chamber of Transwell, while the lower chamber contained DEME medium with 5% fetal bovine serum

for the further 24 h of culture. Then the Transwell chamber was taken out and washed, using the paraformaldehyde for the fixation. It was then stained with the crystal violet. The number of membrane-penetrating cells in five fields was counted under the inverted optical microscope and the mean number of cells for each field was calculated to represent the invasion ability of cells.

2.7. Western blot assay

RIPA lysis buffer was added in the collected cells. It was put in Vortex instrument for 30 s of shaking every 10 min. After 40 min, it was centrifuged at 4 °C and 10000 rpm for 10 min. The supernatant was sucked carefully to obtain the total protein. The protein concentration was measured with BCA kit. The protein loading buffer was treated with SDS gel electrophoresis and then it was transferred with the wet method. Then the film was immersed into the primary antibody solution for the incubation at 4 °C over night. After being washed, it was immersed into the secondary antibody solution for the incubation at the room temperature for 1–2 h. Afterwards, the film was taken out and washed, while ECL reagent was added on the film for the exposure in the gel imaging system. Statistics was performed on the gray value of each antibody band using 'Quantity one' software.

2.8. Statistical analysis

The results were expressed as mean ± SD, with three repeats for each set of data at least. The *t* test was employed and $P < 0.05$ was meant to be significant difference. All data were treated using SPSS 17.0.

3. Results

3.1. Expression of MAPK1 in cervical cancer tissue and adjacent normal tissue detected by immunohistochemical assay

As shown in Figure 1, compared with the adjacent normal tissue, the positive expression of MAPK1 in the cervical cancer tissue was significantly higher [(0.29 ± 0.00) *vs.* (10.21 ± 1.38)] ($P < 0.01$).

3.2. Expression of MAPK1 in cervical cancer tissue and adjacent normal tissue detected by Western blot assay

As shown in Figure 2, similar with the result of immunohistochemistry, compared with the adjacent normal tissue, the expression of MAPK1 protein in the cervical cancer tissue was significantly higher [(0.38 ± 0.05) *vs.* (4.86 ± 0.35)] ($P < 0.01$).

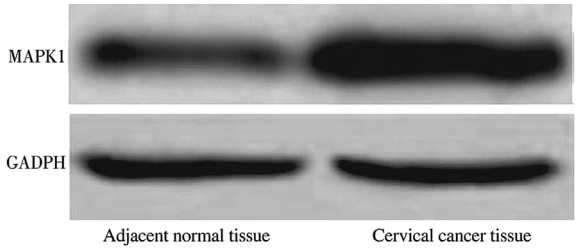

Figure 2. Expression of MAPK1 in cervical cancer tissue and adjacent normal tissue detected by Western blot assay.

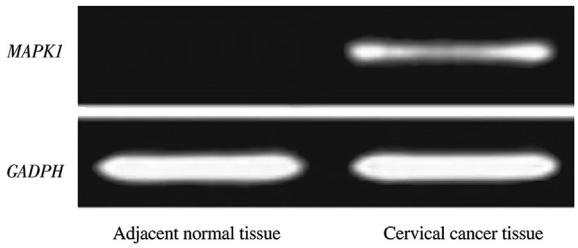

Figure 3. Expression of *MAPK1* in cervical cancer tissue and adjacent normal tissue detected by RT-PCR.

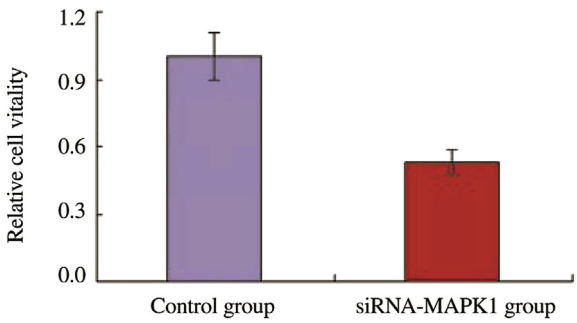

Figure 4. Effect of MAPK1 on vitality of human cervical cancer HeLa cells detected by MTT. Compared with the control group, $^{**}P < 0.01$.

