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MicroRNA-429 inhibits the proliferation and migration of esophageal squamous cell carcinoma cells by targeting RAB23 through the NF-κB pathway

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Abstract. – OBJECTIVE: Esophageal squamous cell carcinoma (ESCC) is the main type of esophageal cancer and is a devastating malignancy. Recent research shows that microRNA-429 (miR-429) has a role in suppressing cell proliferation, cell cycle and promoting apoptosis in many cancers. This study aims to explore the great role of miR-429 in esophageal squamous cell carcinoma.

MATERIAL AND METHODS: The mRNA and protein levels of miR-429 and genes were calculated by using Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) and Western blot. We applied Cell Counting Kit-8 (CCK-8) and transwell assays to measure the proliferative and migratory abilities. Meanwhile, the Kaplan-Meier method was used to calculate the overall survival of esophageal squamous cell carcinoma patients.

RESULTS: MiR-429 was downregulated while RAB23 was upregulated in ESCC tissues and cell lines, and downregulation of miR-429 predicted poor prognosis in ESCC. RAB23 was found to be a direct target gene of miR-429 and its expression was regulated by miR-429 in ESCC. Moreover, miR-429 inhibited the proliferation through nuclear factor-kappa B (NF-κB) pathway and inhibited cell migration-mediated epithelial-mesenchymal transition (EMT) in TE-2 cells. In addition, overexpression of miR-429 suppressed tumor growth of ESCC *in vivo*.

CONCLUSIONS: MiR-429 inhibited the proliferation through the RAB23/NF-κB pathway and the migration-mediated EMT in ESCC. The newly identified miR-429/RAB23 axis provides novel insight into the pathogenesis of ESCC.

Key Words:

MiR-429, Proliferation, Migration, Epithelial-mesenchymal transition (EMT), Esophageal squamous cell carcinoma.

Introduction

Esophageal cancer, a malignant tumor that starts from esophageal mucosa, is the fifth most common cancer in China and it has a high incidence[1,2]. Esophageal squamous cell carcinoma (ESCC), accounts for 90% of esophageal cancer, has a lower 5-year survival rate of less than 10%[3]. ESCC was a devastative malignancy because of the aggressive progress and advanced diagnosis, although it has made great progress in treatment[4]. Therefore, it is urgently necessary to discover novel biomarkers for the early diagnosis and treatment of esophageal squamous cell carcinoma.

MicroRNAs (miRNAs), a class of small non-coding RNAs that are 22-28 nucleotides in length, could target complementary mRNA to down-regulate gene expression at the post-transcription[5]. Increasing evidence has indicated that miRNAs have a huge effect on the proliferation and metastasis of tumors, meanwhile, are greatly related to the development and progression of tumor[6]. Most reports[7-10] have found that several miRNAs that including miR-516b, miR-675, miR-214 and miR-23b, were played important roles in ESCC. In gastric cancer, miR-429 has been reported to act as a tumor suppressor and suppressed cell proliferation[11]. Similarly, miR-429 has been reported to impair cell growth and reduced apoptosis in nephroblastoma[12]. Moreover, Guo et al[13] have revealed that miR-429 eliminated the metastasis and epithelial-mesenchymal transition (EMT) in hepatocellular carcinoma. However, a few studies illuminated the functions of miR-429 in ESCC; thus, the focus of this research is to explore the vital roles of miR-429 in ESCC.

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RAB23, a member of the RAS oncogene family, has been deemed to be the regulator for endocytic recycling and vesicle transportation[14,15]. In osteosarcoma, Jiao et al[16] have revealed that miRNA-16 suppressed the invasion and migration of osteosarcoma by directly inhibiting RAB23. Zhang et al[17] have indicated that RAB23 enhanced cisplatin resistance in ovarian cancer. Moreover, Jiang et al[18] have validated that RAB23 has been facilitated cell proliferation and invasion in human bladder cancer. In our work, miR-429 was validated to suppress the proliferation and migration through directly binding to the 3'-untranslated region (3'-UTR) of RAB23 mRNA in esophageal squamous cell carcinoma. MiR-429 was found to impair the migration-mediated EMT and proliferation through the nuclear factor-kappa B (NF-κB) pathway.

