

Original Article

Downregulation of RKIP by miR-200a promotes the invasive ability of esophageal cancer cells by upregulating the expression of LIN28 and MMP-14

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Abstract: Esophageal cancer (EC) is one of common digestive tract malignant tumors which morbidity and mortality were increased year by year. This study was aimed to investigate the role of microRNA (miR)-200a in EC. Human esophageal squamous cell carcinoma (ESCC) cells TE3 was transfected with miR-200a mimic or scramble control. Cell viability and invasion were assessed by MTT and Transwell assay, respectively. Binding effect of miR-200a on 3'UTR of RKIP was verified by luciferase activity assay. RKIP expression in miR-200a mimic transfected cells was measured. RKIP was overexpressed in miR-200a transfected cells and cell viability and invasion were measured. The expressions of Raf1, ERK, MMP-14, LIN28 and GRK-2 were also measured by qRT-PCR and Western blot analysis, respectively. Results showed that miR-200a mimic transfection increased cell viability and invasion of TE3 cells *in vitro*. miR-200a binding with 3'UTR of RKIP negatively regulated RKIP expression. RKIP overexpression inhibited effects of miR-200a on cell viability and invasion, as well as the increased phosphorylation levels of Raf1 and ERK. miR-200a increased expressions of MMP-14, LIN28 and GRK-2 in TE3 cells, and the up-regulations were inhibited by RKIP overexpression. In conclusion, the up-regulation of miR-200a in TE3 cells promoted cell viability and invasion via negatively regulating RKIP expression. RKIP was a direct target of miR-200a. miR-200a might be involved in activation of MAPK/ERK signaling pathway and expression of MMP-14, LIN28 and GRK-2 which were important factors of intracellular information transduction. Our findings demonstrated that miR-200a regulated ESCC cells via regulating RKIP expression.

Keywords: microRNA-200a, Raf kinase inhibitor protein, MAPK/ERK signal pathway, esophageal cancer

Introduction

Esophageal cancer (EC) is one of the most aggressive digestive tract tumors [1]. In recent years, incidence of human EC was continually increased with the improvement of life standard and impact of eating habit and environment [2, 3]. The esophageal squamous cell carcinoma (ESCC) accounts for more than 90% of all EC cases. ESCC in early stage is usually asymptomatic, most ESCC are diagnosed at advanced stages, following local invasion and lymph node metastasis [4]. In spite of advances in surgical techniques and perioperative management in recent years, mortality of ESCC has not been decreased significantly. The discovery of oncogenes, tumor suppressor or proteins specifically related to esophageal car-

cinogenesis might reveal new diagnostic biomarkers and targets for EC therapy.

MicroRNAs (miRNAs) as a large class of short RNAs (20-24 nucleotides in length) have been proved to be involved in the complex life process, including growth, development, organ formation, cell proliferation and apoptosis, and etc. [5]. miRNA regulates gene expression, especially at the post-transcriptional level. It has key roles in cell development and differentiation by mediating the post-transcriptional regulation of protein-coding genes [6]. Abnormal expression of miRNA was found in many kinds of tumors including EC and was closely related with tumor development and metastasis [7]. Researchers suggested that the deregulation of miRNAs not only affected cancer

progression, but also directly promoted tumor initiation [8]. The development of EC is a complex process involving in many pathogenic factors, multiple stages, and accumulation of multiple gene mutations and interactions. These factors cause dysregulation of oncogenes, tumor-suppressor genes and signaling protein molecules at the molecular level [9]. Previous studies found that miRNAs were frequently deregulated in EC, indicating that miRNAs were important in tumorigenesis of EC [10]. Nowadays, several miRNAs were supported to be potential therapeutic targets for the treatment of ESCC, such as miRNA (miR)-21, miR-375, miR-218 and etc. [10-12]. But there is still little known about the role of miRNAs in EC. As a member of miR-200 family which is becoming a new hotspot in researches of human cancer, miR-200a was also believed to be a tumor suppressor in cancers, but the exactly role of miR-200a in ESCC is still not clear.

In this study, we explored the effects of miR-200a on human ESCC cells by using miR-200a mimic transfection *in vitro*. The probable target gene of miR-200a in ESCC cells was also verified, as well as the potential mechanisms of miR-200a in regulating ESCC cells. Our findings might provide potential treatment targets and novel strategies for ESCC clinical therapy.

