MicroRNA-21 promotes cell metastasis in cervical cancer through modulating epithelial-mesenchymal transition

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Abstract. MicroRNA (miR)-21 is known to act as an oncogene in cervical cancer by promoting cell proliferation and migration; however, the underlying molecular mechanisms have remained to be fully elucidated. The present study revealed that the gene expression levels of miR-21 and epithelial-mesenchymal transition (EMT)-associated transcription factor Zinc finger E-box-binding homeobox 1 (ZEB1), in cervical cancer and lymphatic metastatic carcinoma tissues were significantly higher than those in normal tissues (P<0.05). Furthermore, the gene expression levels of miR-21 and ZEB1 were positively associated with muscular infiltration depth, parametrical invasion and lymph node metastasis in patients with cervical cancer. Immunohistochemistry assays indicated that the expression levels of ZEB1 and the mesenchymal cell marker Vimentin in cervical cancer tissues were significantly higher than those in normal cervical tissues (P<0.05). Overexpression of miR-21 in HeLa and SiHa cells caused the upregulation of the mesenchymal cell markers Vimentin and N-cadherin, and downregulation of the epithelial cell marker E-cadherin at the proteins level. In addition, overexpression of miR-21 enhanced the invasiveness of HeLa and SiHa cells. These results demonstrated that miR-21 was upregulated in cervical cancer tissues and promoted cell metastasis through modulating EMT. A better understanding of the role of miR-21 and EMT may lead to the development of more effective therapies for patients with cervical cancer.

Introduction

Cervical cancer is a primary malignant tumor of the female reproductive system and is associated with high mortality. Its

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development involves multiple steps of genetic aberrations and complex biological processes, and is closely linked to persistent infection with high-risk human papilloma virus (HPV) (1). The overall malignant transformation process includes a number of genetic and epigenetic alterations and the mechanisms of carcinogenesis remain to be fully elucidated.

MicroRNAs (miRNAs) are small non-coding RNAs of 19-25 nucleotides in length, which modulate gene expression either by catalyzing mRNA cleavage or by inhibiting mRNA translation. They may act as tumor suppressors or oncogenes by regulating the expression of various target genes (2,3). MiR-21 has been reported to be associated with a wide variety of human cancers, including cervical cancer (4,5). The upregulation of miR-21 has been observed in cervical cancer cell lines and clinical specimens (6,7). MiR-21 acts as an oncogene by promoting the proliferation and migration of cervical cancer cells, while the underlying molecular mechanisms have remained to be fully elucidated (8,9).

Epithelial mesenchymal transition (EMT) is a biological process that involves the polarization of epithelial cells, which refers to the transformation of epithelial cells to stroma cells accompanied by loss of cell polarity and gain of stroma cell features (10). Previous studies have indicated that EMT plays an important role in cervical cancer progression and metastasis (10). Of note, miR-21 has been confirmed to act synergistically with transforming growth factor β (TGF-β) to accelerate the EMT process in colon carcinoma, involving the direct targeting of Rac GTPase to enhance cell migration and invasion (11). MiR-21 has also been reported to promote the invasion and migration of cholangiocarcinoma cells by inducing EMT (12). However, whether EMT is involved in promoting the invasion and metastasis of cervical cancer cells driven by miR-21 has remained elusive.

This study aims to investigate the role of EMT in the malignant progression of cervical cancer that is driven by the increased expression of miR-21, to provide theoretical basis for individualized treatment and gene targeting interventions in cervical cancer patients.

Materials and methods

Patients and samples. A total of 45 cervical cancer tissue samples, the corresponding adjacent non-neoplastic tissues and

lymph nodes with suspected metastasis were collected from patients who underwent cervical surgical resection without any pre-operative systemic therapy. These 45 patients were diagnosed with cervical cancer at the IB1-IIB stage, according to the 2009 International Federation of Gynecology and Obstetrics (FIGO) Cervical Cancer Staging Guidelines (13). All tissues included in the present study were confirmed by pathological examination. Furthermore, 15 cases of cervical cancer with lymph node metastasis were included. After surgical removal, the tissues were immediately frozen in liquid nitrogen and stored at -80°C.