Material and Methods

Clinical Specimens

Fifty pairs of ESCC tissues and peritumoral normal tissues were gathered from 50 ESCC patients who received treatment in the Qilu Hospital from June 2016 to June 2018. Before surgery, none of the patients have received radiotherapy or chemotherapy. After surgery, specimens were immediately frozen in liquid nitrogen and then stored at -80°C. We obtained informed consent from each patient. This study was approved by the Ethics Committees of the Qilu hospital.

Cell Culture

We purchased the normal epithelial cell line HET-1A and two human gastric cancer cell lines TE-2 and TE-8 from the American Type Culture Collection (ATCC; Manassas, VA, USA). All the cells were incubated in a Roswell Park Memorial Institute-1640 medium (RPMI-1640; HyClone, South Logan, UT, USA) maintained with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a humidified atmosphere of 5% CO_2.

Transfection

The specific miR-429 mimic or the miR-429 inhibitor and their negative control plasmids were designed and synthesized from GenePharma (Shanghai, China). TE-2 cells were incubated in a 6-well plate, which was used to transfect the vectors. According to the instructions, Lipofectamine™ 2000 Reagent (Invitrogen, Carlsbad, CA, USA) was used to perform the transfection, which was diluted by the Opti-MEM/Reduced serum medium (Thermo Fisher Scientific, Waltham, MA, USA). For the stable transfection, the cells were selected by Geneticin (G418; Thermo Fisher Scientific, Waltham, MA, USA), while the cells with transient transfection were harvest after transfected 48 h.

Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR)

The miRNeasy Mini Kit (Qiagen, Hilden, Germany) was applied to extract total miRNAs from tissues or cells. The first complementary deoxyribose nucleic acid (cDNA) chain was synthesized by the TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA); the miRNA-specific TaqMan miRNA Assay Kit (Applied Biosystems, Foster City, CA, USA) was employed to perform the quantitative Polymerase Chain Reaction (qPCR). The relative levels of miRNA were derived using the $2^{-\Delta\Delta Cq}$ method with U6 small nuclear RNA as normalization.

Total RNA was extracted by using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and the first cDNA chain was synthesized by using the Omniscript Reverse Transcription Kit (Qiagen, Hilden, Germany) from total RNA. The QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany) was conducted to perform the RT-qPCR in a Quantitect SYBR Green PCR System (Qiagen, Hilden, Germany). A $2^{-\Delta\Delta Cq}$ method was used for the mRNA quantification, which normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). PCR cycling conditions were as follows: an initial denaturation step at 95°C for 10 min, followed by 40 cycles of denaturing at 95°C for 10 sec, annealing and synthesis at 60°C for 60 sec. The sequences of the primers were as follows: miR-429 forward 5'-GCTAATACTGTCTGGTAAAACCGT-3', reverse 5'-TCGTATCCAGTGCAGGGTC-3'; U6 forward 5'-GTGCTCGCTTCGGCAGCACATATAC-3', reverse 5'-AAAAATATGGAACGCTCACGAATTTG-3'; RAB23 forward 5'-GTAGTAGCCGAAGTGGGA-3', reverse 5'-CCTTTGTTTGTTGGGTCTC-3'; GAPDH forward 5'- ATGGGGAAGGTGAAGGTCGG-3' and reverse 5'- GACGGTGCCATGGAATTTGC-3'.

Western Blot Analysis

Total proteins were lysed by using radioimmunoprecipitation assay (RIPA) Lysis Buffer (Beyotime, Shanghai, China) containing 10% phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich,

St. Louis, MO, USA). The protein separation was made using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then the blots were electro-transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After being blocked in 5% fat-free milk at room temperature for 1 h, the membranes were incubated with primary antibodies. The primary antibodies were against RAB23 (1:1000; Abcam, Cambridge, MA, USA), E-cadherin (1:1000; Abcam, Cambridge, MA, USA), N-cadherin (1:1000; Abcam, Cambridge, MA, USA), Vimentin (1:1000; Abcam, Cambridge, MA, USA), c-Myc (1:1000, Abcam, Cambridge, MA, USA), TAK1 (1:1000, Abcam, Cambridge, MA, USA), p-TAK1 (1:1000, Abcam, Cambridge, MA, USA), TRAF6 (1:1000, Abcam, Cambridge, MA, USA). Subsequently, the blots were incubated by secondary anti-rabbit Horseradish peroxidase (HRP)-conjugated antibody (Cell Signaling Technology, Danvers, MA, USA). Enhanced Chemiluminescence Detection Kit (ECL; Thermo Fisher Scientific, Waltham, MA, USA) was applied to capture the protein signals.

