Original Article
miR-204 inhibits invasion and epithelial-mesenchymal transition by targeting FOXM1 in esophageal cancer

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Abstract: MicroRNAs (miRNAs), endogenous noncoding small RNAs, have been reported to play crucial roles in epithelial-mesenchymal transition (EMT) in cancers. Deregulation of microRNA-204 (miR-204) has been documented in many cancers, but its role in the development of esophageal cancer (EC) has not been studied. Here, we reported the role of miR-204 in invasion and EMT in EC. We identified an inverse correlation between miR-204 expression level and the invasion and EMT phenotype of EC cells, and up-regulation of miR-204 inhibited invasion and EMT phenotype of EC cells. Furthermore, we showed that forkhead box protein M1 (FOXM1) was a direct target gene of miR-204, and miR-204 regulated invasion and EMT in EC by acting directly on the 3'UTR of FOXM1 mRNA and suppressing its protein expression. We also explored the anti-tumor effect of miR-204, and found that overexpression of miR-204 suppressed the growth of esophageal tumors *in vivo*. These findings suggest that miR-204 might be a suppressor of invasion and EMT in EC, which offers a novel potential therapeutic target for EC.

Keywords: Esophageal cancer, miR-204, forkhead box protein M1 (FOXM1), epithelial-mesenchymal transition, invasion

Introduction

Esophageal cancer (EC) is one of the most frequent malignant tumors, which is the fifth and eighth most frequent cause of cancer-related death in male and female worldwide, respectively [1]. Despite the great advances achieved in diagnosis and multimodality therapies recently, the prognosis of EC is still poor with a 5-year survival rate of 26.2% [1, 2]. Besides, over 40% of esophageal carcinoma cases result in recurrence [3]. The high probability of metastasis and recurrence is the major cause of treatment failure, yet the precise molecular mechanism of metastatic dissemination is still not completely clear [4, 5]. Recently, many studies have demonstrated that epithelial-mesenchymal transition (EMT) plays a critical role in cancer metastasis [6]. Forkhead box protein M1 (FOXM1) is one of transcription factors, which plays an important role in the activation of EMT [7-9]. The EMT is a process whereby epithelial cells with a cobblestone morphology obtain mesenchymal cell features with a spindle-shaped fibroblast-like phenotype [9]. With the change of cell morphology, the epi-

thelial markers E-cadherin and α1-catenin decrease, and the mesenchymal markers N-cadherin, fibronectin and vimentin increase [5, 7, 8]. Besides, this process involves a disassembly of cell-cell junctions, which makes mesenchymal phenotypic cells have less cell adhesion capacity, and higher cell migration and invasion capacity, resulting in tumor aggressiveness [9].

MicroRNAs (miRNAs) are derived from characteristic hairpins in primary transcripts, and are a class of endogenous noncoding small RNAs with approximately 19-23 nucleotides, which regulate gene expression by binding to complementary sequences of targeted mRNAs, causing translational inhibition or degradation [10, 11]. miRNAs are involved in various biological processes, such as cellular proliferation, differentiation, apoptosis, aging, stress response, oncogenesis, and tumor suppression [12-15]. Mounting evidence indicates that aberrant expression of miRNAs occurs in many kinds of malignant tumors, some of which function as tumor oncogenes or suppressor genes [16, 17]. Furthermore, some studies have implied that

miRNAs function as critical modulators for EMT [18-20]. miR-204 has been reported to act as a tumor suppressor, and is obviously down-regulated in various types of solid malignant tumors [21-23]. Recent studies have found that the expression level of miR-204 in EC cells is decreased, but also directly related to the prognosis [24]. However, for miR-204, the related target genes and molecular mechanism in EC are still not well elucidated.

In this study, we detected the expression level of miR-204 in primary EC cases and cell lines by quantitative Real-Time PCR (qRT-PCR), and explored the association between miR-204 expression and invasive potential and EMT phenotype of EC cells. We further investigated the molecular mechanism by which miR-204 exerts regulatory effects on invasive potential and EMT phenotype in EC. Furthermore, we performed the animal experiments to explore the anticancer action of miR-204 *in vivo*. These findings will provide a novel potential therapeutic target for EC.