Materials and methods

Cell culture and transfection

Human ESCC TE3 cells were previously established from primary EC and maintained in 90% RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 U/ml penicillin and 100 U/ml streptomycin (GIBCO, Grand Island, NY, USA) at 37°C, in a humidified incubator under 5% CO₂ condition. Cells were passaged at a ratio of 1:3 when 80% confluence was reached [13].

For miRNA transfection, synthetic miR-200a mimic and scramble miRNAs as negative control were purchased from Genepharma (Shanghai, China). For overexpression of Raf kinase inhibitor protein (RKIP), full length of RKIP sequence was cloned into the pBabe retroviral vector (Cell Biolabs, Inc., San Diego, CA, USA) and empty vector was used as negative control. All the transfections were performed by

using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. After 48 h of transfection, cells were selected and collected for the next analysis.

RNA extraction and quantitative real-time PCR (qPCR) analysis

Total RNA of cells was extracted by using TRIzol reagent (Invitrogen) and was quantified by using agarose gel (1%) electrophoresis and spectrophotometric analysis (A_{260}/A_{280} ratio). The RNA (500 ng) was reversely transcribed to cDNA by using NCode miRNA First-Strand cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. The cDNA was used as the template for real-time PCR FastStart Universal SYBR green Master (Roche, Branchburg, NJ, USA) supplemented with the universal reverse primer in the kit. The qPCR analysis was performed on Applied Biosystems real-time detection system (Applied Biosystems, Foster City, CA, USA), and the thermocycling parameters were 95°C for 3 min and 40 cycles of 95°C for 15 s followed by 60°C for 30 s. Each sample was run in triplicate and was normalized to U6 snRNA levels (for miR-200a) or GAPDH levels (for mRNAs) [14]. Melting curve analysis was performed to confirm the specificity of the PCR products. The replicates were then averaged, and fold induction was determined by a $\Delta\Delta CT$ -based fold change calculation [15].

Cell viability assay

After corresponding treatments, cells were seeded in triplicate in 96-well plates at a density of 2×10^3 cells/well and were cultured at 37°C, in a humidified incubator with 5% CO₂. The cell viability was assessed by using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay according to the standard methods. Briefly, on each day of the MTT assay, 50 µg of MTT (Sigma, St. Louis, MO, USA) were added into each well and the mix was incubated for another 4 h at 37°C. At the end of incubation, 100 µl of 0.04 N HCl in 2-propanol (Sigma) was mixed thoroughly into each well by shaking for 10 min. The absorbance of each well of plates was read on a Molecular Devices microplate reader (Sunnyvale, CA, USA) at a wavelength of 570 nm, with a background reading at 650 nm subtracted. Triplicate reading for each sample was averaged.

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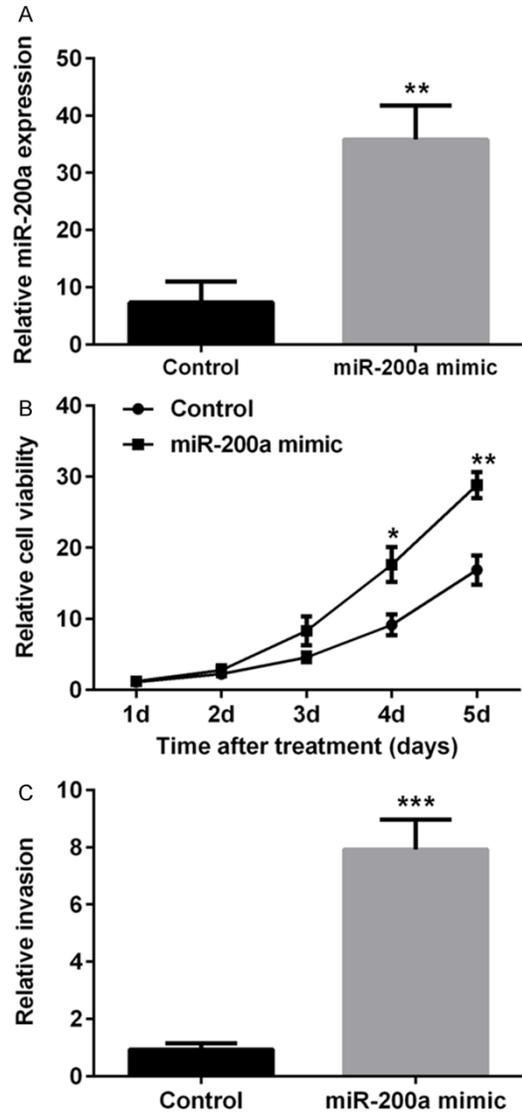


