# Original Article miR-433 inhibits viability, migration and invasion of esophageal cancer cells EC9706 and EC109 possibly via suppressing RAB34

Xinxuan Li<sup>1\*</sup>, Ming Dong<sup>2\*</sup>, Bin Yang<sup>1</sup>, Zheng Huang<sup>1</sup>

<sup>1</sup>Department of Thoracic Surgery, Weinan Central Hospital, Weinan, Shaanxi, China; <sup>2</sup>Department of Surgical Oncology, Affiliated Hospital of Shaanxi University of Chinese Medicine, Xianyang, Shaanxi, China. \*Co-first authors.

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**Abstract:** Esophageal cancer is of high malignancy, causing a great number of deaths worldwide. microRNAs are powerful regulators of gene expression that may play vital roles in esophageal cancer development. This study aims at revealing the roles of miR-433 in esophageal cancer. Human esophageal cancer cell lines EC9706 and EC109 were used to investigate miR-433 expression compared to normal cell line HEEC. miR-433 was overexpressed by lentivirus transfection. The oncogene RAB34 was likely to be regulated by miR-433, and its overexpression and knockdown were achieved by overexpression vectors and short hairpin RNAs, respectively. Cell viability, migration and invasion of the transfected cells were analyzed by MTT, wound healing assay and transwell experiments. Results showed that miR-433 was down-regulated, and RAB34 was up-regulated in both EC9706 and EC109 cells (*P* < 0.01). Overexpression of miR-433 inhibited cell viability, migration and invasion of EC9706 and EC109 cells, and inhibition of RAB34 caused similar effects. RAB34 overexpression in the two cell lines with miR-433 overexpression could reverse the effects of miR-433, leading to the promoted cell viability, migration and invasion. Up-regulation of miR-433 could inhibit both mRNA and protein expression levels of RAB34, which was in consistent with the changes in cell abilities. These results indicated that miR-433 was a suppressor in cell viability, migration and invasion of esophageal cancer cells, possibly via inhibiting RAB34. So miR-433 might provide a promising therapeutic strategy for treating esophageal cancer, but further mechanism research is still necessary.

Keywords: Esophageal cancer, microRNA, miR-433, RAB34, cell invasion

#### Introduction

Esophageal cancer is one of the most malignant cancers with a high morbidity and mortality. It is the fourth leading cause of cancer death in China, with an estimated death of nearly 220 thousands in 2011 [1]. The two predominant forms of esophageal cancer are esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). ESCC is the main cause of esophageal cancer death worldwide, and EAC has increased its incidence in men of high-income countries in recent years [2]. ESCC arises from the stratified squamous epithelial lining, and EAC generates from the columnar glandular cells which can replace the squamous epithelium of the organ [3]. For the high malignancy of esophageal cancer, safe and effective diagnosis and treatment methods are of great importance to reduce the mortality. Efforts have been made on improving the diagnosis and surgery strategies, such as the endoscopic screening and intervention program [4], and endoscopic submucosal dissection [5].

microRNAs are small, noncoding molecules that play significant roles in regulating gene expression, mostly post-transcriptionally. For their modulation of oncogenes, research on microRNAs has become a hotspot in cancer studies, including esophageal cancer. Some microRNAs were aberrantly expressed in esophageal cancer, indicating their potential roles in the development of this disease [6]. For example, microRNA (miR)-141 inhibits the expression of tumor suppressor gene SOX17 [7], and SOX4 can inhibit miR-31 expression by regulating the promoter region of miR-31 [8]. miR-375 can be regulated by hypermethylation in its promoter. thus promoting the expression of its target gene, 3-phosphoinositide-dependent protein



**Figure 1.** Expression of miR-433 and RAB34 in esophageal cancer cells detected by qPCR (n = 3). A. Relative miR-433 expression in HEEC cells and two esophageal cancer cell lines EC9706 and EC109. Significant down-regulation is detected in esophageal cancer cells. B. Relative *RAB34* mRNA expression in HEEC cells and two esophageal cancer cell lines EC9706 and EC109. Significant up-regulation exists in esophageal cancer cells. \*\**P* < 0.01 compared to HEEC.