3.3. Expression of MAPK1 in cervical cancer tissue and adjacent normal tissue detected by RT-PCR

As shown in Figure 3, in line with the trend of protein expression, compared with the adjacent normal tissue, the expression of *MAPK1* mRNA was significantly higher in the

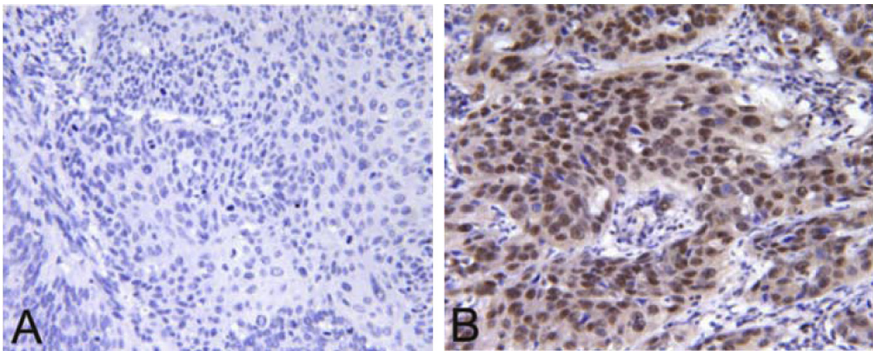

Figure 1. Expression of MAPK1 in cervical cancer tissue and adjacent normal tissue detected by immunohistochemical assay. A: adjacent normal tissue; B: cervical cancer tissue.

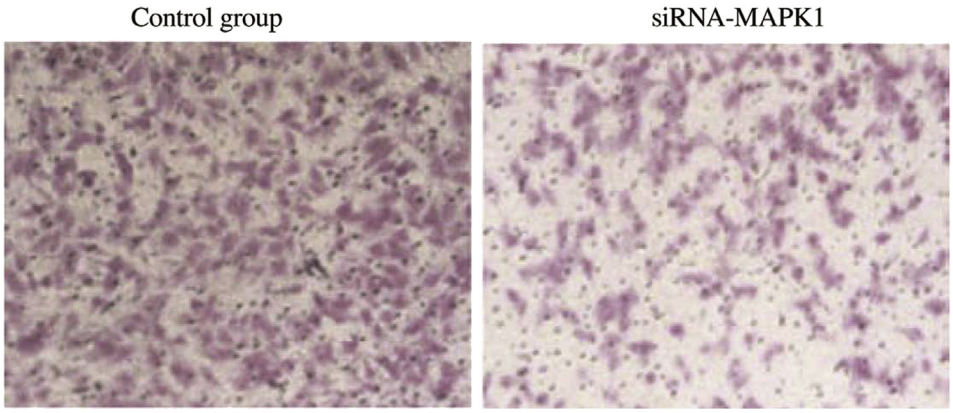

Figure 5. Effect of MAPK1 on the invasion of human cervical cancer HeLa cells detected by Transwell method.
Compared with the control group, $^{**}P < 0.01$.

cervical cancer tissue [(0.02 ± 0.00) *vs.* (0.34 ± 0.01)] ($P < 0.01$).

3.4. Effect of MAPK1 on vitality of human cervical cancer HeLa cells by detected MTT

As shown in Figure 4, after transfecting siRNA-MAPK1 in HeLa cells, the results of MTT assay showed that, compared with the control group, the cell vitality in the transfection group was significantly decreased ($P < 0.01$).

3.5. Effect of MAPK1 on invasion of human cervical cancer HeLa cells detected by Transwell method

As shown in Figure 5, after transfecting siRNA-MAPK1 in HeLa cells, the results of transwell invasion assay showed that, compared with the control group, the number of cell invasion in the transfection group was significantly decreased [(217.34 ± 19.35) *vs.* (93.94 ± 9.97) ($P < 0.01$).

3.6. Effect of MAPK1 on expression of MMP-2 and TIMP-2 in human cervical cancer HeLa cells

As shown in Figure 6, after transfecting siRNA-MAPK1 in HeLa cells, the results of western blot assay showed that, compared with the control group, the expression of MMP-2 was decreased and the expression of TIMP-2 was significantly increased in the transfection group ($P < 0.01$).

3.7. Effect of MAPK1 on expression of MMP-9 and TIMP-1 in human cervical cancer HeLa cells

As shown in Figure 7, after transfecting siRNA-MAPK1 in HeLa cells, the results of western blot assay showed that, compared with the control group, the expression of MMP-9 was decreased and the expression of TIMP-1 was significantly increased in the transfection group ($P < 0.01$).