Figure 1. miR-200a mimic transfection promoted cell viability and invasion of human esophageal squamous cell carcinoma TE3 cells. miR-200a mimics or scramble control (control) were transfected into TE3 cells. Real-time PCR was performed to measure the expression level of miR-200a (A). Cell viability (B) and invasiveness (C) were measured by MTT and Transwell assays, respectively. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared to control group or that in corresponding time point of control group.

Cell invasion assay

Cell invasion was assessed by using 24-well Transwells and Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). In brief, a total of 50 μ l dissolved Matrigel was evenly coated onto the bottom of upper chamber of each Transwell. After 48 h of corresponding administration,

cells were digested and centrifuged. Then cells were re-suspended in culture medium without FBS, adjusting the concentration of cells to 5×10^5 /ml. Then, 20 μ l of cell suspension was added onto the upper chamber, and 600 μ l RPMI-1640 medium containing 10% FBS was added into the lower chamber. Transwells were incubated at 37°C with 5% CO₂ for 48 h. The non-invasive cells on the upper chamber were carefully removed by using cotton swabs. Then Transwell was inverted and 600 μ l anhydrous ethanol was added. After being air-dried and rinsed, cells were stained with crystal violet, counted and photographed under microscope (Leica Microsystems, Wetzlar, Germany).

Luciferase activity assay

The 3' untranslated region (UTR) segment of *RKIP* gene containing the miR-200a binding sites was amplified by PCR and was inserted into the pmirGLO Dual-Luciferase miRNA target Expression vector (Promega, Madison, WI, USA) as *RKIP* 3'UTR wild type (WT). Cells were co-transfected with *RKIP* 3'UTR WT and miR-200a mimic or miRNA scramble control by using Lipofectamine 2000 reagent (Invitrogen). The binding site of miR-200a in *RKIP* 3'UTR WT was mutated via point mutation, which was referred to as negative control (*RKIP* 3'UTR Mt). The Firefly and Renilla luciferase activity were analyzed at 48 h post-transfection by using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. For each transfection, the luciferase activity was averaged from three replicates.

Western blot analysis

The proteins were extracted by RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche). The samples were quantified by using BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). Equal amount of samples were lysed with 1 \times SDS-loading buffer (50 mM Tris-Cl pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol and 0.1% bromophenol blue) as the whole-cell sample and were subjected to SDS-PAGE. All blots were transferred on to the polyvinylidene difluoride (PVDF) membranes. Immunoblots were carried out with primary antibodies at the dilution of 1:1000 including anti-*RKIP* (sc-101504), anti- β -Tubulin (sc-101527), anti-Raf1 (sc-528-