Quantitative real-time PCR. MicroRNA was extracted using the miRNeasy Mini Kit (QIAGEN) and reverse transcription was performed with a miRNA Reverse Transcription Kit (QIAGEN). The relative expression levels of miR-21 in tissues from patients and cell lines were detected using the miScript SYBR Green PCR Kit (QIAGEN), with U6 as the internal control (12). The primers used in the present study were listed in Table SI. The PCR program consisted of three steps: denaturation at 94°C for 15 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec, with 40 cycles in total. For the detection of other genes, total RNA was isolated with TRIzol® (Invitrogen) and reverse transcription was performed with the PrimeScrip RT reagent Kit with gDNA Eraser (TaKaRa Bio). Real-time qPCR was performed using PrimeScript RT Master Mix, with GAPDH as the internal control. The cycling program consisted of one cycle at 95°C for 1 min, and 40 cycles of 15 sec at 95°C, 20 sec at 55°C, plus 60 sec at 72°C. Each sample was evaluated in triplicate. Default threshold settings were used as threshold cycle (Ct) data. Relative quantification of gene expression was performed using $2^{-\Delta\Delta Ct}$ method, which indicates relative fold changes.

Western blot analysis. Total protein was lysed with radio immunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) and the protein concentration of each sample was determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Equal amounts of protein were separated by SDS-PAGE (10% acrylamide gel) and transferred to polyvinylidene fluoride membrane. After blocked with 1% BSA, membranes were incubated with the primary mouse monoclonal antibodies (Santa Cruz Biotechnology) (dilution, 1:1,000) for E-cadherin, Vimentin and N-cadherin at 4°C overnight, and then with the corresponding secondary anti-mouse antibodies (TaKaRa Bio) (dilution, 1:5,000) at 37°C for 1h. Digital images were acquired using an enhanced chemiluminescence reagent from EMD Millipore.

Immunohistochemistry. The tissue sections were de-paraffinized and rehydrated through a series of graded alcohols. Endogenous peroxidase activity was blocked with H_2O_2. Primary antibodies were titrated to determine the optimal antibody concentration for staining with no background. A negative control was run on each tissue, where the primary antibody was substituted with antibody diluent buffer. In addition, positive control tissues known to express the antigen in question were run in conjunction with each batch of slides. The tissues were incubated with mouse-derived primary antibody (Santa Cruz Biotechnology) (dilution, 1:300) at room temperature for 1 h. After washing three times with PBS, the tissues were incubated with the secondary anti-mouse antibody (TaKaRa Bio) (dilution, 1:1,000) at room temperature for 15 min. The staining for ZEB1, E-cadherin and Vimentin was evaluated semi-quantitatively according to staining intensity as previously reported (14).

miR-21 transfection. The cervical cancer cell lines (HeLa and SiHa) were employed in the present study. Mimics control, miR-21 mimics, and inhibitor control or miR-21 inhibitor were transfected into the cells using Lipofectamine 2000 reagent as previously reported (5). Quantitative real-time PCR was used to confirm the expression levels of miR-21in the cell lines after transfection.

Invasion assay. The cell invasion assay was performed using Matrigel-coated upper chambers (BD Biosciences). Briefly, cells ($1x10^5$) in 200 μl serum-free medium were seeded in the upper chambers, and medium containing 10% serum as a chemo-attractant was used in the lower chambers. After 48 h of incubation, non-invading cells were gently removed, and cells at the outer surface of the insert membranes were fixed with menthol and, stained with 1% crystal violet. Images were captured under 200x magnification. Cells were counted from 5 random fields of view. All experiments were performed three times independently.

Statistical analysis. The quantitative variables in symmetric distributions were presented as means ± standard deviation (SD) and comparisons between groups were performed through the Student's t test. The miR-21 expression was expressed as median (interquartile range, IQR) and comparisons between groups were performed through the Wilcoxon rank sum test (two groups) or Kruskal-Wallis test (multiple groups). If the Kruskal-Wallis test was significant, Dunn's test was performed as a post-hoc analysis to determine which levels of the independent variable differ from each other. Protein expression was semi-quantified using an IHC scoring system, and comparisons between groups on protein expression were also performed through Wilcoxon rank sum test. Correlations between laboratory indicators and clinical parameters in specimens were calculated using Spearman's rank correlation test. Data analysis was carried out with Intercooled Stata v.11 for Windows; P<0.05 was considered to indicate a statistically significant difference.