kinase-1 in esophageal cancer [9]. Uncovering the roles of microRNAs in esophageal cancer is still a complex issue to be solved.

miR-433 is reported to be a suppressor on hematopoietic cell proliferation in myeloproliferative neoplasms via inhibiting its target guanylate binding protein 2 [10]. It also plays tumor-suppressive roles in oral squamous cell carcinoma by directly inhibiting histone deacetylase 6 [11]. However, the relationship between miR-433 and esophageal cancer remains elusive. This study aimed at revealing the role of miR-433 in esophageal cancer cells. The expression of miR-433 was compared between human normal esophageal epithelial cell line HEEC and two human esophageal cancer cell lines EC9706 and EC109. The lentivirus of miR-433 and the specific short hairpin RNA (shRNA) of RAB34, member RAS oncogene family, as well as the overexpression vector of RAB34, were transfected to these cells for cell viability, migration and invasion analyses. This study would offer evidence for miR-433 functioning in esophageal cancer, and provide information for microRNA research in cancers.

# Materials and methods

### Cell culture

The human normal esophageal epithelial cells HEEC and human esophageal cancer cells EC9706 and EC109 were purchased from ATCC (Manassas, VA). HEEC cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Gibco). EC9706 and EC109 cells were cultured in Dulbecco modified Eagle medium (DMEM, high glucose, Gibco) supplemented with 10% FBS. All cells were incubated in humidified atmosphere with 5% CO<sub>2</sub> at 37°C.

### Cell transfection

The cultured EC9706 and EC109 cells were divided into seven groups: control (with no transfection), miR-433 (transfected with the lentivirus to overexpress miR-433), miR-SCR (transfected with the lentivirus containing scramble miRNAs as a control), sh-RAB34 (transfected with the lentivirus containing the shRNA for RAB34), sh-control (transfected with the lentivirus containing the lentivirus containing control shRNA), miR-433 + RAB34 (transfected with miR-433 lentivirus and then RAB34 overexpression vector), and miR-433 + vector (transfected with miR-433 lentivirus and then blank vector as a control).

At one day before the transfection, the cells were counted and seeded in 24-well plates at 1  $\times$  10<sup>5</sup> cells per well. Before transfection, the medium in plates was changed by fresh medium with 6 µg/mL Polybrene (Sigma-Aldrich, Shanghai, China) and the corresponding lentivirus for miR-433 overexpression, RAB34 knockdown or the controls (GenePharma, Shanghai, China) according to the manufacturer's instruction, and the cells were incubated at 37°C for 4 h. Then the medium was replaced by fresh medium for further culture. For RAB34 overexpression, the coding sequence of RAB34 was cloned into pcDNA3.1 vector (Thermo Scientific, Carlsbad, CA) and the correct clone was screened by PCR and sequencing. Then the overexpression vector (1 µg) or blank vector was transfected into cells overexpressing miR-

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**Figure 2.** miR-433 inhibits RAB34 expression in esophageal cancer cells. control, cells without transfection. miR-SCR, cells transfected with the control lentivirus. miR-433, cells transfected with lentivirus overexpressing miR-433 (n = 3). A. qPCR shows that miR-433 is significantly up-regulated after transfection of miR-433 lentivirus compared to miR-SCR. B. qPCR shows that *RAB34* mRNA expression is inhibited when miR-433 is overexpressed. \**P* < 0.05. \*\**P* < 0.01. C. Western blot shows that RAB34 protein expression is inhibited by miR-433 overexpression. GAPDH is used as an internal control.

433 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction.