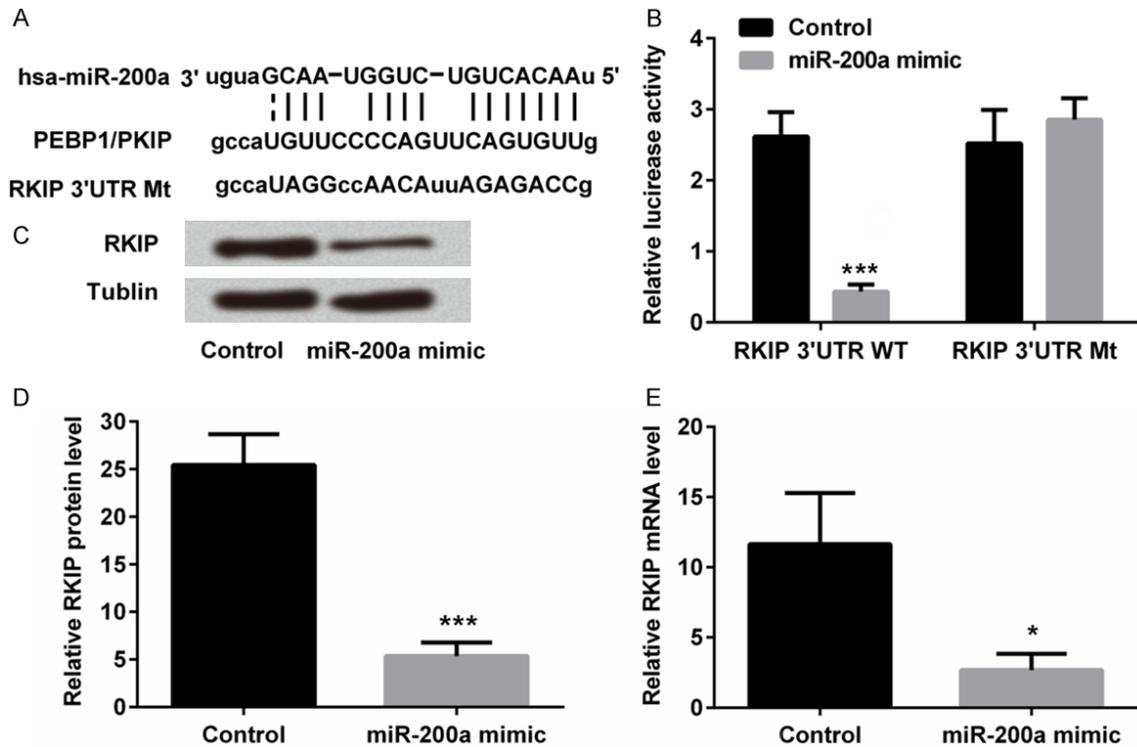


Figure 2. miR-200a directly regulated the expression of RKIP in human esophageal squamous cell carcinoma TE3 cells. The potential miR-200a binding sites on RKIP 3'UTR wild type (WT) and mutant (Mt) as shown (A). miR-200a and 3'UTR of RKIP were co-transfected into TE3 cells and luciferase activities were performed to assess binding effect of miR-200a on 3'UTR of RKIP (B). miR-200a mimic or scramble control (control) were transfected into TE3 cells. Western blot analysis and real-time PCR were performed to determine the protein (C and D) and mRNA (E) expressions of RKIP. *, $P < 0.05$; ***, $P < 0.001$ compared to control group.

27), anti-phosphorylated (p)-Raf1 (sc-81513), anti-ERK1/2 (sc-94), anti-p-ERK1/2 (sc-1017-60), anti-matrix metalloproteinase (MMP)-14 (sc-377097), anti-LIN-28 (sc-293120) and anti-G-protein-coupled receptor kinase (GRK)-2 (sc-13143) (Santa Cruz, USA), respectively. Then the membranes were incubated with secondary antibody marked by horseradish peroxidase (HRP) (sc-516102, Santa Cruz), at the dilution of 1:5000. The intensity of blots were detected by enhanced chemiluminescence (ECL-plus, Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) and quantified by using Image Lab™ Software (Bio-Rad, Shanghai, China).

Statistical analysis

The results of multiple experiments were presented as mean + standard deviation (SD) except for the results of cell viability, which were presented as mean ± SD. Statistical analysis was performed by using GraphPad Prism 6

software (GraphPad Software, San Diego, CA, USA). The P -values were calculated by using a one-way analysis of variance (ANOVA) for multiple groups, and two-tailed Student's t-test between two groups. P value of < 0.05 was considered to indicate statistically significant.

Results

miR-200a promoted cell viability and invasion of TE3 cells

To verify the effect of miR-200a on human ESCC, TE3 cells were transfected with miR-200a mimic or scramble miRNAs as negative control. The transfection efficiency was measured by qPCR. Results in **Figure 1A** showed that miR-200a mimic transfection significantly increased miR-200a expression in TE3 cells compared with control group ($P < 0.01$), suggesting that miR-200a expression level in TE3 cells was effectively increased after miR-200a mimic transfection.