Materials and methods

Clinical samples

Esophageal cancer samples and the corresponding adjacent normal esophageal tissues were obtained from 21 patients with EC who underwent radical esophagectomy in the First Affiliated Hospital of Qiqihar Medical University. These 21 patients did not receive any treatment prior to surgery, and cases were confirmed by histopathological examination. All samples were stored in the refrigerator of -70°C after liquid nitrogen freezing. This study was approved by the Ethical Committee of Qiqihar Medical University, and all patients had written informed consent.

Cell lines and cell transfection

Two EC cell lines EC109 and TE10 were obtained from American Type Culture Collection (ATCC, Manassas, VA). Human esophageal epithelial cells (HEEpiC) were purchased from Sciencell (Carlsbad, CA, USA). These cells were grown in RPMI 1640 medium (Hyclone, USA) with 10% fetal bovine serum (FBS, Hyclone, USA), 100 µg/ml of streptomycin, and 100 IU/ml of penicillin at 37°C in 5% CO_2.

miR-204 mimics, negative control mimics (NC), miR-204 inhibitors (anti-miR-204 mimics), neg-

ative control inhibitors (anti-NC), and FOXM1 siRNAs were purchased from GenePharma Company (Shanghai, China). Cell transfection was carried out with Lipofectamine 2000 (Invitrogen, CA, USA) following the manufacturer's suggestions.

*Quantitative real-time PCR (qRT-PCR)*

Total RNA samples were isolated from cultured cells or tissues using Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions, and both mRNA and miRNA were inversely transcribed to cDNA. The expression level of miR-204 was detected by TaqMan stem-loop RT-PCR method with a mirVana miRNA Detection Kit and the gene-specific primer (Invitrogen). Expression of FOXM1 mRNA was examined by SYBR Green real-time PCR (RT-PCR). RT-PCR was carried out by ABI 7500 Fast Real-Time PCR system (ABI, CA, USA). GAPDH was used for normalization.

Cell invasion assay

Cell invasion assay was performed by using transwell chambers. 2×10^4 cells were added into the top chamber with matrigel-coated membrane (24-well insert; 8 µm pore size; BD, San Jose, Biosciences, MA, USA). Top chamber contained serum-free medium, and full medium supplemented with 10% FBS was used as a chemoattractant in the bottom chamber. After 24 h incubation at 37°C, the cells which did not invade through the pores were carefully wiped out by a cotton-tipped swab. Cells on the lower surface of the membrane were immobilized with 4% paraformaldehyde, stained with 0.5% crystal violet, then rinsed with PBS and subjected to microscopic inspection. Invaded cells were counted (five high-power fields/chamber) using an inverted microscope (Olympus, Tokyo, Japan).

Western blot analysis

Total proteins were extracted from cultured cells or tissues by using a RIPA buffer with 0.5% sodium dodecyl sulfate (SDS) in the presence of protease inhibitor cocktail (Beytime, Shanghai, China). Then the standard techniques polyacrylamide gel electrophoresis (PAGE), tank-based transfer to Immobilon Hybond-C membranes (Abcam, Cambridge, UK), and immunological detection were performed in turn. Primary antibodies against
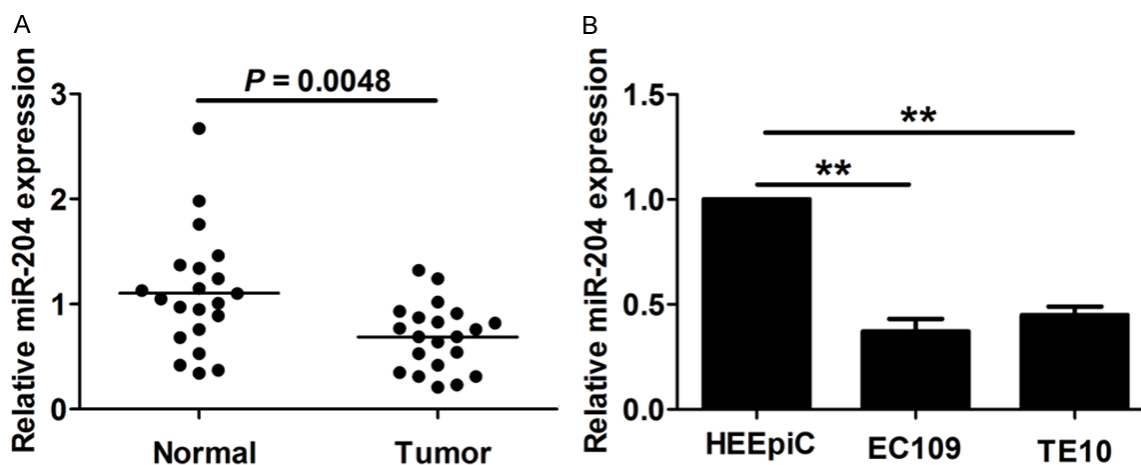
Figure 1. The expression level of miR-204 in primary EC cases and cell lines. A. qRT-PCR shows that miR-204 was frequently up-regulated in 21 primary ES tissues compared with their adjacent normal tissues. B. Up-regulated miR-204 was detected in EC109 and TE10 cell line compared with human esophageal epithelial cells (HEEpiC). **$P < 0.01$.

