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MicroRNA-16 suppressed the invasion and migration of osteosarcoma by directly inhibiting RAB23

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Abstract. – OBJECTIVE: Osteosarcoma is the most frequent primary bone malignancy that affects young adults and adolescents around the world. Increasing evidence suggests that dysfunctions of microRNAs (miRNAs) used to play an important role in human cancers. We aimed at evaluating the potential function of miR-16 and verify its influence on the function of RAB23 in osteosarcoma.

PATIENTS AND METHODS: miR-16 expressions in osteosarcoma tissues and cell lines were examined using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Transwell chambers were conducted to detect the miR-16 effects on osteosarcoma cells migration and invasion. Meanwhile, Western blot and luciferase assays were performed to validate RAB23 as miR-16 targets.

RESULTS: miR-16 was down-regulated in osteosarcoma cell lines (MG63, SAOS-2, U2OS, and SOSP-9607) and osteosarcoma specimens, while RAB23 expression was higher in tumor tissues. Ectopic over-expression of miR-216 in osteosarcoma cells could inhibit cells migration and invasion. RAB23 was confirmed as a direct target of miR-16 and the inverse relationship between them was also observed. Over-expression of RAB23 ablates the inhibitory effects of miR-16.

CONCLUSIONS: miR-16 inhibited cancer migration and invasion, and promoted the RAB23 expression in osteosarcoma. This newly identified miR-16/RAB23 axis may provide new insight into the pathogenesis and represents a potential therapeutic target for osteosarcoma.

Key Words:
miR-16, RAB23, Migration, Invasion, Osteosarcoma.

Instruction

As the most frequent primary bone malignancy affects adolescents and young adults, especially those aged from 15 to 19, human osteosarcoma is characterized by occurring at the extremities of long bones, where osteoblasts transform into mature bone tissue[1]. Despite the advancements of treatment methods including adjuvant chemotherapy, wide tumor excision and radiotherapy in past decades, the prognosis and 5 years survival rate for patients diagnosed with osteosarcoma remains poor[2-4]. The putative molecular mechanisms underlying osteosarcoma carcinogenesis have not been completely deciphered and are still a challenge. Thus, there are urgent needs to elucidate these potential molecular mechanisms that mediate the progression and initiation of osteosarcoma for exploring its therapeutic strategies.

Ras-related protein 23 (RAB23), originally identified as one member of the Rab family of Ras small GTPases superfamily, can facilitate vesicular transportation and control the endocytic progression to lysosomes[5,6]. RAB23 is taken into account as one oncogene on account of its high expression in a great range of tumors including gastric carcinomas and hepatocellular carcinoma (HCC)[7,8]. A previous research[9] has suggested that over-expression of RAB23 promoted the esophageal squamous cell carcinoma growth, migration and metastasis. However, its underlying molecular mechanisms and the biological functions for its oncogenic roles in osteosarcoma remain unknown. microRNAs (miRNAs) are a class of highly conserved, non-coding and endogenous RNAs (ranging in 18-23 nucleotides length)[10,11], which can modulate the physiological process or pathogenesis through partial complementary binding to the 3′-UTR of mRNAs[12]. Dysfunctions of miRNAs occurred in a group of tumors such as gastric cancer[13] and osteosarcoma[14], and can regulate cancer progressions that are involved in cell differentiation, proliferation, metastasis, and apoptosis[15]. miR-16 could act as either the suppressor or promoter in different cancers, and it can be considered quite contradictory. On one hand, miR-16 is observably down-regulated and

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functions as suppressor in tumors, including lung cancer[16], breast cancer[17], glioma[18] and so on. On the other hand, it was found that miR-16 was up-regulated as promoter in different kinds of tumors such as renal cell carcinoma[19], gastric cancer[20], and pancreatic cancer[21], indicating its potential role in the progression of tumors as an oncomiR. Despite all these progresses in miR-16 research, the detailed functions of miR-16 in osteosarcoma have not been examined yet.

We provide evidence that miR-16 is aberrantly down-regulated in osteosarcoma cell lines and tissues. Over-expression of miR-16 inhibits epithelial-mesenchymal transition (EMT), migration and invasion of osteosarcoma cells. RAB23 was a functional target of miR-16. The newly identified miR-16/RAB23 axis may provide a new therapeutic target for osteosarcoma treatment.