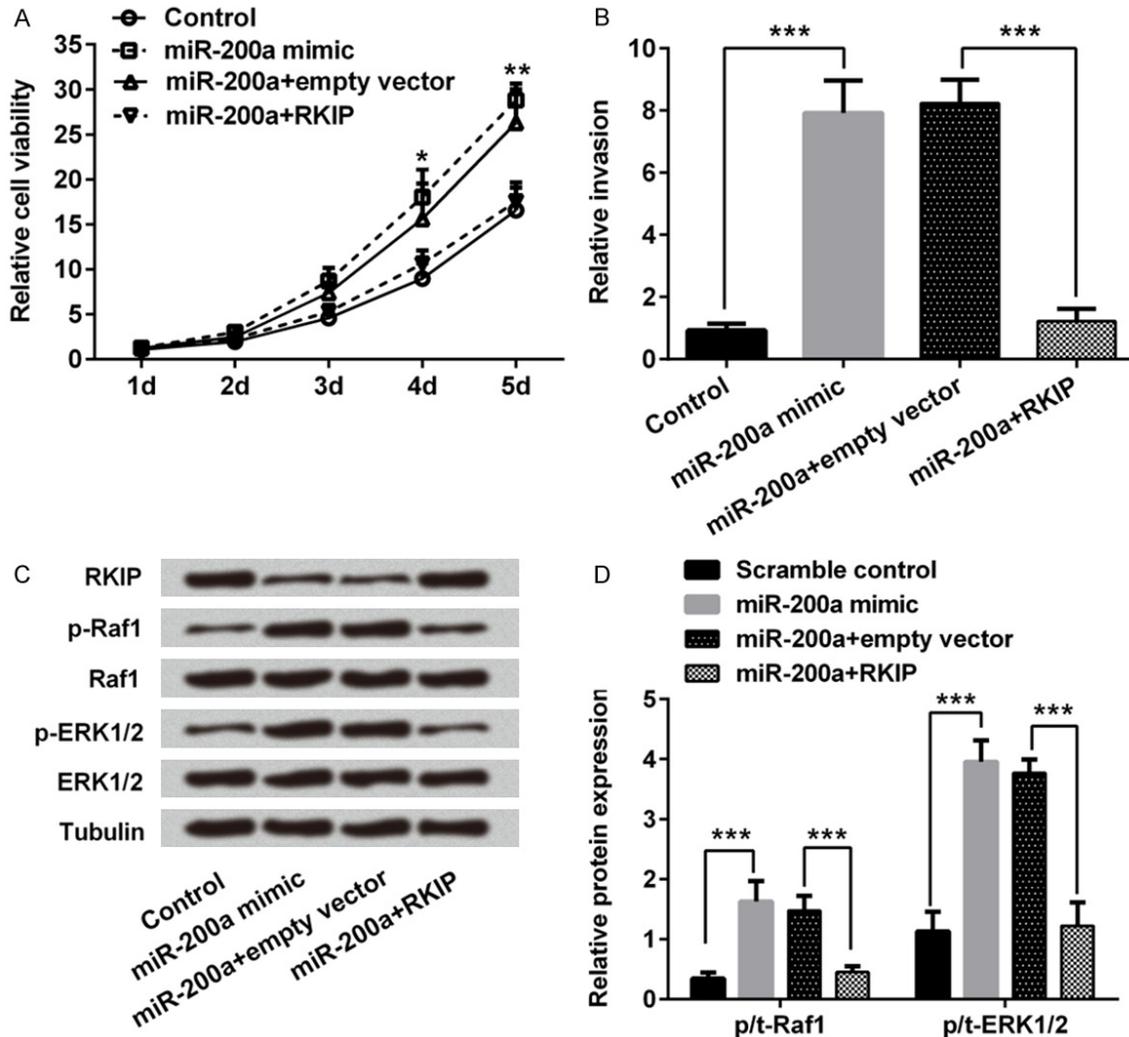


Figure 3. RKIP overexpression inhibited miR-200a induced increases of cell viability and invasion in human esophageal squamous cell carcinoma TE3 cells via inactivating MAPK/ERK pathway. Cells were transfected with RKIP overexpression vector (RKIP) and/or miR-200a mimic. MTT and Transwells assays were performed to determine cell viability at corresponding time (A) and invasiveness (B). Protein expressions of RKIP, Raf1, phosphorylated (p)-Raf1, ERK-1/2 and p-ERK1/2 were measured by western blotting (C). Statistical results of phosphorylation levels about Raf1 and ERK1/2 to assess the activation of MAPK/ERK pathway in TE3 cells (D). *, $P < 0.05$; **, $P < 0.01$; compared to that in corresponding time point of control group. ***, $P < 0.001$ compared to the corresponding negative control.