Cell Counting Kit-8 (CCK-8) Assay

The cell proliferation ability was measured by using the Cell Counting Kit-8 assay (CCK-8; Dojindo, Kumamoto, Japan). Briefly, TE-2 cells transfected with special vectors were incubated in 96-well plates and cultured for 24 h, 48 h, 72 h and 96 h at 37°C in a humidified atmosphere of 5% CO_2; then, we added 10 μL of CCK-8 solution into each well and incubated for 2 hours. Finally, the absorbance at 450 nm was measured to use a microplate reader.

Transwell Assay

To perform the cell migration ability, the transwell insert (8 μm membrane, Corning, Lowell, MA, USA) was placed in a 24-well plate and formed upper and lower chambers. The 200 μL of TE-2 cells suspension by RPMI-1640 medium was added in the upper chamber. Meanwhile, the lower chamber was filled with 500 μL of the medium containing 15% FBS, which acted as an attractant. After incubating the cells at 37°C for 24 h, the non-invasive cells that still on the upper surface, were removed by a cotton swab. For the invasive cells, we fixed and then stained by 4% paraformaldehyde and 10% crystal violet respectively; then, we counted the cells under a microscope (Olympus Corporation, Tokyo, Japan).

MiRNA Targets Prediction and Dual-Luciferase Reporter Assay

TargetScan (http://www.targetscan.org) was used to predict the target gene of miR-429 and RAB23 was predicted to be one potential target gene. To validate that miR-429 directly targeted to RAB23 in ESCC, the binding sequences were mutated from GUGCAAUA to GCCCAACA in TE-2 cells. Subsequently, the wild type and the mutant 3'-UTR of RAB23 were inserted into the Dual-Luciferase reporter vectors, which were designated as WT or MUT. For the Luciferase assay, Lipofectamine 3000 Reagent (Invitrogen, Carlsbad, CA, USA) was applied to co-transfect the miR-429 mimic and the WT or MUT plasmid into TE-2 cells. Then, the Luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical Analysis

All the statistical analysis was performed using the Statistical Product and Service Solutions (SPSS) 16.0 software (IBM, Armonk, NY, USA). All the data were presented as mean ± SD (Standard Deviation) from at least three independent experiments. Comparison between groups was made using One-way analysis of variance (ANOVA) test followed by Post-Hoc Test (Least Significant Difference). The association between the expression of miR-429 and the overall survival for ESCC patients were assessed by the Kaplan-Meier curve and log-rank test. $p<0.05$ was considered statistically significant.

Results

Downregulation of MiR-429 Predicted Poor Prognosis of ESCC

The mRNA level of miR-429 was evaluated in 50 pairs of ESCC and peritumoral normal tissues. We found that the expression of miR-429 was lower in ESCC tissues than corresponding peritumoral normal tissues ($p<0.0001$; Figure 1A). Moreover, the Kaplan-Meier method revealed that overexpression of miR-429 was associated with poor 5-year overall survival of ESCC patients ($p=0.0360$; Figure 1B).

MiR-429 Inhibited the Proliferation and Migration in TE-2 Cells

We calculated the expression of miR-429 in two ESCC cell lines (TE-2 and TE-8) and a normal epithelial cell line HET-1A. The expression

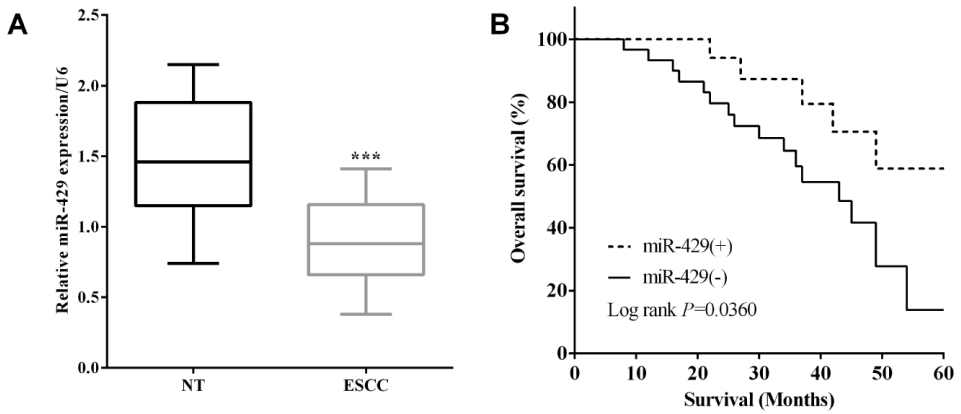

Figure 1. Downregulation of miR-429 predicted poor prognosis of ESCC. **A,** MiR-429 was low expressed in ESCC tissues versus corresponding peritumoral normal tissues. **B,** Downregulation of miR-429 predicted poor 5-year overall survival in ESCC.