#### Cell viability assay

The seven groups of cells in the logarithmic phase were seeded in 96-well plates ( $1 \times 10^4$ / well). 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was performed at 0, 24, 48 and 72 h after transfection. Briefly, 20 µL MTT (0.5 mg/mL, Sigma-Aldrich)

4 h. Then 200 μL dimethyl sulfoxide (DMSO, Sigma-Aldrich) was added to each well and the plates were shaken until the formazan was dissolved. Optical density (OD) at 492 nm was measured using a microplate reader (Bio-Rad, Hercules, CA). The experiment on each sample was performed in triplicate.
Wound healing assay
The seven groups of cells

was added to each well and

the plates were incubated for

were seeded in 6-well plates (5 × 10<sup>5</sup>/well) at 12 h after transfection and cultured until the confluency was 100%. A scratch was made with a 200 mL pipette tip across the cells. The floating cells were removed by washing with phosphate buffer saline (PBS) for two times, and the serum-free medium was added. Then the cells were incubated in humidified atmosphere with 5% CO2 at 37°C for 24 h. Photos were taken before and after incubation, respectively, for analysis using ImageJ 1.49 (National Institutes of Health, Bethesda, MD). The wound areas at the two time points were indicated as  $\rm S_{_{\rm O}}$  and S1, and wound healing rate was calculated as  $(S_0-S_1)/S_0 \times$ 100%. The experiment on each sample was performed in triplicate.

#### Cell invasion assay

Cell invasion assay of the seven cell groups was performed using Millicell Standing Cell Culture 24 well (Millipore, Billerica, MA) and Matrigel (BD Biosciences, San Jose, CA) at 12 h after transfection. The chambers coated with gel were first hydrated in serum-free medium for 2 h, and then the culture medium with 20% FBS was added to the lower chamber. The cells (5 ×  $10^5$ ) in medium with 1% bovine serum albumin (BSA) were added to the upper chamber, after which the chambers were incubated in humidi-



**Figure 3.** Inhibition and overexpression of RAB34 in esophageal cancer cell lines EC9706 and EC109 (n = 3). A. qPCR shows that *RAB34* mRNA expression is inhibited after sh-RAB34 transfection. Control, cells without transfection. sh-control, cells transfected with the control lentivirus. sh-RAB34, cells transfected with sh-RAB34 lentivirus. B. qPCR shows that *RAB34* mRNA expression is promoted in miR-433-transfected cells after RAB34 overexpression. miR-433, cells transfected with miR-433 lentivirus. miR-433 + vector, cells transfected with miR-433 lentivirus and then blank vector. miR-433 + RAB34, cells transfected with miR-433 lentivirus and then RAB34 overexpression vector. \**P* < 0.05. \*\**P* < 0.01. \*\*\**P* < 0.001.

fied atmosphere with 5%  $\rm CO_2$  at 37°C for 12 h. After the incubation, cells left in the upper chamber were removed with a cotton swab, and the invaded cells on the lower surface of the membrane were fixed with methanol and stained in crystal violet (Beyotime, Shanghai, China) for 30 min. Then the stained cells were dissolved in 33% acetic acid and OD at 570 nm was measured with the microplate reader. The experiment on each sample was performed in triplicate.

### Real-time quantitative PCR

The seven groups of cells were sampled for total RNAs and microRNAs extraction. For total RNAs extraction, the samples were lysed in TRIzol (Invitrogen) and RNAs were extracted

according to the manufacturer's instruction, after which 1 µg of RNAs were used for the complementary DNAs (cDNAs) synthesis using PrimeScript 1<sup>st</sup> Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). For microRNAs extraction, the cells were lysed in RNAiso (TaKaRa) and reversetranscripted using Prime-Script Reverse Transcriptase (TaKaRa) and the specific primer for miR-433 (5'-CTC AAC TGG TGT CGT GGA GTC GGC AAT TCA GTT GAG ACA CCG AG-3'). Real-time quantitative PCR (qPCR) was performed on LightCycler 480 (Roche, Basel, Switzerland) with the specific primers for miR-433 (Fw: 5'-ACA CTC CAG CTG GGA TCA TGA TGG GCT CCT-3' and Rv: 5'-TGG TGT CGT GGA GTC G-3') or RAB34 (Fw: 5'-ACT CGA GGC CCT GTA GCC-3' and Rv: 5'-CTA CCA TTA CAG AGC GGC CC-3'). The gPCR on each sample was conducted in triplicate, and data were analyzed with the  $2^{-\Delta\Delta Ct}$  method.