Patients and Methods

Tissues and Cell Lines

Osteosarcoma tissues and the corresponding normal tissues of 48 patients were obtained at Rizhao Hospital of Traditional Chinese Medicine from 2014 to 2016. All patients signed the informed consent and the study was approved by the Ethics Committee of Rizhao Hospital of Traditional Chinese Medicine. The normal human osteoblastic cell line hFOB and osteosarcoma cell lines (MG63, SAOS-2, U2OS, and SOSP-9607) were cultured in the Dulbecco's modified eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA).

Cell Transfection

The cells were transfected with miR-16 mimics/inhibitor as well as the corresponding control using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA). MG-63 cells were used for cell migration and invasion assays after transfection. All transfection was conducted in three times. miR-16 mimics/inhibitor were obtained from GenePharm (Shanghai, China).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from plasma and tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). qRT-PCR was conducted with SYBR Premix Ex Taq™ (TaKaRa, Dalian, China). U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) acted as the internal control for the miR-16 and RAB23 expression. The relative expressions of miR-16 and RAB23 were calculated according to the $2^{-\Delta\Delta ct}$ method. The primer of RAB23 and GAPDH were synthesized from Invitrogen (Carlsbad, CA, USA). The transcription primer and PCR primer of miR-16 and U6 were purchased from RiBoBio (Guangzhou, China).

Primers were as follows: miR-16 forward, 5′-GCCGTAGCAGCACGTAAATAA-3′, reverse, 5′-GTGCAGGGTCCGAGGT-3′; U6 forward, 5’-GCTTCGGCAGCACATATACTA-AAAT-3’, reverse, 5’-CGCTTCACGAATTT-GCGTGTCAT-3’; RAB23, forward 5′-AGC-GAGACTCCGTCTTCAAA-3′; reverse, 5′-CACCCCTAAGGTACGCATGT-3′; GAPDH, forward 5′-GCACCGTCAAGGCTGAGAAC-3′; reverse, 5′-TGGTGAAGACGCCAGTGGA-3′.

Western Blotting

Proteins were isolated from cells with different transfection using radioimmunoprecipitation assay (RIPA) lysis buffer phenylmethylsulfonyl fluoride (PMSF) (Beyotime, Shanghai, China). The measurement of protein concentration was done useing Protein bicinchoninic acid (BCA) Assay Kit (Bio-Rad, Hercules, CA, USA). The protein was transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membranes were blocked by 5% bovine serum albumin (BSA) and incubated with specific primary antibody against RAB23 (Abcam, Cambridge, MA, USA), or glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Abcam, Cambridge, MA, USA). After that, the membrane was incubated in the secondary antibody. The membrane was measured by electrochemiluminescence (ECL) reagent (Applygen, Beijing, China).

Transwell Assay

The transwell assay with or without Matrigel (Clontech, Palo Alto, CA, USA) was chosen to measure the migration and invasion. In transwell assay, the upper chamber contained 200 μL serum-free medium, the lower chambers contained 800 μL of medium with 15% FBS containing 1×10^5 MG63 cells. The cells on the low chambers were removed. The cells on the top chambers were fixed with methanol, stained with 0.05% crystal violet. The stained cells were counted using Image J software (Bethesda, MD, USA).

Luciferase Assay

The bioinformatics analysis software TargerScan (http://www.targetscan.org/) was chosen

for predicting the targets of miR-16. The 3'-UTRs of RAB23 were amplified with polymerase chain reaction (PCR) from genomic DNA. The wild-type (WT) and mutant type (MUT) 3'-UTR of RAB23 were cloned into the pRL-CMV Renilla luciferase vector (Ambion, Austin, TX, USA) and verified by sequencing. For the luciferase assay, the cells were co-transfected with miR-16 mimics and WT or MUT 3'-UTR of RAB23 luciferase reporter plasmid. Next, we used Dual-Luciferase Assay Kit (Promega, Madison, WI, USA) for measuring the relative luciferase activity.