of miR-429, same results with that in tissues, was higher in HET-1A cells than TE-2 (p=0.0004) and TE-8 (p=0.0012) cells (Figure 2A). To explore the function of miR-429, the miR-429 mimic and the miR-429 inhibitor were applied to up- (p<0.0001) or down-regulate (p=0.0015) miR-429 in TE-2 cells measured by RT-qPCR (Figure 2B).

The CCK-8 assay elucidated that the proliferation was the inhibited by the miR-429 mimic (p=0.0448, 0.0057 and 0.0019 of 72 h and 96 h), while it was improved by the miR-429 inhibitor (p=0.0233, 0.0026 and 0.0009 of 48 h, 72 h and 96 h) in TE-2 cells (Figure 2C). In addition, the transwell assay showed that the miR-429 mimic

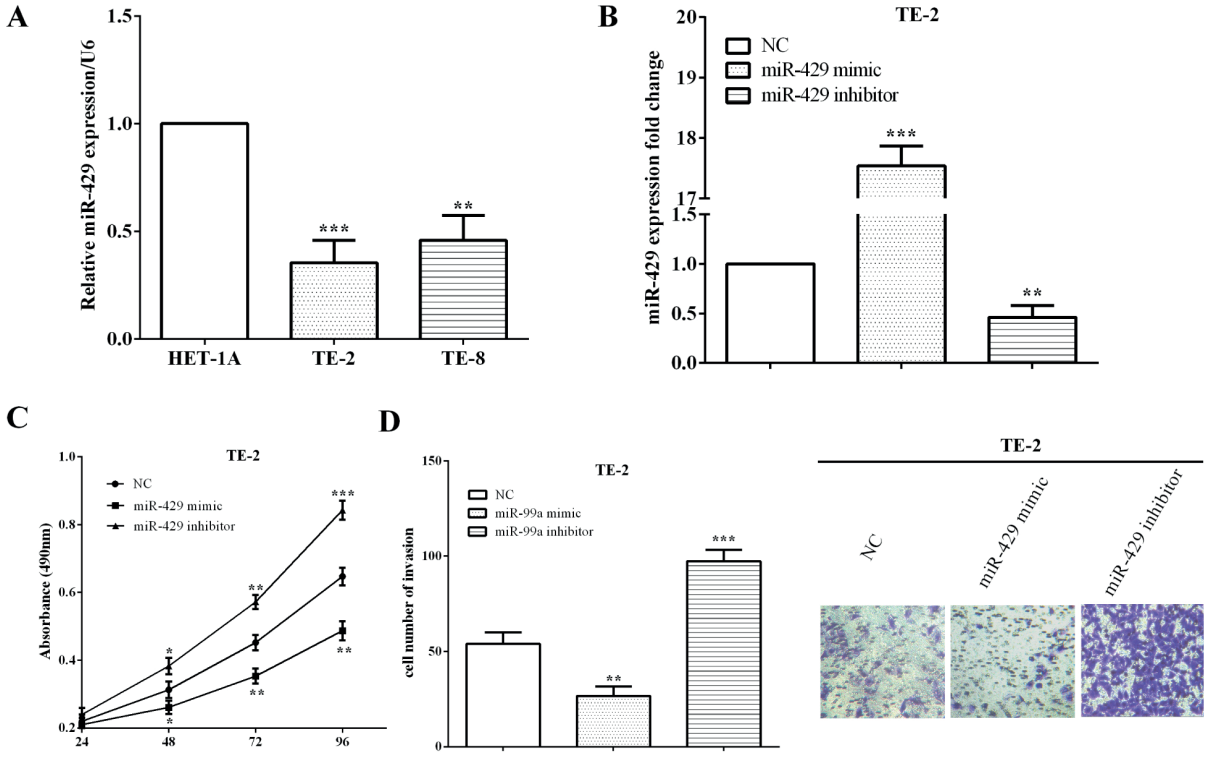

Figure 2. MiR-429 inhibited the proliferation and migration in TE-2 cells. **A,** The expression of miR-429 was higher in HET-1A cells than that in TE-2 and TE-8 cells. **B,** RT-qPCR revealed that the miR-429 mimic and the miR-429 inhibitor were conducted to up- or down-regulate miR-429 in TE-2 cells. **C,** The CCK-8 assay showed that the miR-429 mimic suppressed the proliferation, while the miR-429 inhibitor promoted the proliferative ability in TE-2 cells. **D,** The transwell assay revealed the miR-429 mimic inhibited the migratory ability whereas miR-429 inhibitor promoted in TE-2 cells (magnification: 40×).