#### Western blot

Total protein of the cells was extracted using Radio Immu-

noprecipitation Assay Lysis Buffer (Beyotime), and the same amount of protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein on the gel was transferred to a polyvinylidene fluoride membrane, and the membrane was blocked in 5% skim milk for 2 h at room temperature, after which it was incubated in the specific primary antibodies for RAB34 (ab73383, Abcam, Cambridge, UK) at 4°C overnight, Anti-GAPDH (ab181602, Abcam) was used as an internal control. Then the membrane was washed in Tris buffered saline with Tween 20 for three times, and incubated in horse reddish peroxidaseconjugated secondary antibodies for 1 h at room temperature. Signals were developed using EasyBlot ECL Kit (Sangon Biotech,



**Figure 4.** miR-433 inhibits viability and RAB34 promotes viability of esophageal cancer cell lines EC9706 and EC109. MTT detecting cell viability is performed at 0, 24, 48 and 72 h after transfection (n = 3). A. The viability of cells overexpressing miR-433 is inhibited. B. The viability of cells with RAB34 knock-down is inhibited. C. The inhibited viability of cells by miR-433 is promoted when RAB34 is overexpressed. \*\*P < 0.01. \*\*\*P < 0.001.

Shanghai, China) and the relative density was analyzed using Image J 1.49.

# Statistical analysis

Results were indicated as the mean  $\pm$  standard deviation. Data were analyzed using *t* test and one-way analysis of variance in SPSS 20 (IBM, New York, USA). Difference was considered significant if *P* < 0.05.

### Results

miR-433 is down-regulated and RAB34 is upregulated in esophageal cancer cells

To begin with, the expression pattern of miR-433 and RAB34 in esophageal cancer cells compared to normal cells was analyzed by qPCR. Results showed that miR-433 was significantly down-regulated in esophageal cancer cells EC9706 (P < 0.01) and EC109 (P < 0.001, **Figure 1A**) compared to normal esophageal epithelial HEEC cells. On the contrary, *RAB34* mRNA was obviously up-regulated in the two esophageal cancer cells (P < 0.01, **Figure 1B**) compared to HEEC. These data drew the attention that miR-433 and RAB34 were likely to function in the pathogenesis and progression of esophageal cancer, or to regulate esophageal cancer cells.

miR-433 inhibits RAB34 in esophageal cancer cells

Since microRNAs are critical suppressors of gene expression, and miR-433 and RAB34



**Figure 5.** miR-433 inhibits migration and RAB34 promotes migration of esophageal cancer cell lines EC9706 and EC109. Wound healing assay is performed at 12 h after transfection (n = 3). A. The cells overexpressing miR-433 have a lower wound healing rate. B. Inhibiting RAB34 results in a lower wound healing rate. C. The wound healing rate is higher when RAB34 is overexpressed. \*\*P < 0.01. \*\*\*P < 0.001.

possessed distinct changing patterns in esophageal cancer cells, so their regulatory relationship was investigated by detecting the change in RAB34 expression when miR-433 was up-

regulated. Before that, miR-433 overexpression by the lentivirus transfection was validated, with the miR-433 overexpression group showing a significant up-regulated level of miR-433 compared to the transfection control group, in both EC9706 (P < 0.01) and EC109 cells (P < 0.05, Figure 2A). In the cells with miR-433 overexpression, RAB34 mRNA was significantly inhibited compared to the transfection control group (P < 0.01 and P < 0.05, Figure 2B), indicating that miR-433 overexpression could decrease RAB34 mRNA. Moreover, western blot showed a consistent result with qPCR, that RAB34 protein levels in the two cell lines were also inhibited (Figure 2C). It could be inferred that miR-433 was capable of reducing RAB34 mRNA, as well as suppressing the production of RAB34 protein.