E-cadherin (Genetex, San Antonio, Southern California USA), α1-catenin (Genmed Scientifics, Arlington, MA, USA), N-cadherin (Abcam), fibronectin (Abcam), vimentin (Sigma, St. Louis, MO, USA), FOXM1 (Sigma) and GAPDH (Abcam), and secondary antibody peroxidase-conjugated anti-IgG (Genmed Scientifics) were used in western blot analysis. Signals were visualized by a chemiluminescent detection system (Pierce ECL Substrate Western blot detection system, Thermo, Pittsburgh, PA) and then exposed in Molecular Imager ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA). The integrated density of the band was quantified by Image J software (version 1.6 NIH).

Target prediction analysis

PicTar, TargetScan, and MicroRNA.org were used to perform bioinformatics-based target prediction analysis.

Luciferase reporter assay

The fragments including the 3'UTR regions (3'UTR-WT) or mutant 3'UTR regions (3'UTR-MUT) of FOXM1 were inserted into XhoI/NotI-digested vector psiCHECK-2 (Promega, Madison, WI) with a firefly and renilla luciferase reporter gene. Then, the psiCHECK-2 vectors including 3'UTR regions or mutant 3'UTR regions of FOXM1 were transfected into miR-204-overexpressing EC109 cells and control cells, separately. Twenty-four hours later, cells were collected, and the firefly and renilla luciferase activities were detected using a dual-luciferase reporter assay system (Promega, USA) according to the manufacturer's suggestions.

In vivo tumor formation assay

Animal experiments were carried out complying with the United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) guidelines for the Welfare of Experimental Animals. For *in vivo* study, EC109 cells (1×10^4) transfected with miR-204 mimics or negative control mimics were subcutaneously injected into the dorsal flanks of nude mice. The volume of subcutaneous tumors was measured weekly by manual caliper measurements. Tumor volumes were calculated using the formula: Tumor volume = length × width^2/2 [25]. After 4 weeks, tested mice were sacrificed, and tumor weight was measured. In addition, the expression levels of E-cadherin, α1-catenin, N-cadherin, fibronectin, vimentin and FOXM1 protein in xenografts were detected by western blot analysis.