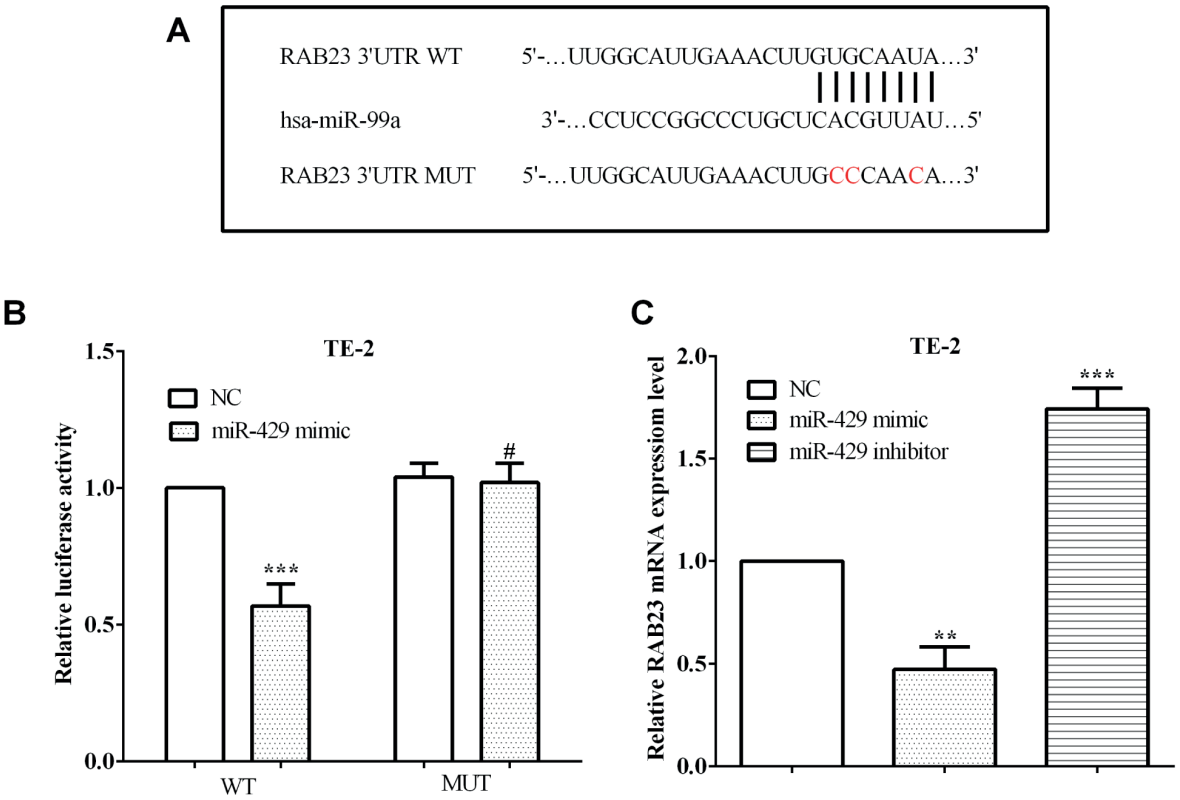

Figure 3. MiR-429 regulated the expression of RAB23 by binding to 3'-UTR of RAB23 mRNA. **A,** TargetScan predicted RAB23 was a target gene of miR-429. **B,** MiR-429 reduced the Luciferase activity of TE-2 cells that transfected wild type 3'-UTR of RAB23 mRNA, while did not alter the Luciferase activity of cells transfected mutant RAB23 3'-UTR. **C,** Overexpression of miR-429 inhibited the mRNA level of RAB23, while knockdown of miR-429 promoted the expression of RAB23 in TE-2 cells.

inhibited (p=0.0038) the invasive ability whereas miR-429 inhibitor promoted it (p=0.0009; Figure 2D). All the results revealed miR-429 impaired the abilities of proliferation and migration in ESCC cell line TE-2.