Statistical Analysis

Statistical product and service solutions (SPSS) version 18.0 (SPSS Inc., Chicago, IL, USA) were used to analyses the data. All the results were present as mean ± SD. Comparison between groups was done using One-way ANOVA test followed by Least Significant Difference (LSD). Statistical significance was considered when $p<0.05$.

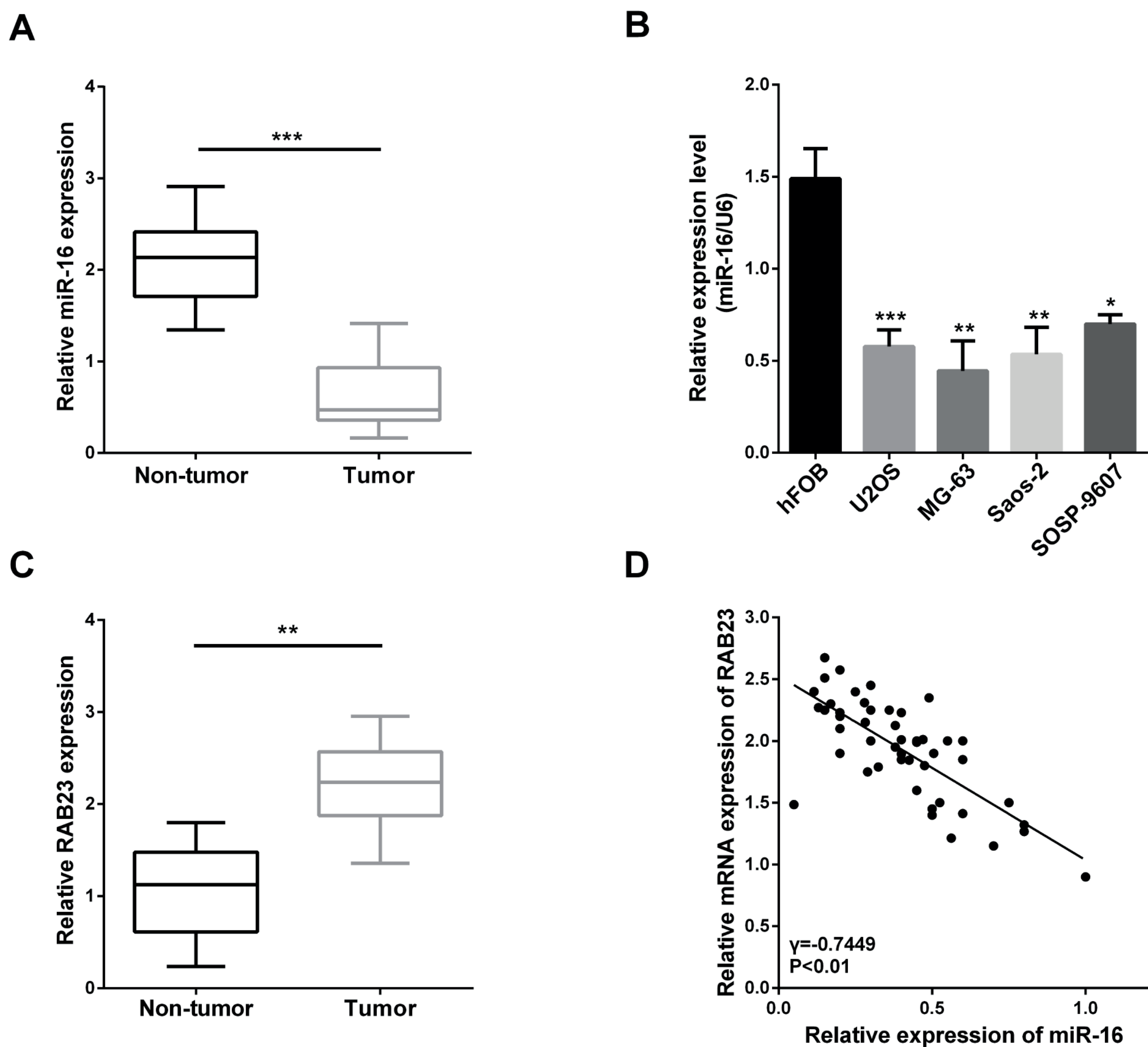

Figure 1. miR-16 was down-regulated and inversely connected with RAB23. A, miR-16 expression in 48 pairs of samples and their adjacent normal tissues. The miR-16 expression was detected by RT-qPCR using U6 as the internal control. B, miR-16 levels in MG63, SAOS-2, U2OS, and SOSP-9607 cell lines compared with the normal cell hFOB. C, Relative mRNA expression of RAB23 in osteosarcoma tissues. D, Spearman correlation analysis of miR-16 and RAB23 level in osteosarcoma tissues (γ=-0.7499, p<0.01). ** $p<0.01$, * $p<0.05$.