Results

miR-21 expression in clinical samples. Quantitative real-time PCR indicated that the relative gene expression levels of miR-21 in cervical cancer and lymphatic metastatic carcinoma tissues were significantly higher than those in normal cervical tissues (P<0.05). Furthermore, the expression levels of miR-21 were significantly higher in those cervical cancer tissues with more severe and invasive characteristics, including those with deeper muscular infiltration depth, more severe parametrical invasion and more extensive lymph node metastasis (P<0.05; Table I), while no significant association was identified between the expression levels of miR-21 and the tumor size or pathological type (P>0.05; Table I). Further correlation

Table I. Association between miR-21 expression and pathological characteristics in cervical cancer.

Parameters	Cases, n	miR-21[a]	P-value[b]
Age, years			
≥50	32	3.636 (0.687-5.123)	0.980
<50	13	2.166 (1.043-5.151)	
Muscular infiltration			
≥1/2	29	4.419 (1.562-6.062)	0.001
<1/2	16	0.860 (0.484-2.166)	
Tumor size, cm			
>4	20	4.301 (0.975-5.807)	0.150
≤4	25	2.046 (0.561-4.187)	
Parametrial invasion			
Yes	10	4.514 (2.947-11.459)	0.036
No	35	2.046 (0.712-4.434)	
Pathologic types			
Squamous	38	3.636 (0.961-5.289)	0.316
Adenocarcinoma	7	2.166 (0.503-3.574)	
Lymph node metastasis			
Yes	15	3.613 (1.005-4.860)	0.045
No	30	2.123 (0.704-5.289)	
HPV16 infection			
Yes	15	3.992 (2.166-4.434)	0.027
No	30	1.451 (0.659-5.564)	

[a]Data are presented as the median (interquartile range); [b]Kruskal-Wallis rank sum test, and P<0.05 was considered to indicate a statistically significant difference. miR-21, microRNA-21.

analysis revealed that the expression level of miR-21 was positively correlated with ZEB1 gene expression in cervical cancer (Spearman's correlation test, rs=0.841, P<0.05).

EMT-associated protein expression in clinical samples. Immunohistochemistry assays indicated that the expression level of ZEB1 in cervical cancer tissues was significantly higher than that in normal tissues (P<0.05; Fig. 1), and was also significantly higher in cervical cancer with deeper muscular infiltration or more extensive lymph node metastasis (P<0.05; Table SII). Similarly, the expression level of Vimentin was also significantly higher in cervical cancer vs. normal tissues (P<0.05; Fig. 1) and was positively associated with lymph node metastasis (Table SIII) (P<0.05). In contrast, the expression level of E-cadherin in cervical cancer tissues was significantly lower than that in normal cervical tissue (P<0.05) and was significantly decreased in cancer tissues with more extensive lymph node metastasis (P<0.05) (Table SIV). Further statistical analysis also revealed that the protein expression level of ZEB1 was negatively associated with E-cadherin in cervical cancer tissues (Spearman's correlation test, rs=-0.862, P<0.05).