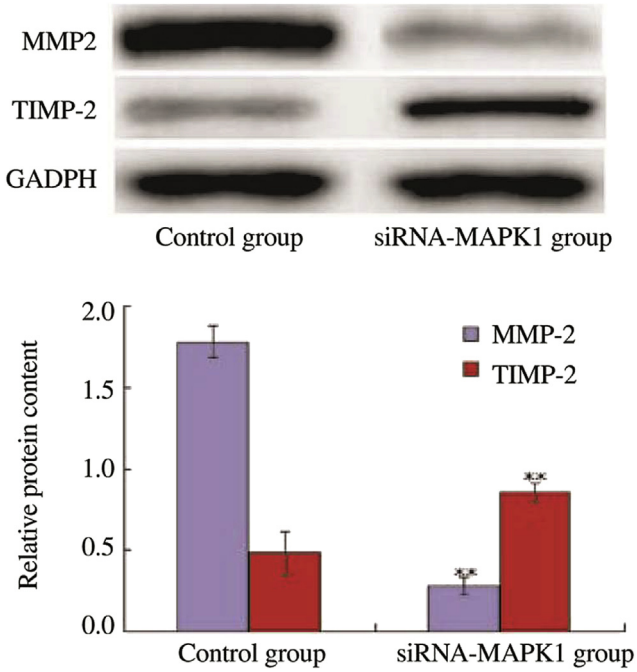

Figure 6. Effect of MAPK1 on expression of MMP-2 and TIMP-2 in human cervical cancer HeLa cells.
Compared with the control group, $^{**}P < 0.01$.

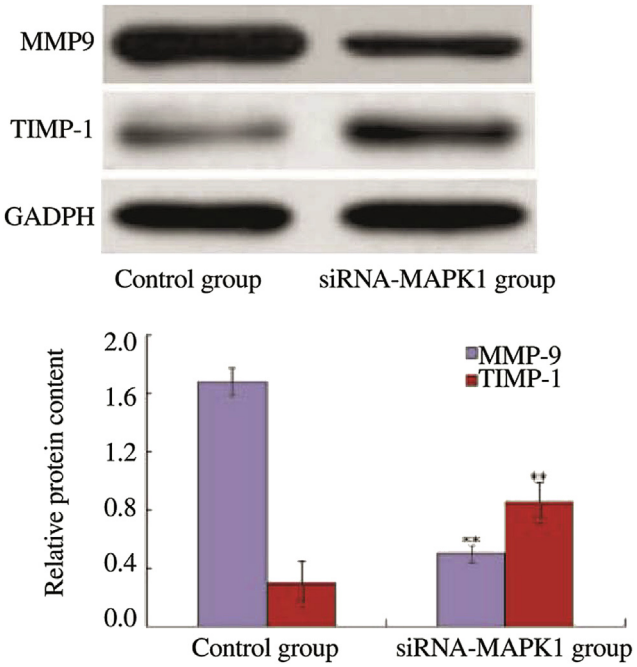

Figure 7. Effect of MAPK1 on expression of MMP-9 and TIMP-1 in human cervical cancer HeLa cells.
Compared with the control group, $^{**}P < 0.01$.

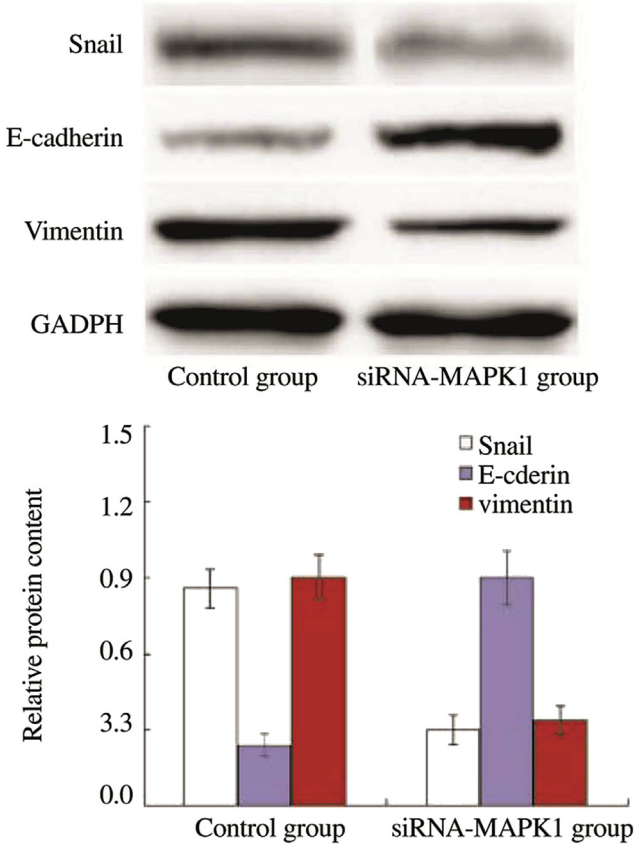

Figure 8. Effect of MAPK1 on expression of Snail, E-cadherin and vimentin in human cervical cancer HeLa cells.
Compared with the control group, $^{**}P < 0.01$.