MiR-429 Regulated RAB23 Expression by Binding to the 3'-UTR of RAB23 mRNA

Through TargetScan, RAB23 was predicted to be a target gene of miR-429, and the binding site was located at the 3'-UTR of RAB23 mRNA. To validate that miR-429 directly binding to the putative binding site of RAB23 mRNA 3'-UTR, the potential binding sequences were mutated from GUGCAAUA to GCCCAACA to evaluate the Luciferase activity (Figure 3A). The Luciferase reporter assay proved that miR-429 reduced the Luciferase activity of TE-2 cells that transfected wild type 3'-UTR of RAB23 mRNA (p=0.0007), however, it makes no difference on the Luciferase activity of cells that co-transfected the mutant RAB23 3'-UTR (p=0.7078; Figure 3B). Moreover, we assessed the mRNA levels of RAB23 after transfecting the miR-429 mimic or the miR-429 inhibitor in TE-2 cells. Not unexpectedly, overexpression of miR-429 inhibited (p=0.0011) the mRNA level of RAB23, while knockdown of miR-429 promoted (p=0.0002) the expression of RAB23 in TE-2 cells (Figure 3B). All the results indicated that miR-429 regulated the expression of RAB23 by directly binding to its mRNA 3'-UTR in ESCC cells TE-2.

MiR-429 Inhibited the Migration-Mediated EMT and the Proliferation through the PI3K/AKT Signal Pathway

RT-qPCR was used to calculate the expression of RAB23 in tissues and cells. The RAB23 in ESCC tissues was lowly expressed compared with that in matched normal tissues (p<0.0001; Figure 4A). Similarly, the expression of RAB23 was lower in ESCC cell lines TE-2 (p=0.0002) and TE-8 (p=0.0016) than that in normal epithelial cell HET-1A (Figure 4B). Moreover, Western