miR-21 modulates EMT in cervical cancer cells. Quantitative real-time PCR analysis indicated that the expression levels of miR-21 were significantly increased in HeLa and SiHa cells after transfection with miR-21 mimics (P<0.05; Fig. 2), and the expression level of miR-21 significantly decreased
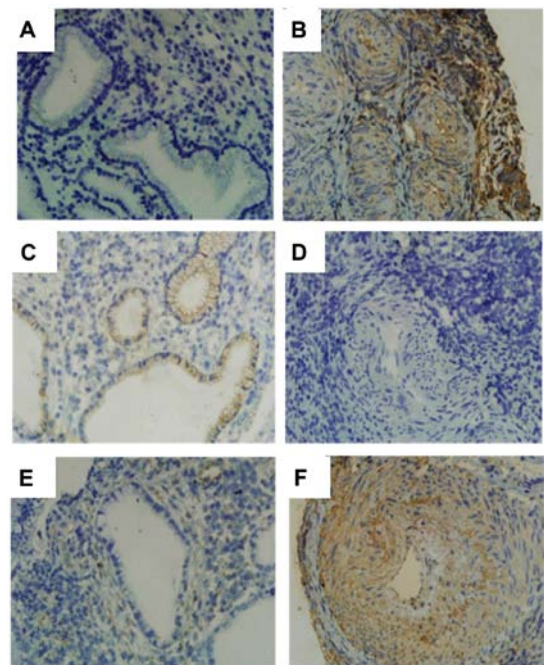

Figure 1. Immunohistochemical staining for ZEB1, E-cadherin and Vimentin in cervical cancer tissue and corresponding normal tissues. Magnification, x40. Immunohistochemical staining for ZEB1 in (A) normal cervical