3.8. Effect of MAPK1 on expression of Snail, E-cadherin and vimentin in human cervical cancer HeLa cells

As shown in Figure 8, after transfecting siRNA-MAPK1 in HeLa cells, the results of western blot assay showed that, compared with the control group, the expression of Snail and vimentin was decreased and the expression of E-cadherin was increased in cells, with the statistically significant difference ($P < 0.01$).

4. Discussion

The cervical cancer is the common gynecologic malignant tumor and its morbidity ranks the second place that is second only to the breast cancer and the mortality the first place among malignant tumors in women. The invasion and metastasis are still the major reasons for the treatment failures and death of most patients. Therefore, it is of critical importance to discuss the malignant biological behavior of cervical cancer cells and the specific molecular biological mechanism of occurrence and development.

The invasion and metastasis of tumor is the biological process that involves multiple steps and factors and the complicated process with the interaction between the tumor cells, host cells and extracellular matrix. In such process, the proliferation of tumor cells is increased, the adhesion decreased and components of extracellular matrix degraded. Accordingly, the tumor cells are migrated and its invasion is enhanced to induce the tumor for the motion along the distant and deep direction. MMPs is the most important set of proteases to degrade the extracellular matrix, which play a key role in the invasion and metastasis of tumor cells and the key enzyme to be involved in these processes. MMP-2 and MMP-9 are two MMPs that have been studied thoroughly. MMP-2 can not only degrade the gelatin IV collagen in the extracellular matrix and promote the infiltration of tumor cells around along the injured basement membrane, but also promote the invasion and metastasis of tumor through the new capillaries, as the indicator for the infiltration and invasion of malignant tumor. MMP-9 is some kind of proteolytic enzyme that is secreted by many cells. It is the enzyme with the largest molecular weight among MMPs, which can degrade the extracellular matrix and basement membrane and thus enhance the ability of cell motion and promote the spread and metastasis of tumor. TIMP is the specific inhibitor of MMP, which can maintain the normal physiological functions of extracellular matrix and has the abnormal expression in the tumor tissue [10]. The expression of MMP-2 and MMP-9 was extremely high in the samples of cervical cancer, which was closely related to the prognosis of patients [11]. MMP-2 had the high expression, but TIMP-2 the low expression. Besides, TIMP-2 was the independent predicator of univariate survival [10]. The high expression of TIMP-1 in HeLa cells could inhibit the invasion [12]. By up-regulating the expression of TIMP-1 and down-regulating the expression of MMP-9, it could significantly inhibit the migration of Hela cells [13], which indicated that the down-regulated expression of MMPs and the up-regulated expression of TIMP could significantly inhibit the invasion and metastasis of cervical cancer cells.

EMT was firstly proved in the process of embryonic development. More and more evidences have shown that EMT plays a critical role in the tumorigenesis, including the local infiltration and the metastasis and spread through the circulatory system, as the characteristics of tumor cells with the strongest ability of invasion. Besides, when the malignant tumor cells had EMT, it could be regarded as the beginning of infiltration and metastasis [14,15]. The characteristics of molecular marker for EMT is the down-regulated expression of intercellular adhesion molecule E-cadherin, and the up-regulated expression of a series of mesenchymal markers, including N-cadherin, vimentin and fibronectin. The loss of E-cadherin contributes to the falling of tumor cells from the primary site. Meanwhile, with the increased degree of malignancy of tumors, the invasion is increased and the recovery of E-cadherin function can reduce the invasion ability of many kinds of tumor cells [16]. The vimentin is a protein of intermediate filaments, which is mainly expressed in the stromal tumors. The increased expression of vimentin contributes to the invasion and metastasis of tumors and can also reduce the E-cadherin-mediated intercellular adhesion, which can thus promote the infiltration and metastasis of cells [17]. In the cervical squamous cell carcinoma, the low expression of E-cadherin and high expression of vimentin are all closely related to the metastasis of tumor cells [18]. Therefore, the reversed expression of EMT-related protein can inhibit the invasion and metastasis of cancer cells. The zinc finger transcription factor Snail was found in the fruit fly, as the member of transcription repressor Snail superfamily. The human *Snail* gene is located in 20th chromosome. Snail can be integrated with E-box sequence in the promoter region of E-cadherin to inhibit the expression of E-cadherin. The interference of RNA in the expression of Snail could significantly inhibit the generation of human lens epithelial cells EMT that was induced by the transforming growth factor

[19]. In the HepG2 cells of hepatocellular carcinoma that were transfected with siRNA-Snail, the expression of E-cadherin was increased and the ability of cell metastasis was decreased [20]. In the hepatocellular carcinoma, Snail could up-regulate the expression of MMP-2 and down-regulate the expression of E-cadherin [21], which indicated that the down-regulated expression of Snail in the cervical cancer cells could significantly inhibit the generation of EMT-related proteins and decrease the metastasis ability of tumor cells.