The cell viability and invasion of TE3 cells were also assessed after miRNA transfection. Results of MTT assay were shown in **Figure 1B**, cell viability was increased compared with control group at the corresponding test time ($P < 0.05$ or $P < 0.01$). The results of invasion assay in **Figure 1C** showed that miR-200a mimic transfection significantly increased cell invasion compared with that in control group ($P < 0.001$). These results suggested that miR-200a mimic transfection promoted human ESCC TE3 cell viability and invasion *in vitro*.

miR-200a regulated RKIP expression in TE3 cells via binding with RKIP 3'UTR

According to the prediction analysis, the segment of RKIP 3'UTR had the potential binding sites of miR-200a as shown in **Figure 2A**. We assessed the binding effect of miR-200a on RKIP 3'UTR, luciferase assay results in **Figure 2B** showed that the fluorescence signal intensity was significantly inhibited in RKIP 3'UTR WT + miR-200a mimic transfection group compared with RKIP 3'UTR WT + miRNA scramble

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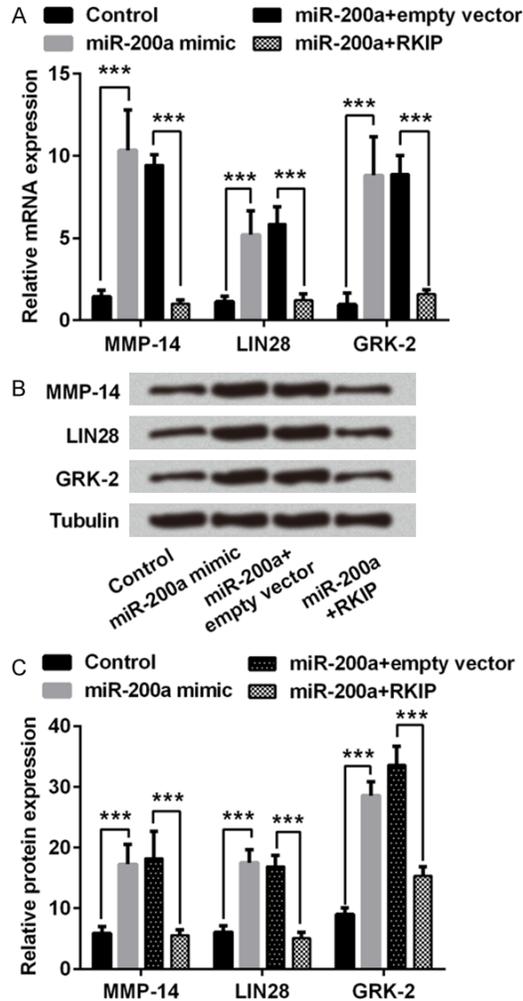


Figure 4. miR-200a increased the expressions of matrix metalloproteinase (MMP)-14, LIN28 and G protein-coupled receptor (GPCR) kinase 2 (GRK-2) in TE3 cells. Cells were transfected with RKIP overexpression vector (RKIP) and/or miR-200a mimic. The mRNA expression level was measured by real-time PCR (A) and protein expression level was assessed by Western blotting (B). Statistical results of immunoblot analysis (C). ***, $P < 0.001$ compared to the corresponding negative control.

control transfection group ($P < 0.001$), while in RKIP 3'UTR Mt groups, there were no significant differences. These results suggested that RKIP might be a direct target of miR-200a in TE3 cells.

Meanwhile, expression of RKIP in TE3 cells after miR-200a transfection was also measured. RKIP protein (Figure 2C and 2D) and mRNA (Figure 2E) expression levels in TE3 cells after transfection were measured, respectively. RKIP

expression was decreased after miR-200a mimic transfection in both mRNA level ($P < 0.05$) and protein level ($P < 0.001$), suggesting that miR-200a negatively regulated expression of RKIP in TE3 cells.