epithelial tissue and (B) in cervical cancer tissue. E-cadherin in (C) normal cervical epithelial tissue and (D) in cervical cancer tissue. Vimentin in (E) normal cervical epithelial tissue and (F) in cervical cancer tissue. ZEB1, zinc finger E-box binding homeobox 1.
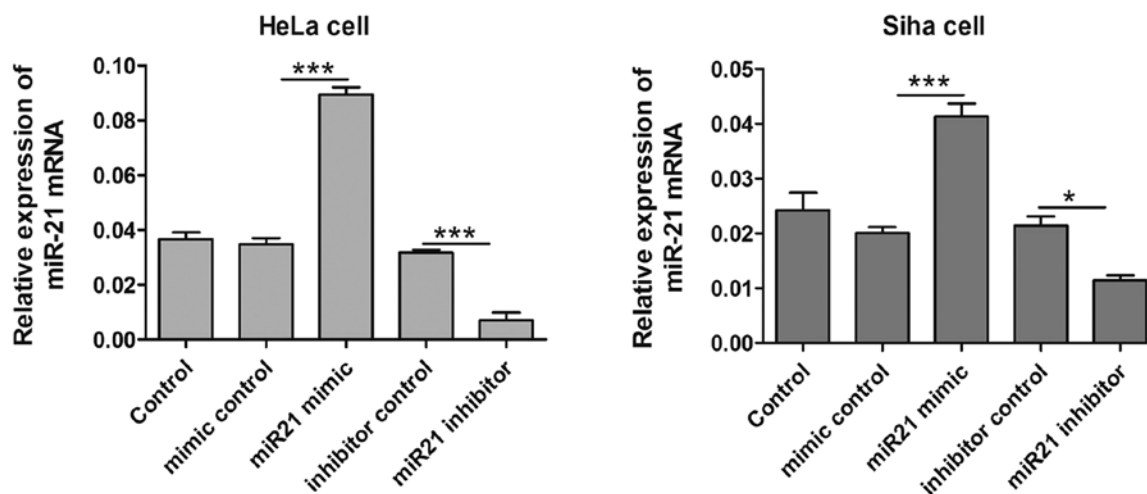
Figure 2. Expression levels of miR-21 in HeLa and SiHa cells after transfection with miR-21-mimics or miR-21-inhibitor. *P<0.05; ***P<0.001 (Kruskal-Wallis test). miR, microRNA. miR-21, microRNA-21.
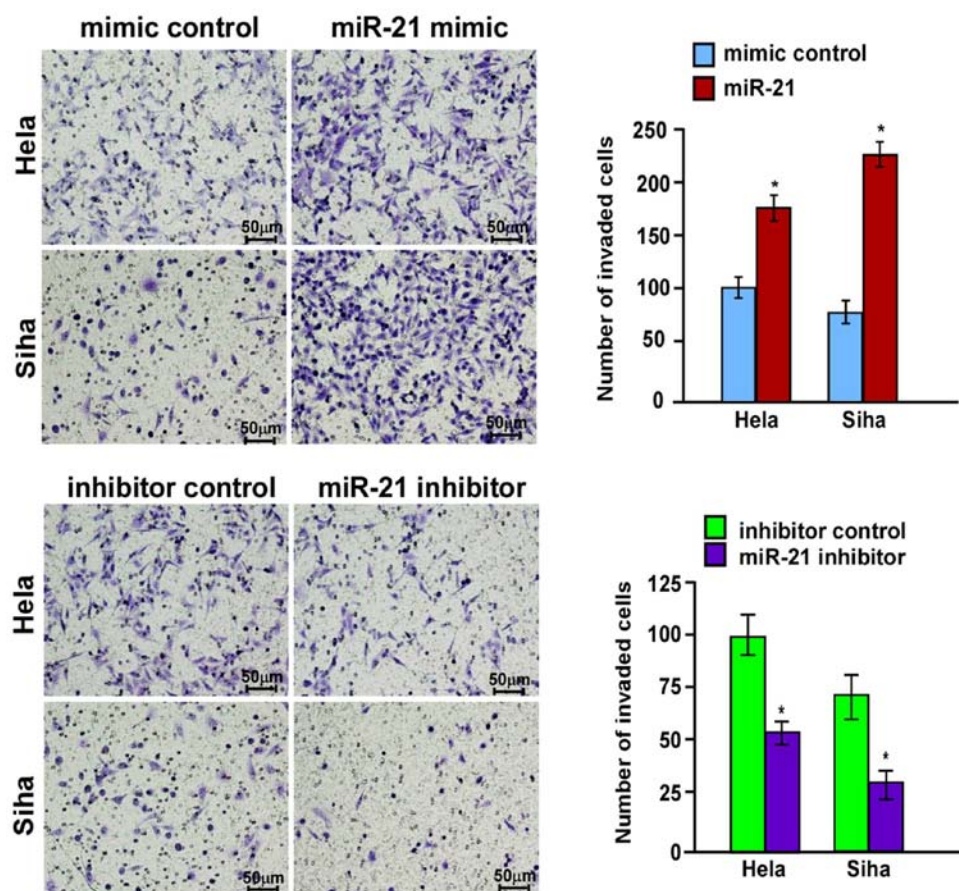