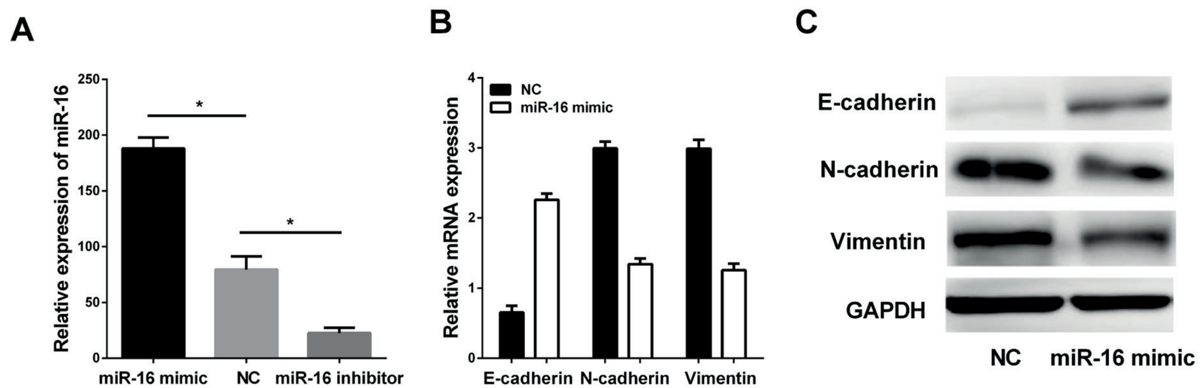

Figure 2. miR-16 overexpression suppressed the osteosarcoma cell EMT. ***A,*** miR-16 levels were determined with qRT-PCR after transfected miR-16 mimic/inhibitor and NC. ***B,*** The mRNA expression of E-cadherin, N-cadherin and vimentin in MG-63 cells was measured using qRT-PCR. ***C,*** The protein level of E-cadherin, N-cadherin and vimentin in MG-63 cells was detected by Western blot. *$p<0.05$.

Results

miR-16 was Down-Regulated and Inversely Correlates with RAB23 Expression

To investigate whether the miR-16 was altered in osteosarcoma tissues, qRT-PCR was performed in 48 pairs of cervical tissues. The osteosarcoma tissues showed a significantly decreased expression of miR-16 compared to the normal specimens (Figure 1A, $p<0.001$). Furthermore, we examined the difference of miR-16 expression in osteosarcoma cell lines (MG63, SAOS-2, U2OS and SOSP-9607) compared to the normal cell hFOB. The results showed the miR-16 expression in osteosarcoma cell lines were prominently reduced relatively to the normal human osteoblastic cell line hFOB (Figure 1B, $p<0.05$, $p<0.01$, $p<0.001$).

Furthermore, the RAB23 expression levels in 48 pairs of osteosarcoma tissues were also examined. The RAB23 expression was significantly higher in osteosarcoma tissues as shown in Figure 1C ($p<0.01$). Spearman's rank correlation analysis was conducted to reveal the correlation between these two factors. We found there was a negative correlation between miR-16 level and the RAB23 level in these clinical specimens (Figure 1D). This correlation may play an essential part in the osteosarcoma progression.

miR-16 Inhibited MG63 Cell EMT, Migration and Invasion

We established stable cells to identify the functions of miR-16 in the progression of osteosarcoma in MG63 cells. In Figure 2A, the miR-16 expression was markedly higher when cells transfected with mimics, and the miR-16 expression was markedly reduced when transfected with inhibitor ($p<0.05$). High expression of miR-16 promoted the E-cadherin level and suppressed the N-cadherin and vimentin levels in the MG-63 cell (Figure 2B). Over-expression of miR-16 increased the E-cadherin level and suppressed the N-cadherin and vimentin level in the MG-63 cells at protein level (Figure 2C).