RKIP overexpression inhibited effects of miR-200a on cell viability and invasion as well as activation of MAPK/ERK pathway in TE3 cells

RKIP was overexpressed in TE3 cells combined with miR-200a mimic transfection to further verify the effect of miR-200a. In Figure 3A, MTT assay results showed that miR-200a mimic transfection increased cell viability compared with control ($P < 0.05$ or $P < 0.01$ at corresponding time point), while combined with RKIP overexpression, cell viability was decreased with no significant difference with control group. Meanwhile, the invasion analysis results in Figure 3B showed that even miR-200a mimic transfection increased cell invasion compared with control group ($P < 0.001$), overexpression of RKIP in miR-200a transfected TE3 cells also significantly decreased cell invasion compared with miR-200a + empty vector group ($P < 0.001$). It suggested that as the direct target of miR-200a, overexpression of RKIP in TE3 cells inhibited the effects of miR-200a mimic transfection on cell viability and invasion.

RKIP was identified as an endogenous inhibitor of MAPK/ERK signaling, we then verified the potential mechanism of miR-200a-associated modulation of TE3 cells. The expression of Raf1 and ERK1/2 was determined in miR-200a mimic and/or RKIP transfection cells. Western blot assay results in Figure 3C showed that expression of RKIP was decreased in miR-200a mimic transfected groups, while phosphorylated Raf1 and ERK 1/2 expressions were increased after miR-200a mimic transfection. According to the statistical analysis results as shown in Figure 3D, phosphorylation levels of Raf1 and ERK1/2 were significantly increased in miR-200a transfected cells compared with control group ($P < 0.001$), while overexpression of RKIP in miR-200a mimic transfected cells significantly decreased phosphorylation levels of Raf1 and ERK1/2 compared with that in miR-200a mimic + empty vector group ($P < 0.001$). All these results suggested that miR-200a might be related with activity of MAPK/ERK pathway via RKIP in human EC TE3 cells.

miR-200a induced the expression of MMPs in TE3 cells

To further explore the role of miR-200a in human EC, expressions of some related factors were also analyzed. Considering that metalloproteinases are related to extracellular matrix (ECM) degradation which is closely associated with tumor cell migration and metastasis, we measured the expression of MMP-14. Meanwhile, LIN28 and GRK-2 which have been proved to play important roles in tumor development were also assessed. The qPCR analysis results in **Figure 4A** showed that miR-200a mimic transfection significantly increased mRNA expressions of MMP-14, LIN28 and GRK-2 compared with scramble control group ($P < 0.001$); while overexpression of RKIP in miR-200a mimic transfected cells significantly decreased these factors mRNA expression level compared to the miR-200a mimic + empty vector group ($P < 0.001$). Western blot assay results shown in **Figure 4B** and **4C** suggested the inducible effect of miR-200a mimic transfection on these factors expression ($P < 0.001$), as well as that RKIP overexpression inhibited protein expressions compared with miR-200a + empty vector group ($P < 0.001$). According to these results, we speculated that miR-200a could elevate expressions of MMP-14, LIN28 and GRK-2 in TE3 cells and might regulate cell bioactivity via its target.

Discussion

EC, especially ESCC, is one of the most common malignant tumors. Most patients with ESCC were diagnosed at an advanced stage [16]. Despite recent improvements in treatment of ECSS, the clinical outcome of ESCC patients remains unsatisfactory [17]. The carcinogenesis of ECSS and its development process is involved in a variety of oncogenes and tumor suppressor genes involved in the abnormal mutation and mutual regulation of complex process. It is urgently needed to investigate the underlying molecular markers to improve the outcome of patients with ESCC [18]. In this study, we made primary researches about the effects of miR-200a on ESCC cells *in vitro*, and also verified that RKIP was the direct target of miR-200a in TE3 cells. The up-regulation of miR-200a in ESCC TE3 cells promoted cell viability and invasion, as well as suppressed its target RKIP expression via binding with the

3'UTR of RKIP, which was also increased the activation of MAPK/ERK signal pathway. Abnormal expression of miR-200a was probably related with TE3 cell bioactivity.