Figure 3. Invasion assay for HeLa and SiHa cells transfected with miR-21-mimics or miR-21-inhibitor. *P<0.05 vs. control (Wilcoxon rank sum test). All experiments were repeated three times. Scale bar, 50 µm. miR-21, microRNA-21.

after transfection with miR-21 inhibitor (P<0.05; Fig. 2). Furthermore, the gene expression level of ZEB1 and Snail in HeLa and SiHa cells also significantly increased following transfection with miR-21 mimics. Transwell assays also indicated that the invasiveness of HeLa and SiHa cells was significantly enhanced after transfection with miR-21 mimics (P<0.05; Fig. 3). In contrast, the invasive ability of HeLa and

SiHa cells significantly decreased after transfection with the miR-21 inhibitor (P<0.05; Fig. 3).

Quantitative real-time PCR analysis indicated that the gene expression level of ZEB1 and Snail increased significantly in HeLa and SiHa cells after transfection with miR-21 mimics (P<0.05; Fig. 4). Significantly decreased protein levels of E-cadherin and increased protein levels of Vimentin
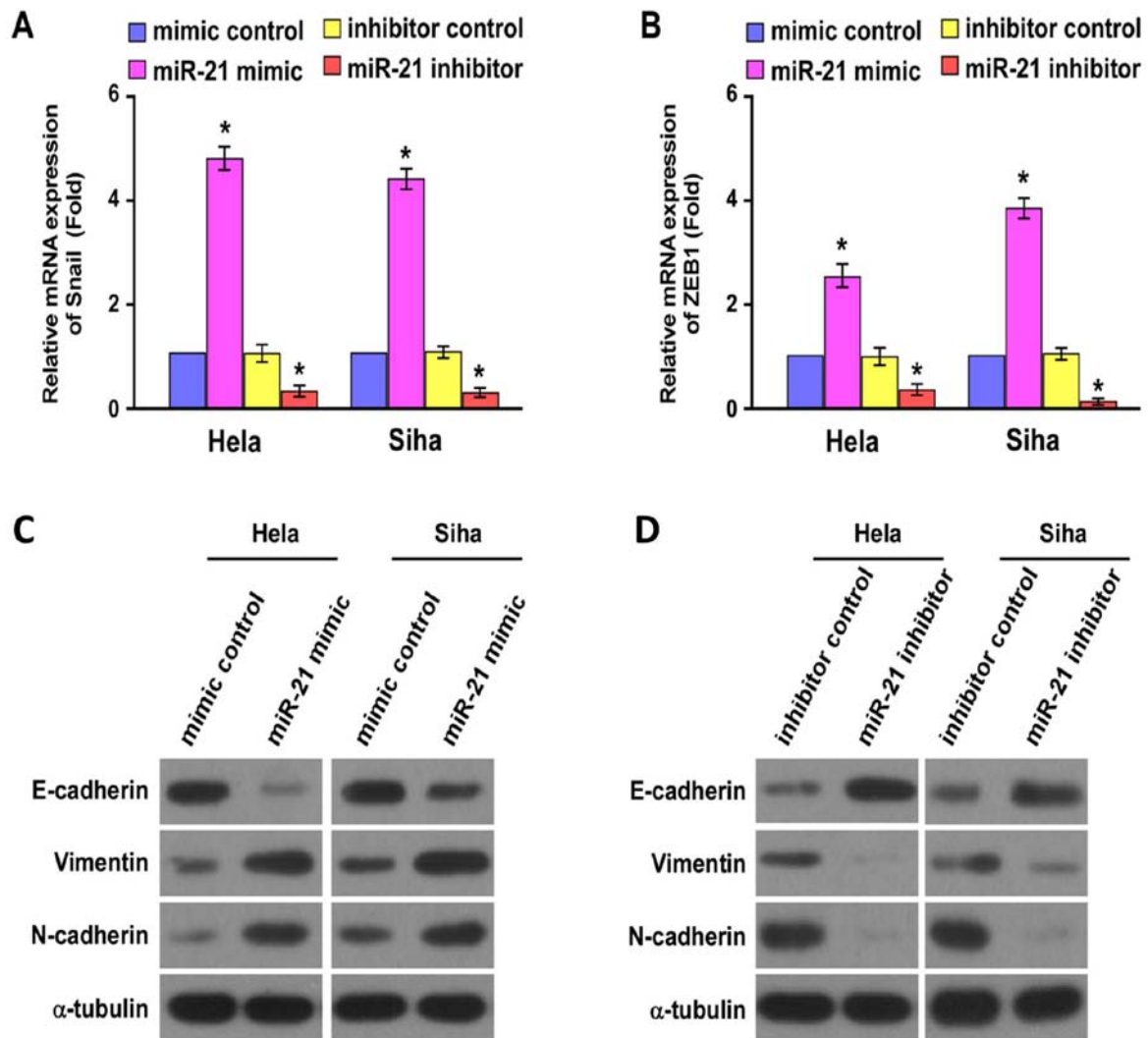
Figure 4. Expression of epithelial-mesenchymal transition-associated factors in cervical cancer cells after transfection with miR-21-mimics or miR-21-inhibitor. Changes in gene expression of (A) Snail and (B) ZEB1 in HeLa and SiHa cells after transfection with miR-21-mimics or miR-21-inhibitor. *P<0.05 vs. control (Wilcoxon rank sum test). Expression of E-cadherin, Vimentin and N-cadherin in HeLa and SiHa cells after transfection with (C) miR-21-mimics or (D) miR-21-inhibitor. miR-21, microRNA-21; ZEB1, zinc finger E-box binding homeobox 1.

and N-cadherin were observed in HeLa and SiHa cells after transfection with miR-21 mimics by western blot analysis (Fig. 4). On the contrary, significantly increased protein levels of E-cadherin and decreased protein levels of Vimentin and N-cadherin were observed in HeLa and SiHa cells after transfection with miR-21 inhibitor (Fig. 4).