Statistical analysis

GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. Results were presented as mean ± standard deviation (SD). Student's *t*-test was used to analyze the differences between groups. Statistical significance was presumed when $P < 0.05$.
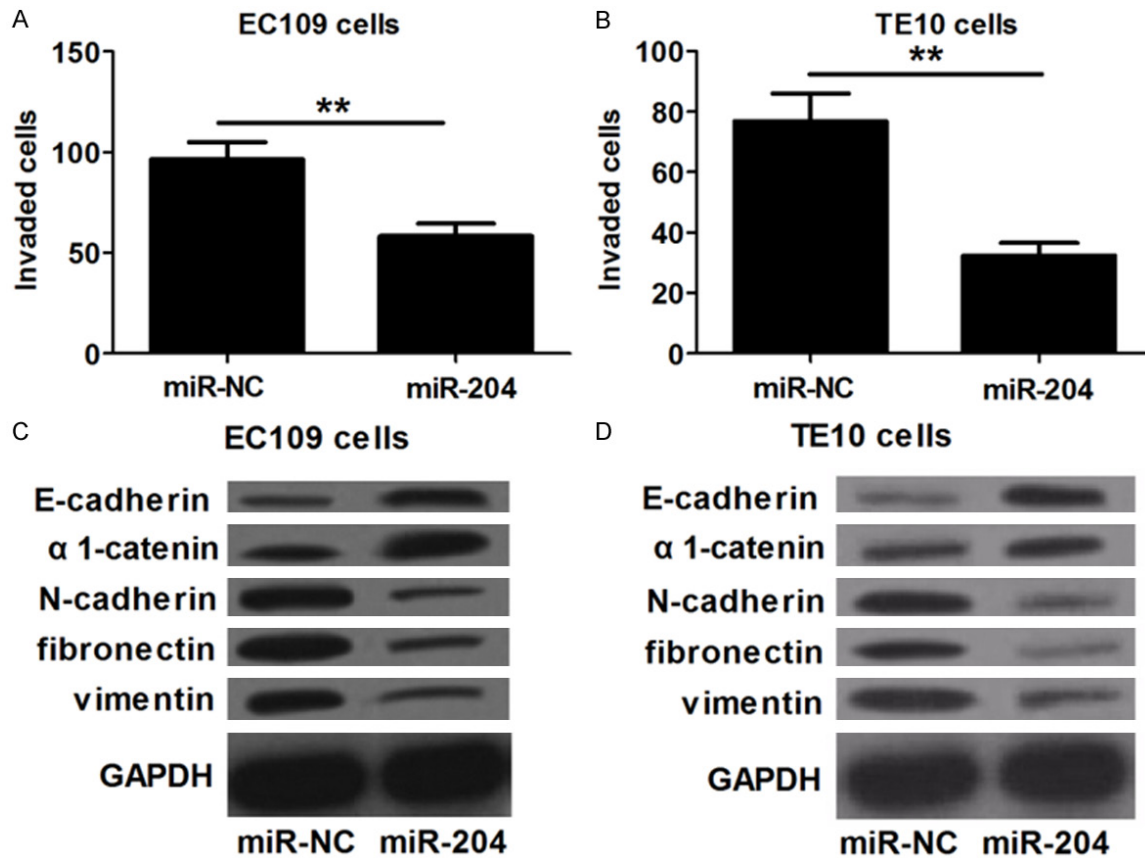
Figure 2. miR-204 inhibited invasive and EMT of EC cell lines. A and B. EC109 and TE10 cells transfected with miR-204 mimics had a significantly lower invasion capacity than the control group. C and D. Compared with the control group, EC109 and TE10 cells transfected with miR-204 mimic had dramatic decreases in N-cadherin, fibronectin and vimentin expression and significant increases in E-cadherin and α1-catenin expression. **$P < 0.01$.

Results

Down-regulation of miR-204 is frequently detected in EC

We compared expression level of miR-204 between tumor and corresponding adjacent normal esophageal tissue in 21 EC patients. The result showed that the expression level of miR-204 in tumor tissues was significantly lower than that in their corresponding normal tissues (**Figure 1A**). Expression level of miR-204 in 2 human EC cell lines was also detected by qRT-PCR and the result showed that miR-204 was obviously down-regulated in EC109 and TE10 cell lines compared with that in HEEpiC (**Figure 1B**).