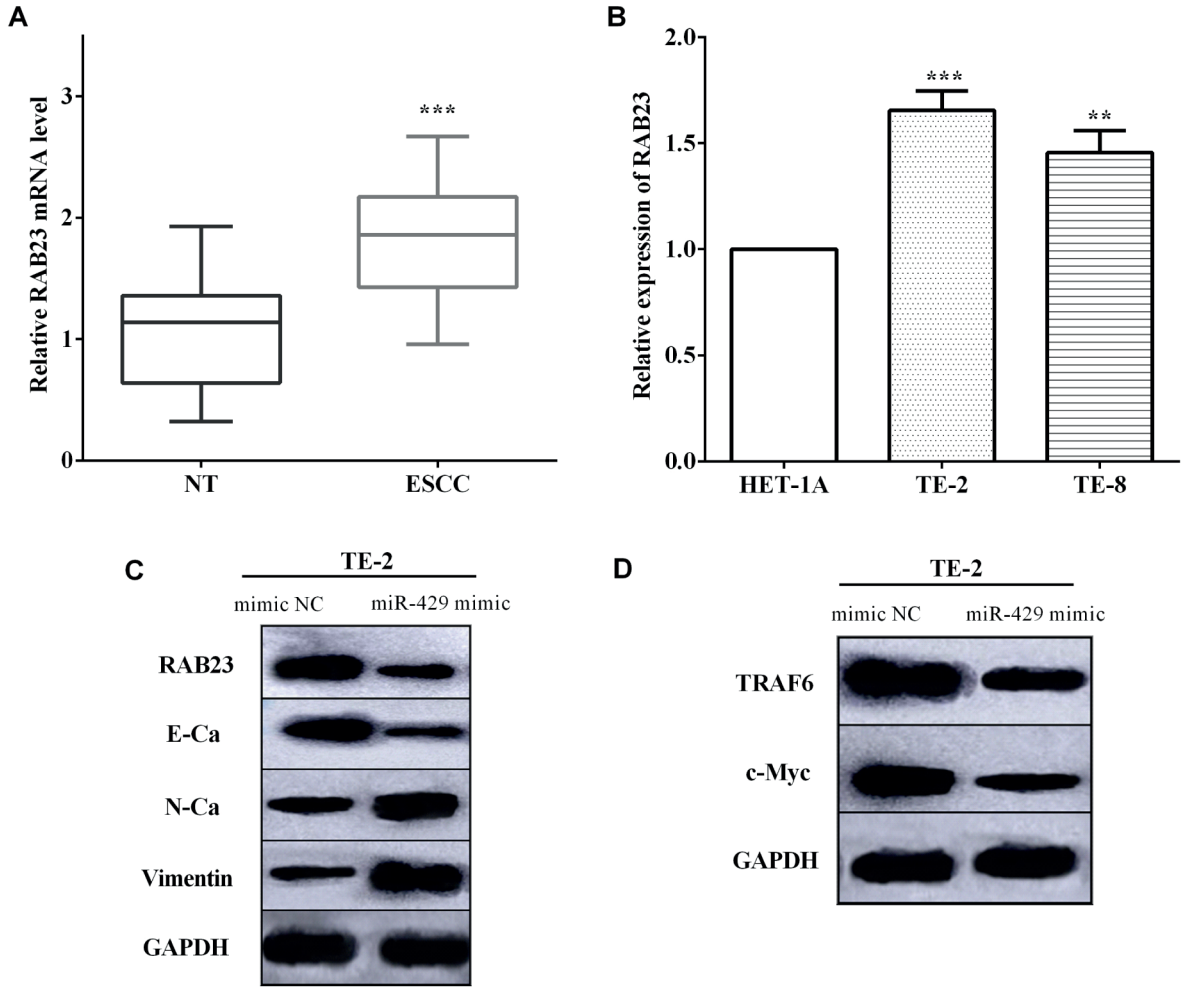

Figure 4. MiR-429 inhibited the migration-mediated EMT and the proliferation through the NF-κB signal pathway. **A,** The relative positive rate of RAB23 in ESCC tissues and peritumoral normal tissues was calculated. **B,** The expression of RAB23 was lower in TE-2 and TE-8 cells than HET-1A cells. **C,** MiR-429 inhibited the migration-mediated EMT. **D,** MiR-429 suppressed the proliferation through the NF-κB signal pathway.

blot was used to calculate the expression of proteins associated with EMT and NF-κB pathway in TE-2 cells. The research found that the miR-429 mimic suppressed the expression of RAB23 and E-cadherin, while improved the expression of N-cadherin and Vimentin in TE-2 cells (Figure 4C), which suggested that miR-429 inhibited the EMT by directly targeting to the 3'-UTR of RAB23 mRNA. In addition, overexpression of miR-429 suppressed the expression of RAB23, TRAF6 and c-Myc in TE-2 cells (Figure 4D), which proved that miR-429 inhibited the proliferation through the NF-κB pathway. All the results revealed that miR-429 suppressed the migration-mediated EMT and the proliferation through the NF-κB signal pathway.

MiR-429 Suppressed the Xenograft Growth In Vivo

TE-2 cells that stably transfected the miR-429 mimic or the control plasmid were applied to inject into the nude mice at subcutaneous. After establishing a transplant tumor model, the volumes of xenograft tumors were calculated every 3 days. And as we expected, the group of transfecting the miR-429 mimic had a slower growth rate than that of the control group (Figure 5A). After cultivating for 26 days, all mice were killed and the volumes of the resulting tumors were measured. We discovered that it had a smaller tumor volume of cells that overexpressed miR-429 than that of the control group (p=0.0002), which indicated that overexpression of miR-429 inhibited the tu-

mor growth of esophageal squamous cell carcinoma *in vivo* (Figure 5B).

Discussion

Esophageal squamous cell carcinoma is the most frequent type of esophageal cancer that severely affects the health of patients[19]. Therefore, identifying novel biomarkers for the early diagnosis and treatment of ESCC is urgent.

Recently, increasing evidence indicated that miRNAs play great roles in the proliferation and metastasis of tumors and are associated with the development and progression of tumor[6]. MiR-429 has been found to act as a tumor suppressor and inhibited the proliferation, metastasis and EMT in renal cell carcinoma cells[20]. Similarly, Deng et al[21] have revealed that miR-429 suppressed the proliferation, migration, invasion and EMT of osteosarcoma cells. Consistent with all above, we proposed that miR-429 was downregulated and miR-429 downregulation inhibited cell proliferation and migration in ESCC. In addition, we found that upregulation of miR-429 could inhibit the growth of ESCC both *in vitro* and *in vivo.* Wu et al[22] have indicated that miR-429 was downregulated and its expression was correlated with poor survival in bladder cancer. Our results, consistent with Wu et al[22], showed that downregulation of miR-429 predicted poor 5-year overall survival of ESCC patients. Moreover, miR-429 inhibited the proliferation through the NF-κB pathway in TE-2 cells and suppressed the growth of ESCC *in vitro* and *in vivo*, which was consistent with the findings in HCC[23]. However, miR-429 promoted cell growth of NSCLN[24]; thus, we believe that miR-429 may have tissue-specificity.