Additionally, to demonstrate the effect of miR-16 in MG-63 cells migration and invasion, transwell chambers were conducted to detect the abilities of MG-63 cells with different transfection. Ectopic expression of miR-16 inhibited MG63 cells migration, whereas inhibiting miR-16 expression promoted MG63 cells migration (Figure 3A-B; $p<0.05$, $p<0.01$). As the cell invasion assay showed, elevated expression of miR-16 dramatically suppressed invasion in the MG63 cell, whereas inhibition of miR-16 expression promoted invasion in the MG63 cell (Figure 3C-D; $p<0.05$, $p<0.01$). Thereby, miR-16 plays an essential role in the migration and invasion of osteosarcoma cells.

RAB23 was the Direct Target of miR-16

The Target Scan database was used to find the potential target of miR-16. The putative binding sites for miR-16 and RAB23 were found at the 3′-UTR (Figure 4A). Luciferase assay showed miR-16 over-expression decreased luciferase activity of WT 3′-UTR of the RAB23 vector, whereas miR-16 inhibitor increased luciferase activity in WT RAB23, but the luciferase activity

has no changes in cells transfected with RAB23 3′-UTR-containing MUT miR-16-binding sites ($p<0.05$) (Figure 4B). Over-expression of miR-16 decreased the protein level of RAB23 in the MG63 cell, while down-regulated expression of miR-16 enhanced RAB23 expression (Figure 4C). Together, these results demonstrated miR-16 negatively regulated endogenous RAB23 expression in the MG63 cell.

Over-Expression of RAB23 Ablated the Inhibitory Effects of miR-16

Given evidence has indicated RAB23 was the direct target of miR-16 in the MG63 cell, and that RAB23 might take part in miR-16-mediated inhibition of osteosarcoma cells migration and invasion. To detect whether over-expression of RAB23 would simulate miR-16-mediated effects, miR-16 mimic and an RAB23 over-expressing vector were transfected into the MG63 cell. qRT-PCR and Western blot assays suggested both the RAB23 protein and mRNA levels were reduced by miR-16 mimic, and the levels were restored after con-transfected RAB23 and miR-16 mimic (Figure 5A-B; $p<0.05$, $p<0.01$). Next, we calculated the functions of RAB23 over-expressing on the inhibition of miR-16 in MG63 cell migration and invasion. Over-expressing of RAB23 reversed the inhibitory effects of miR-16 on MG63 cell migration (Figure 5C; $p<0.05$, $p<0.01$) as well as invasion (Figure 5D; $p<0.05$, $p<0.01$).

Discussion

Dysregulation of miRNAs may lead to uncontrolled and progressive cancer growth and has been thoroughly reported in almost all kinds of human malignancies[22,23]. Increased investigation on the gene interaction mediated by miRNAs may provide