Exogenous expression of miR-204 inhibits invasion of EC cells

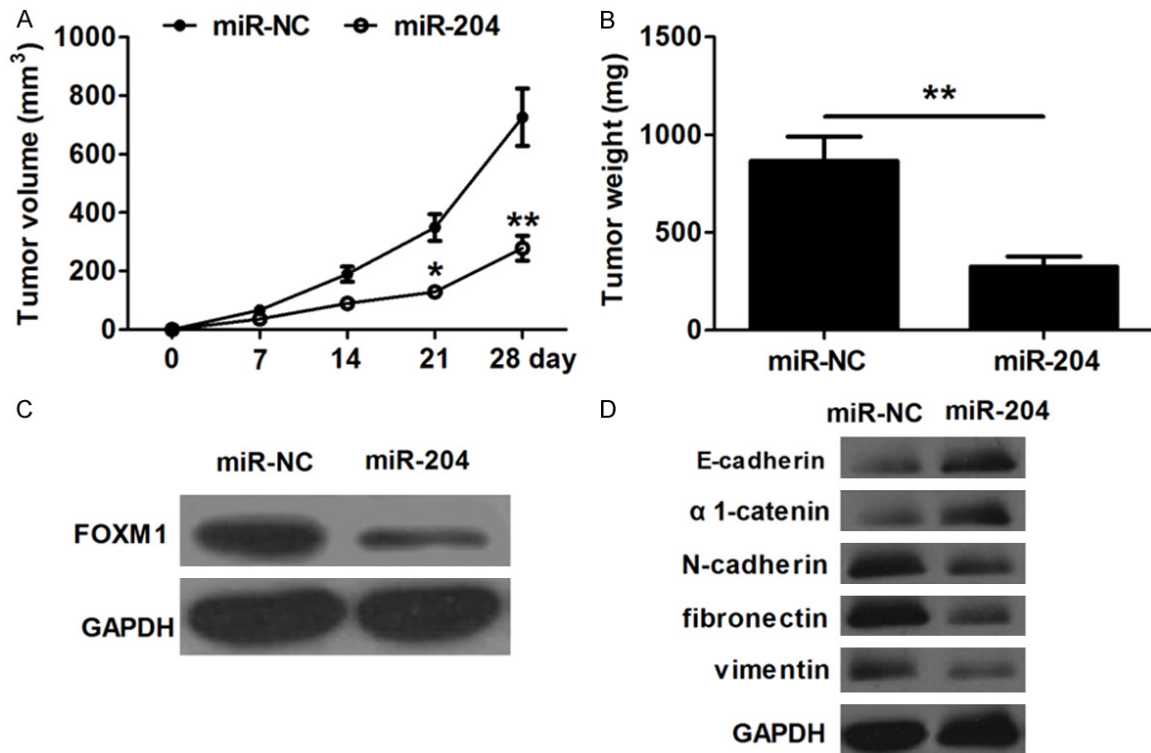To investigate the effect of miR-204 on the invasive capability in EC cells, we transfected

EC109 and TE10 cells with miR-204 mimics or negative control mimics, respectively, and then performed invasion assays by transwell methods. Results showed that EC109 and TE10 cells transfected with miR-204 mimics had a significantly lower invasion capacity than control, which indicated that miR-204 efficiently inhibits invasion of EC cells (**Figure 2A** and **2B**).

miR-204 inversely regulates EMT phenotype of EC cells

Epithelial-mesenchymal transition (EMT) has been identified to contribute to cancer metastasis by transformation of adherent epithelial cells into motile and invasive mesenchymal cells [6]. To investigate whether miR-204 could regulate EMT in EC cells, the expression of epithelial markers (i.e. E-cadherin and α1-catenin) and the mesenchymal markers (i.e. N-cadherin, fibronectin and vimentin) were determined in EC109 and TE10 cells with miR-204 mimics or negative control mimics. The results showed

Figure 3. FOXM1 is a direct target of miR-204. A. Bioinformatics-based target prediction analysis showed that FOXM1 is a potential target gene of miR-204 and the binding site is on the 3'UTR of FOXM1 mRNA. B. Luciferase reporter assay showed that in the miR-204 group, the luciferase activity driven by 3'UTR of FOXM1 mRNA is significantly lower than that in the miR-NC or 3'UTR-MUT group. C. A negative correlation was observed between miR-140 and FOXM1 mRNA in clinical samples (r = -0.861; P < 0.0001). D. The miR-204 group had a significantly low expression of FOXM1 protein compared with the miR-NC group. Relatively, the anti-miR-204 group had an obvious high expression of FOXM1 protein compared with the anti-miR-NC group. E. The transwell invasion assay showed that in the Si-FOXM1 group, the number of invaded cells decreased significantly compared to cells transfected with scramble siRNA (Si-control). F. The Si-FOXM1 group had dramatic decreases in N-cadherin, fibronectin and vimentin expression and significant increases in E-cadherin and α1-catenin expression compared to the Si-control group. $**P$ < 0.01, $***P$ < 0.001.