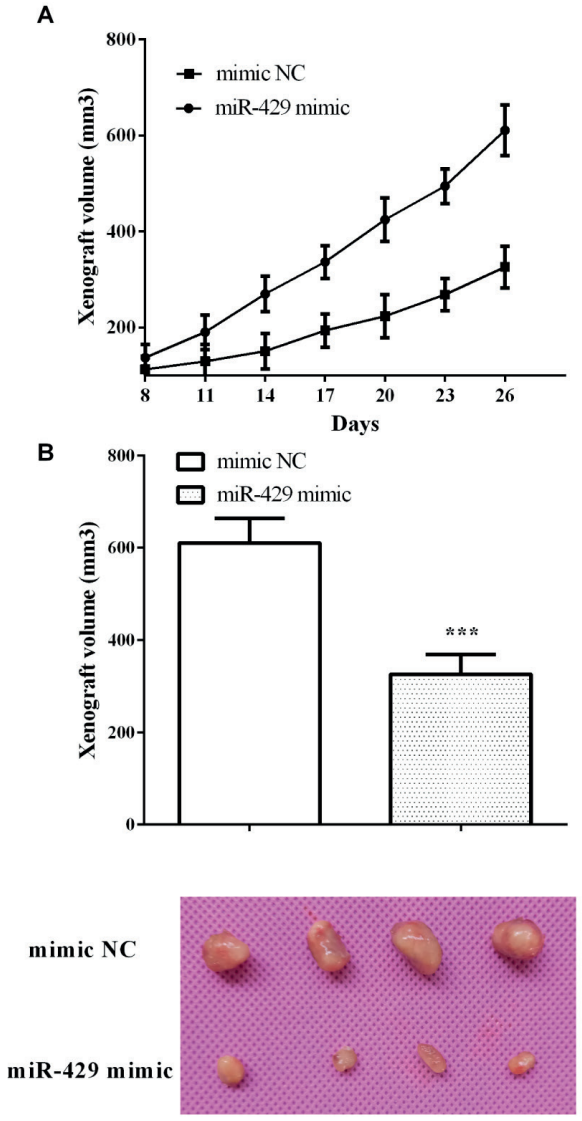

Figure 5. MiR-429 suppressed the xenograft growth *in vivo.* **A,** It had a smaller tumor volume of cells overexpressed miR-429 than the control group. **B,** Overexpression of miR-429 inhibited ESCC xenograft growth.

RAB23 has been reported to act as an oncogene and enhanced cell growth and metastasis in several tumors including prostate cancer, breast cancer, hepatocellular carcinoma and gastric cancer[25-28]. In astrocytoma, Wang et al[29] have demonstrated that RAB23 was overexpressed and enhanced the migration and invasion. Similarly, Jian et al[30] have indicated that RAB23 improved the migration and invasion in cutaneous squamous cell carcinoma cells. Moreover, RAB23 inactivation inhibited the motility and invasion in pancreatic duct adenocarcinoma[31]. Consistent with all the findings, RAB23 was upregulated in ESCC tissues and cell lines in comparison with non-tumor tissues and normal cell line. RAB23 was a direct target gene of miscellaneous miRNAs that including miR-16, miR-802, miR-665 and miR-367[32-35]. Consistent with the results in HCC[36], we revealed that RAB23 was a direct target gene of miR-429 in ESCC, and miR-429 regulated the proliferation, migration and EMT by regulating the expression of RAB23 in TE-2 cells.

Conclusions

MiR-429 was significantly downregulated in ESCC tissues and cell lines, and downregulation of miR-429 predicted poor prognosis of ESCC

patients. MiR-429 regulated the expression of RAB23 by directly targeting the 3'-UTR of RAB23 mRNA in ESCC. Moreover, miR-429 inhibited the proliferation through the NF-κB pathway and inhibited the migration-mediated EMT in TE-2 cells by directly targeting to RAB23. In addition, overexpression of miR-429 suppressed the growth of ESCC *in vivo*.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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