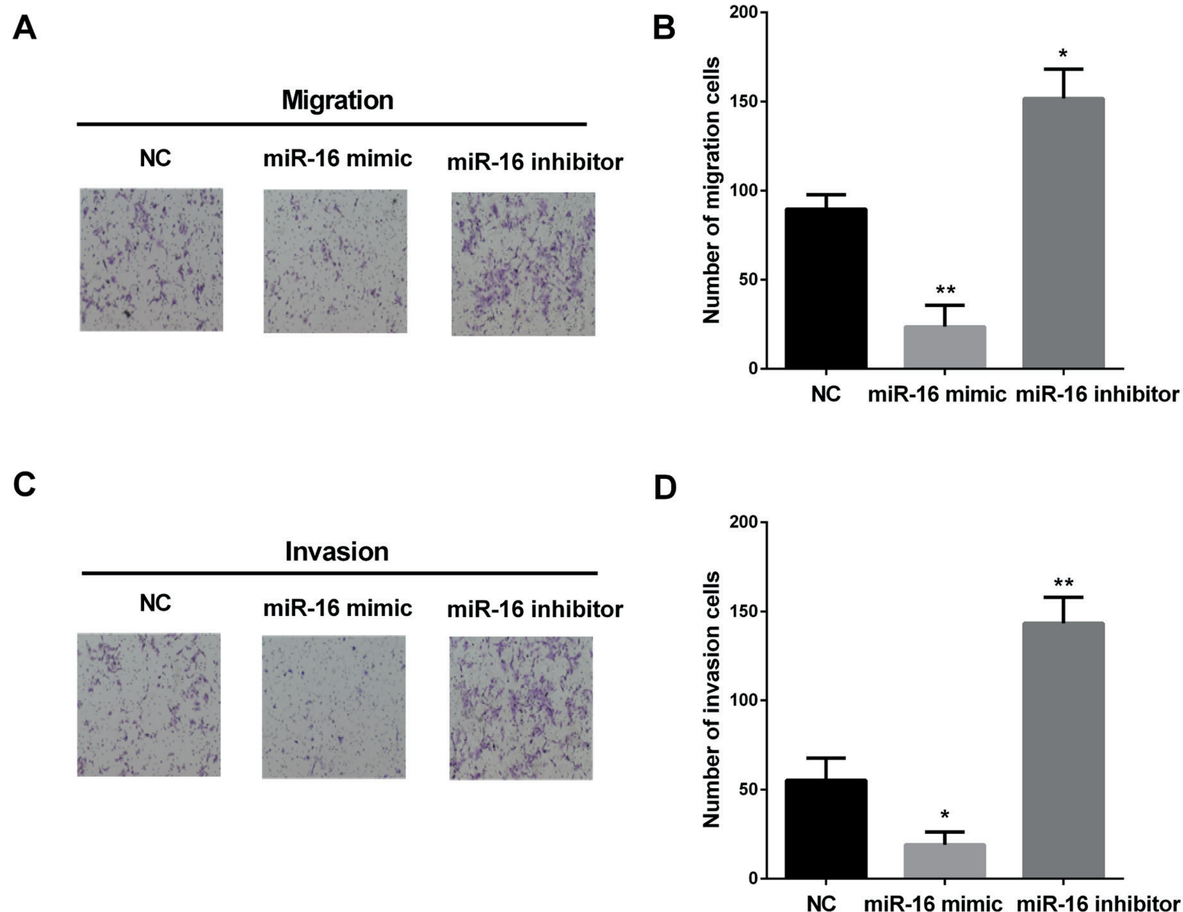

Figure 3. miR-16 inhibited osteosarcoma invasion and migration. A-B, Ectopic miR-16 expression significantly suppressed MG-63 cell migration, whereas inhibiting miR-16 expression promoted MG-63 cell migration. C-D, miR-16 over-expression dramatically inhibited MG-63 cell invasion, whereas inhibition of miR-16 expression significantly promoted MG-63 cell invasion. **$p<0.01$, *$p<0.05$.

further therapeutic targets and potential biomarkers for patients suffering from malignancy. In the present research, preliminary evidence was presented about the negative regulation of miR-16 in osteosarcoma invasion and migration through targeting RAB23. We identified that miR-16 expression levels were significantly lower in osteosarcoma tissues and cell lines (MG63, SAOS-2, U2OS, and SOSP-9607). Ectopic expression of miR-16 inhibits EMT, migration and invasion of osteosarcoma cells. We demonstrated RAB23-3′-UTR carried the binding sites for miR-16 and inversely correlated with miR-16 expression. Rab23 expression levels were higher in the osteosarcoma tissues and over-expression of RAB23 ablated the inhibitory function of miR-16. These results revealed miR-16 might contribute to the development of osteosarcoma.

miR-16 was altered in osteosarcoma and its functional role was extremely tangle as it could act as promising targets for therapeutic purposes in previous study[24]. The miR-16 expression was down-regulated in various kinds of tumors, for example lung cancer[16], breast cancer[17] and so on, but the biological effect of miR-16 remains poorly understood in osteosarcoma. This research firstly demonstrated the synthesized analysis of miR-16 effects on osteosarcoma. miR-16 was significantly down-regulated in osteosarcoma cell lines and tissues, and its expression was inversely correlated with RAB23. Further analyses revealed that miR-16 inhibits osteosarcoma cells EMT, migration and invasion. These results together demonstrated miR-16 could be a powerful anti-osteosarcoma candidate.