Discussion

MiR-21 is a well-known oncogenic miRNA involved in the development of cervical cancer (4), and previous studies have indicated the upregulation of miR-21 in cervical cancer cell lines and clinical specimens (6,9). The present study also indicated that the expression of miR-21 in cervical cancer and lymphatic metastatic carcinoma tissues was significantly higher than that in normal cervical tissues. Furthermore, the expression level of miR-21 was positively correlated with muscular infiltration, parametrical invasion and lymph node metastasis. Together with the results of previous studies, these results demonstrated that miR-21 played a key role in the development and metastasis of cervical cancer.

As the key transcription factor involved in EMT (15), the gene expression of ZEB1 in cervical cancer tissues was significantly higher than in normal cervical tissues. Furthermore, the gene expression level of ZEB1 was also positively correlated with muscular infiltration depth and lymph node metastasis. Combined with results regarding increased protein expression of Vimentin and decreased E-cadherin, it is apparent that the EMT process is involved in the development and biological behavior changes of cervical cancer. Furthermore, primary cervical cancers with an EMT phenotype exhibit increased tumor progression, invasion and metastasis in epithelial integrity (10). Since EMT plays a major role in metastasis and resistance to chemotherapy, it is necessary to investigate the EMT process in cervical cancer.

The results of the present *in vitro* study confirmed that the overexpression of miR-21 in cervical cancer cell lines promoted their motility and invasiveness. Furthermore, upregulation of the EMT-associated transcription factors ZEB1 and Snail was observed in cervical cancer cells after

overexpression of miR-21. In addition, increased expression of Vimentin and N-cadherin, and decreased expression of E-cadherin were observed. From these results, it may be deduced that miR-21 promotes metastasis of human cervical cancer by enhancing EMT. To date, the direct targets of miR-21 involved in the EMT process had remained elusive. The present study demonstrated that the gene expression level of ZEB1 increased significantly in cervical cancer cell lines after overexpression of miR-21. It is clear that miR-21 exerts its gene regulation activity at the post-transcriptional level; therefore, the increased gene expression of ZEB1 suggested that it may not be the direct target of miR-21. MiR-21 probably targets other signaling pathways, which then exert an influence on ZEB1 expression.

Previous studies have indicated that several signal pathways are involved in EMT process, including NF-κB (16), Wnt/β-catenin (17), interleukin-6/signal transducer and activator of transcription (STAT)3 (18) and AKT/glycogen synthase kinase (GSK)-3β (19). STAT3 was indicated to be a potential target of miR-21 in chronic lymphocytic leukemia (20). Similarly, miR-21 may negatively regulate the gene expression of STAT3 and promote the EMT process in cervical cancer. Phosphatase and tensin homolog (PTEN) was also reported to be a direct target of miR-21 in breast phyllodes tumors (21). The ability of miR-21 to induce myofibroblast differentiation in phyllodes tumors was determined to be mediated via modulation of the expression of Smad7 and PTEN, which regulate cell migration and proliferation, respectively. In this way, activation of the AKT/GSK-3β pathway by miR-21 may downregulate the gene expression of PTEN and result in the upregulation of ZEB and Snail, which may promote the EMT process in cervical cancer (19).

In summary, the present study indicated that miR-21 expression was upregulated in cervical cancer tissues and promoted metastasis in cervical cancer through enhancing EMT, while the direct targets of miR-21 still remain elusive. The present results provide novel insight into the molecular mechanisms of miR-21 and the EMT process, and suggest that miR-21 may serve as a potential target in cervical cancer. Assessment of miRNA levels in cervical cancer cells certainly opens novel possibilities for studying molecular markers in the context of screening programs. Clearly, future work should focus on clinically relevant samples and predictive markers expressed in pre-cancerous tissue may be identified for further validation.

Authors' contributions

YT and YZ performed the experiments and prepared the manuscript. JR collected and analyzed clinical data. YW participated in the experimental design and revision of the manuscript. YT and YW supervised the research work, participated in designing the study and revised the manuscript. All authors read and approved the final manuscript.

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