Previous studies found that miRNAs were frequently deregulated in ESCC, indicating that the deregulation of miRNAs played important roles in the tumorigenesis of ESCC [19]. As a member of miR-200 family which has been proved to act as tumor suppressors, miR-200a is significantly involved in inhibition of epithelial-to-mesenchymal transition (EMT), repression of cancer stem cells (CSCs) self-renewal and differentiation, modulation of cell division and apoptosis, and reversal of chemoresistance [20-22]. Experimental evidence revealed that the majority of human miRNA genes were located at fragile sites and genomic regions involved in cancer, functioning as oncogenes or tumor suppressor genes [23]. In this study, we found that upregulated miR-200a could prompt cell viability and invasion *in vitro*, suggesting that up-regulation of miR-200a might be related with metastasis of ESCC.

As known, in miRNA biology, seed sequences, in terms of the complementary sequences to binding sites of miRNA within the 3'UTRs of the target genes, play a crucial role in regulatory effects of miRNA on gene expression [24]. In our study, we found that RKIP was a direct target of miR-200a in TE3 cells, miR-200a binding with 3'UTR of RKIP, negatively regulated expression of RKIP *in vitro*. RKIP is a member of the phosphatidylethanolamine-binding protein (PEBP) family and plays a pivotal modulatory role in several protein kinase signaling cascades [25]. RKIP inhibits Raf-1-mediated phosphorylation of MEK through binding to Raf-1. It is a negative regulator of the Raf/MEK/ERK signaling pathway [26]. Research had proved that ectopic expression of RKIP antagonized the kinase activity of B-Raf, and expression levels of RKIP in melanoma cancer cell lines were low relative to primary melanocytes [27]. The regulatory role of RKIP in cell signaling is reflected in its role in physiology and pathophysiology and thus also be related with regulation of cancer cells [28]. Our results suggested that miR-200a regulated cell viability and invasion of ESCC cells *in vitro* via regulating its target gene RKIP expression. This regulatory effect was also involved in the MAPK/ERK signaling pathway, that the upregulation of miR-200a in TE3

cells, which inhibited the expression of RKIP as an inhibitor of Raf/MEK/ERK pathway, could effectively increase the phosphorylation levels of Raf and ERK, and thus increased activity of MAPK/ERK signaling pathway.

In the present study, we also found that miR-200a could increase the expression of MMP-14, the RNA-binding protein LIN28 and GRK-2 in both mRNA and protein levels which were inhibited by RKIP in EC TE-3 cells. RKIP was proved to prevent the invasion of cancer cells via controlling the expression of MMPs [29]. MMPs overexpression might indicate a higher risk of poor prognosis in cancers [30]. MMP-1 or MMP-2 in RKIP knockout cancer cells was reverted invasiveness and metastatic potential to normal levels [31]. In the researches about RKIP effect on esophageal TE-1 cancer cells, overexpression of RKIP inhibited MMP-14 expression and reduced transcription of LIN28 which is posttranscriptional repressor of let-7 miRNA biogenesis, and thus reduced the invasiveness of esophageal TE-1 cancer cells [29]. The Lin28-mediated downregulation of let-7 might also play a key role in development, stem cell programming, and tumorigenesis [32]. Meanwhile, GRK-2 is a ubiquitous member of the GRK family that appears to play a central, integrative role in signal transduction cascades. GRK-2 is a negative feedback inhibitory protein for G protein-coupled receptors (GPCRs) which has been shown to phosphorylate GPCRs, and separate G protein from GPCRs [33, 34]. In addition, emerging evidence indicates that alterations in GRK-2 levels or functionality participated in the development of several tumor or inflammatory diseases [34]. The binding conversion from Raf-1 to GRK-2 suggests that RKIP serves as a signal regulator in ESCC TE-3 cells, and miR-200a was involved in regulation of the signaling pathways via RKIP.

In conclusion, our findings demonstrated that miR-200a promoted cell viability and invasion of human EC cells via downregulating RKIP, which could inactivate MAP/ERK signaling pathway, and regulated expressions of MMP-14, LIN28 and GRK-2. However, further studies about the expression of miR-200a in ESCC tissues, as well as the usage of animal models simulated *in vivo* conditions will help to understand the exact role of miR-200a, and provide a potential clinical therapeutic strategy for treatment of ESCC.

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Disclosure of conflict of interest

None.

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