that compared with control, EC109 and TE10 cells transfected with miR-204 mimics both had dramatic decreases in N-cadherin, fibronectin and vimentin expression and significant increases in E-cadherin and α1-catenin expression, which indicated that miR-204 inversely regulates EMT phenotype of EC cells (**Figure 2C** and **2D**).

miR-204 directly targets FOXM1

miRNA target analysis tools PicTar, TargetScan, and MicroRNA.org were used to explore potential targets of miR-204, and FOXM1 was predicted to be a target of miR-204 (**Figure 3A**). Dual luciferase reporter assay was used to identify whether the 3'UTR of FOXM1 mRNA

Figure 4. miR-204 suppressed tumorigenesis of EC *in vivo*. A. In the miR-204 group, the tumor volume was significantly decreased than that in the control group. B. After 4 weeks, the tumor weight was significantly decreased in the miR-204 group than that in the control group. C. miR-204 inhibited the expression of FOXM1. D. miR-204 decreased N-cadherin, fibronectin and vimentin expression and increased N-cadherin, fibronectin and vimentin expression compared to the control group. *$P < 0.05$, **$P < 0.01$.

was a binding target of miR-204. The results showed that in the miR-204 group, the luciferase activity driven by FOXM1 3'UTR was significantly lowered compared with that in negative control cells, while miR-204-mediated inhibition of luciferase activity was abolished by the mutant presumptive binding site, which suggested that FOXM1 is indeed a direct target of miR-204 (**Figure 3B**). Furthermore, an inverse correlation was directly observed between miR-204 and FOXM1 mRNA (r = -0.861; *P* < 0.0001; **Figure 3C**) in EC samples. To confirm the regulatory effect of miR-204 on FOXM1, western blot assay was performed to detect the expression level of FOXM1 in response to the change of miR-204 expression in EC109 cells. As shown in **Figure 3D**, the results showed a negative regulatory effect of miR-204 on FOXM1. Up-regulated miR-204 in EC109 cells significantly decreased FOXM1 protein expression compared with cells transfected with negative control mimics. Relatively, down-regulation of miR-204 by inhibitors increased FOXM1 protein expression level. To fully explore the roles of

FOXM1 in EC, EC109 cells were transfected with FOXM1 siRNAs (Si-FOXM1) to silence the expression of FOXM1. The transwell invasion assay as illustrated in **Figure 3E** showed that in the Si-FOXM1 group, the number of migrated cells decreased significantly compared to the Si-control group, which indicated that knockdown of FOXM1 represses invasion of EC109 cells. Western blot analysis showed that Si-FOXM1 cells had dramatic decreases in N-cadherin, fibronectin and vimentin expression and significant increases in E-cadherin and α1-catenin expression compared to the Si-control group, which indicated that knockdown of FOXM1 inhibits EMT phenotype of EC109 cells (**Figure 3F**). Similar effect of Si-FOXM1 was observed in miR-204 mimics treated cells, suggesting that Si-FOXM1 could mimic the effect of miR-204. Taken together, these results suggested that FOXM1 is one of the direct targets of miR-204, and functional effect of miR-204 on EC cell lines is dependent on FOXM1.

Overexpression of miR-204 suppresses growth of esophageal tumors in vivo

To explore the anti-tumor effect of miR-204, we subcutaneously injected EC109 cells (1×10^4) transfected with miR-204 mimics or negative control mimics into the dorsal flanks of nude mice. We measured the volume of subcutaneous tumors weekly and found that in the miR-204 group, the tumor growth was significantly inhibited than that in the control group (**Figure 4A**). After 4 weeks, tested mice were sacrificed, and tumor weight was measured. Accordingly, in the miR-204 group, the tumor weight was significantly lower than that in the control group (**Figure 4B**). In addition, we detected the expression of FOXM1, epithelial markers (*i.e.* E-cadherin and α1-catenin), and the mesenchymal markers (*i.e.* N-cadherin, fibronectin and vimentin) in xenografts by western blot. As shown in **Figure 4C**, compared with the control group, the miR-204 group had an obviously low expression of FOXM1. Furthermore, the miR-204 group had dramatic decreases in N-cadherin, fibronectin and vimentin expression and significant increases in E-cadherin and α1-catenin expression compared with the control group (**Figure 4D**). Taken together, these results indicated that overexpression of miR-204 suppresses growth of esophageal tumors *in vivo*.