miR-433 and RAB34 regulate esophageal cancer cell viability, migration and invasion

The effects of miR-433 and RAB34 on esophageal cells were analyzed from cell viability, migration and invasion. The RAB34-specific shRNAs and overexpression vectors were used to inhibit and promote RAB34 levels, respectively, and the desired results were achieved. RAB34 mRNA was inhibited by sh-RAB34 compared to the sh-control in both cell lines (P < 0.001 and P < 0.05, Figure 3A), and promoted even in the cell lines overexpressing miR-433 (P < 0.01 and P < 0.05, Figure 3B),

implying that the RAB34 overexpression vector used in this experiment could reverse the effect of miR-433 overexpression, thereby the two cell lines were valid for overexpressing RAB34.



**Figure 6.** miR-433 inhibits invasion and RAB34 promotes invasion of esophageal cancer cell lines EC9706 and EC109. Transwell experiments are performed at 12 h after transfection (n = 3). A. Cell invasion is inhibited by miR-433 overexpression. B. Cell invasion is inhibited when RAB34 is suppressed. C. Cell invasion is promoted when RAB34 is overexpressed. \*\*P < 0.01.

MTT assay showed that both EC9706 and EC109 cells miR-433 overexpression exhibited a decrease in cell viability after the transfection (**Figure 4A**), and significant differences from the control groups were detected at 48 h after transfection in EC9706 cells (P < 0.01) and at 24 h after transfection in EC109 cells (P < 0.001), indicating miR-433 overexpression

could inhibit viability of EC-9706 and EC109 cells. As the abovementioned results that RAB34 was inhibited by miR-433, the role of RAB34 in esophageal cell viability was also analyzed, and results showed that inhibiting RAB34 led to a significant suppression in cell viability of both EC9706 and EC109 (P < 0.01, Figure 4B), while RAB34 overexpression could up-regulate the miR-433-suppressed cell viability (P < 0.01, Figure 4C). These results implied that miR-433 inhibited, and RAB34 promoted cell viability in esophageal cancer cells, which was in consistent with their regulatory relationship that miR-433 inhibited RAB34.

Would healing assay and transwell experiments indicated that the migration and invasion of EC9706 and EC109 cells were both changed in similar patterns. Briefly, miR-433 overexpression or RAB34 inhibition resulted in a decrease in cell wound healing rate (Figure 5A and 5B) and cell invasion ability (Figure 6A and 6B), and RAB34 overexpression led to the increased cell migration and invasion ability (Figures 5C and 6C). Taken together, miR-433 functioned as a suppressor in esophageal cancer cell viability, migration and invasion, possibly via inhibiting RAB34, a factor facilitating esophageal cancer cells in all these aspects.

# Discussion

microRNAs and gene therapies are promising strategies for the treatment of various diseases. In this study, roles of miR-433 and RAB34 are analyzed in esophageal cancer cell lines EC9706 and EC109. miR-433 and RAB34 are both aberrantly expressed in esophageal cancer cells compared to normal cells, and RAB34 expression can be inhibited by miR-433. Moreover, the two factors have distinct effects on esophageal cancer cells, with miR-433 suppressing cell viability, migration and invasion, and RAB34 promoting these abilities.

miR-433 has been reported regulating the expression of cancer-related genes, functioning as a tumor suppressor in liver cancer, gastric cancer, amongst others. miR-433 overexpression in gastric cancer cells induces the suppression of KRAS signaling and the inhibition in cell viability, migration and invasion [12]. Other validated targets of miR-433 include p21 activated kinase and cAMP response elementbinding protein in hepatocellular carcinoma [13, 14], and growth factor receptor-bound protein 2 in gastric cancer cells [15, 16]. In consistent with these studies, miR-433 was also down-regulated in esophageal cancer cell lines EC9706 and EC109, and its overexpression suppressed cell viability, migration and invasion, indicating its roles as an esophageal cancer suppressor.