MAPK family includes eight subfamilies of p38MAPK, extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), ERK3, big mitogen-activated protein kinase-1 (ERK5/BMK1), ERK-7, NLK and ERK8. The signal is transmitted through MAPK pathway to activate the downstream substrate finally and then start the expression of related genes, which will cause the activation or transformation of biological behaviors such as the cell proliferation, invasion and metastasis. With the in-depth researches on the subfamilies of MAPK, Huang *et al.* [7] adopted siRNA to interfere the expression of MAPK1 and found that it could significantly regulate the growth, apoptosis and cytoskeleton of HeLa cells. Lwin *et al.* [8] also proved that after the interference of siRNA in MAPK1, it could significantly inhibit the proliferation of HeLa cells, which indicated that the expression of MAPK1 was closely related to the proliferation of cervical cancer cells. However, there are limited researches on the relationship between the invasion and metastasis of cervical cancer cells. In consequence, on such basis, this study would discuss the expression of MAPK1 in the cervical cancer tissue and adjacent normal tissue and the effect of *MAPK1* gene silencing on the epithelial-mesenchymal invasion and metastasis of cervical cancer cells.

In this study, the immunohistochemistry, western blot and RT-PCR method were employed to detect the expression of MAPK1 in the cervical cancer tissue and adjacent normal tissue. Results showed that the expression of MAPK1 in the cervical cancer tissue was higher than the one in the adjacent normal tissue, which was in accordance with the findings of Wang *et al.* [6] who found the overexpression of MAPK1 in the follicular lymphoma through the gene analysis. Erkutlu *et al.* [22] proved the low expression of MAPK1 in the glioblastoma multiforme. It indicates that MAPK1 has the different functions in the different tumor tissues, which can promote and also inhibit the cancer gene, just playing the role of promotion in the cervical cancer. The further interference of RNA in the expression of MAPK1, the results of MTT assay showed that the expression of MAPK1 was down-regulated and it significantly reduced the cell vitality, which was similar with the findings of Huang and Lwin. Afterwards, the transwell assay was employed to detect the MAPK1-siRNA transfected HeLa cells. Results showed that the invasion ability was significantly reduced. Referring to the previous research, the protein tyrosine phosphatase receptor R activated MAPK signaling pathway to inhibit the metastasis of cervical cancer [23]. The prion protein PrPc could promote the generation of EMT in cervical cancer cells through MAPK1 signaling pathway [9], which indicated that the expression of MAPK1 was closely related to the invasion and metastasis of cervical cancer cells and also related to the generation of EMT. Accordingly, the western blot assay was employed to further detect the expression of proteins related to the invasion and metastasis of tumors. Results showed that the decreased expression of MAPK could inhibit the expression of MMP-2, MMP-9 and vimentin in HeLa cells and increase the expression of TIMP-1, TIMP-9 and E-cadherin. It means that the down-regulated expression of MAPK1 could interfere the expression of MMPs/TIMP, interfere the generation of EMT and inhibit the invasion and metastasis of HeLa cells. According to previous researches, it targeted at the MAPK/Snail signaling pathway to inhibit the metastasis of salivary adenoid cystic carcinoma cells and siRNA-MAPK1 could significantly inhibit the activity and transcription expression of *Snail2* gene promoter [24]. MAPK1/2/ERK1/2 inhibitor PD98059 could significantly down-regulate the expression of Snail in the PDGF-BB-induced mesenchymal stem cells and thus inhibit the cell invasion [25], which indicated that siRNA-MAPK1 could also down-regulate the expression of Snail to inhibit the invasion and metastasis of tumor cells. Therefore, the results of in-depth study in this experiment also proved that the down-regulated expression of MAPK1 could significantly reduce the expression of Snail. It indicates that the *MAPK1* gene silencing can regulate the expression of MMPs/TIMP and inhibit the Snail-mediated EMT generation and expression of related proteins, in order to inhibit the invasion and metastasis of tumor cells.

In conclusion, MAPK1 has the overexpression in the cervical cancer tissue. The interference in the expression of MAPK1 can significantly inhibit the invasion and metastasis of cervical cancer HeLa cells, which is related to the regulation on the expression of MMPs/TIMP and the inhibition against the expression of Snail-mediated EMT-related proteins.

Conflict of interest statement

We declare that we have no conflict of interest.

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