Discussion

EC is one of the common causes of cancer-related death worldwide. The main reason of cancer death is complications arising from metastasis, while EMT is the key process driving cancer metastasis [6, 26]. Losses of E-cadherin and α1-catenin expression and increases of N-cadherin, fibronectin and vimentin expression are identified as the most important molecular markers of EMT [5, 7, 27, 28]. Emerging evidences have showed that miRNAs play critical roles in the regulation of EMT of cancer cells [29]. A variety of miRNAs have been identified as the promoters for EMT. miR-23a inhibits E-cadherin expression and promotes EMT under the stimulation of TGF-β1 [30]. miR-31 represses the tumor suppressor expression and stimulates EMT [31]. miR-9 promotes tumor metastasis via inhibiting E-cadherin expression in esophageal squamous cell carcinoma [5]. Conversely, many miR-

NAs have been identified as the suppressors for EMT. Particularly, miR-149 and miR-134 inhibit non-small-cell lung cancer cells EMT by targeting FOXM1, and miR-140 and miR-625 repress EC cells EMT and invasion by targeting Slug and Sox2, respectively [7, 8, 32, 33].

Recent researches have demonstrated the essential role of miR-204 in the progression of various types of malignant tumors. miR-204 is down-regulated in some tumors, and functions as a tumor suppressor. For example, loss of miR-204 expression promotes glioma migration and stem cell-like phenotype, and miR-204 represses tumor growth through inhibition of MAP1LC3B-mediated autophagy in renal clear cell carcinoma [21, 23]. Recent studies have also found that miR-204 expression is decreased in EC cells, but also directly related to the prognosis [24]. However, the role of miR-204 in EC is still not well elucidated. Our study demonstrated that up-regulation of miR-204 inhibits invasion and EMT in EC.

FOXM1 is one of transcription factors characterized by the presence of a DNA-binding domain named the forkhead box or winged helix domain, which is detected in many tumor cell lines and regulates the expression of genes related to cell cycle [34]. Overexpression of FOXM1 has been found in various malignant tumors, such as esophagus, breast, lung, colon, and pancreas cancer [7, 35-37]. Interestingly, some studies on FOXM1 have implicated its participation in invasion and EMT of the tumor cells, the key processes contributing to cancer metastasis. Ke *et al.* and Li *et al.* found that FOXM1 stimulates invasive and EMT phenotype of non-small cell lung cancer cells [7, 8]. Bao *et al.* demonstrated that overexpression of FOXM1 leads to EMT and cancer stem cell phenotype in pancreatic cancer cells [9]. In this study, we obtained that FOXM1 is a potential target gene of hsa-miR-204 by bioinformatics analysis. Luciferase assay confirmed that miR-204 targets FOXM1 directly. An inverse correlation is also found between miR-204 and FOXM1 mRNA in clinical patients with EC. Furthermore, knockdown of FOXM1 by siRNA reverses invasion and EMT in EC cells. Li *et al.* observed a similar result that knockdown of FOXM1 by siRNA reversed EMT in non-small cell lung cancer cells [8]. These findings implied that miR-204 suppresses FOXM1 protein expression by

directly binding on the 3'UTR of FOXM1 mRNA to negatively regulate invasion and EMT in EC. We also explored the anti-tumor effect of miR-204 *in vivo*. We reached the conclusion that overexpression of miR-204 suppresses growth of esophageal tumors.

In summary, down-regulation of miR-204 is frequently detected in EC, and miR-204 inversely regulates invasion and EMT phenotype of EC cell. We also provided evidence to prove that miR-204 regulates invasion and EMT in EC by acting directly on the 3'UTR of FOXM1 mRNA. Due to the limit on the number of EC samples and cell types, further comprehensive study will be necessary for exploring the potential role of miR-204 in EC development. miR-204-FOXM1 pathway that we studied might be exploited by a therapeutic strategy for EC treatment in future.

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Disclosure of conflict of interest

None.

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