In contrast, RAB34 was found promoting the viability, migration and invasion in EC9706 and EC109 cells, having the distinct effects on esophageal cancer cells compared to miR-433. Most of the studies on RAB34 focus on its association with the interaction between phagosomes and endosomes or lysosomes [17, 18], but recent research has revealed the pivotal roles of RAB34 in cancer cells. Its expression is related to glioma grade progression [19], and it can be regulated by miR-124a and miR-148a in prostate cancer [20]. Moreover, RAB34 is negatively modulated by miR-9, thus affecting esophageal cancer cell metastasis [21]. Similarly in this study, RAB34 was proved to promote esophageal cancer cells in cell viability, migration and invasion, with its overexpression abrogating the effects of miR-433 overexpression, indicating its significance in the progression of esophageal cancer.

In addition, this study tried to investigate the regulatory mechanism of miR-433 in esophageal cancer cells by associating miR-433 with RAB34, and found that miR-433 inhibited RAB34 expression in both EC9706 and EC109 cells. However, the direct interaction of miR-433 and RAB34 could not be predicted by online databases such as TargetScanHuman

7.0 or miRTarBase, so there was a great possibility that miR-433 inhibited RAB34 indirectly. through the regulation of other factors and signaling pathways. In renal fibrosis and the fibrogenesis of other organs, for example, miR-433 directly suppresses AZIN1 expression, thus leading to the amplification of the TGF- $\beta$ / SMAD3 signaling [22, 23]. The TGF-B/SMAD signaling is an important pathway of gene regulation because of the core role of SMAD transcription factors, which are translocated to the nucleus to modulate gene expression at the transcriptional level [24]. It might be the supportive information that TGF-B receptor 1 was predicted to be a target for miR-433 by microR-NA.org (www.microrna.org), which implied the possibility that miR-433 functioned through the TGF-β/SMAD signaling to regulate expression of genes including RAB34. Of course, other pathways between miR-433 and RAB34 could not be ruled out. Further validation would be helpful for the understanding of miR-433 regulatory mechanisms.

Ever since the research in 2008 that microR-NAs are potential in affecting esophageal cancer risk [25] and identifying esophageal cancer [26], a bunch of studies on microRNAs in esophageal cancer have emerged. For example, RNASEN, a RNase III endonuclease mediating the procession of pri-microRNAs, may play functional roles in the development of esophageal cancer [27]. microRNA expression disparities help to explain why esophageal cancer patients at the same tumor node metastasis may have different prognoses [28]. In addition, several microRNAs and their regulatory mechanisms are elucidated [29]. So besides the commonly used adjuvant chemotherapy and chemoradiotherapy [30, 31], microRNAs are perspective therapeutic strategies for esophageal cancer. In this study, miR-433 was shown to be an effective inhibitor for esophageal cancer, suppressing esophageal cancer cell viability, migration and invasion, as well as the expression of oncogene RAB34. With further mechanism studies and a more profound understanding of the regulatory pathways, miR-433 would offer a promising therapeutic strategy for treating esophageal cancer, through modulating RAB34.

To sum up, this study indicates miR-433 to be a suppressor on cell viability, migration and invasion in esophageal cancer cell lines EC9706

and EC109, which is possibly related to its inhibition of RAB34. These results provide basic information for the potential usages of miR-433 in treating esophageal cancer, but further studies on the regulatory mechanisms remain necessary.

# Disclosure of conflict of interest

None.

Address correspondence to: Zheng Huang, Department of Thoracic Surgery, Weinan Central Hospital, Shengli Street, Weinan 714000, Shaanxi, China. E-mail: huangzheng99663@126.com

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