Ras-Related Protein 23 (RAB23), which is well recognized as a member of the Rab family of Ras small GTPases superfamily, has been found involving in the regulation of tumor biological behaviors[6,25]. RAB23 has been reported as a target of miR-16; Chen et al[26] reported miR-16 relieved CFA-induced chronic inflammatory pain by targeting RAB23. Another research[27] demonstrated that miR-665 acted as suppressor in the invasion

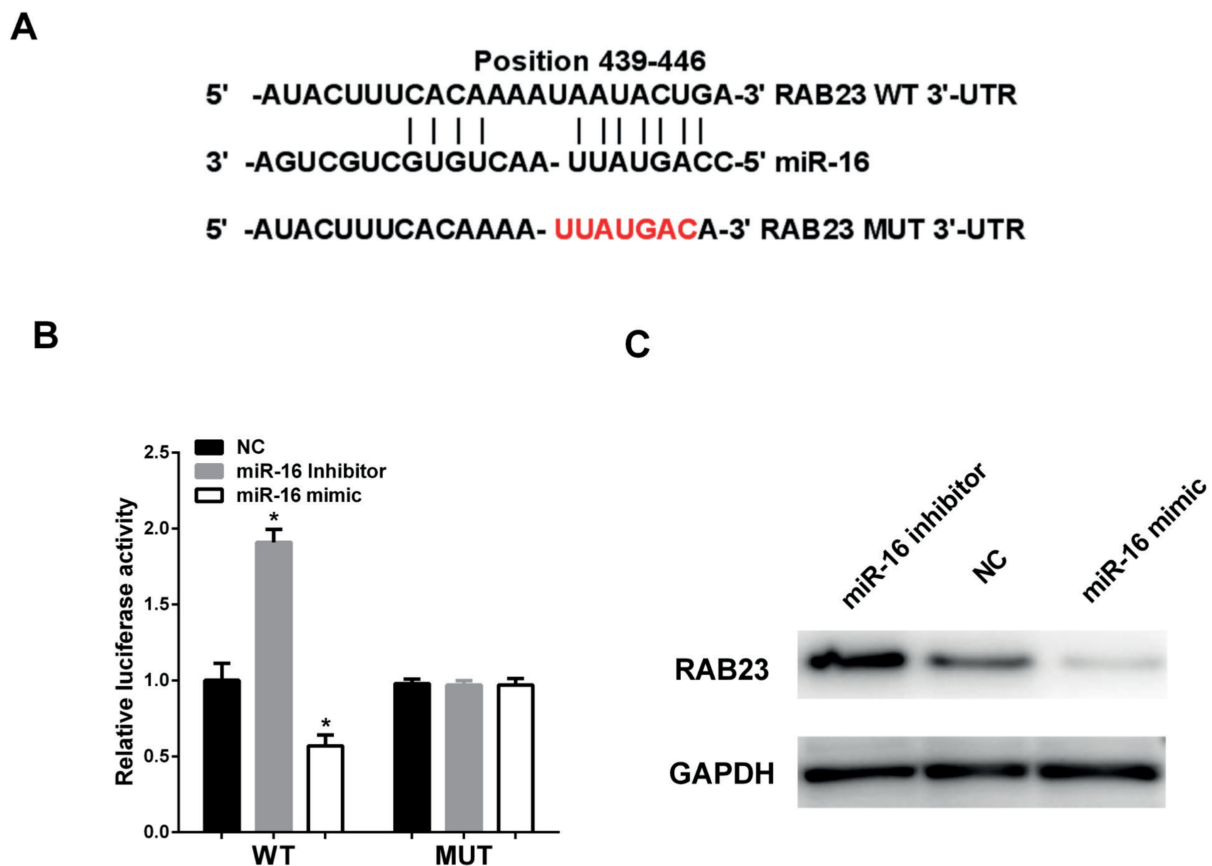

Figure 4. RAB23 is the direct target of miR-16. ***A,*** The binding sites of miR-211 on RAB23-3′-UTR. ***B,*** Luciferase reporter assay with the pGL3-RAB23-3′-UTR-WT or pGL3-RAB23-3′-UTR-MUT were co-transfected with miR-16 mimics/inhibitor/NC. ***C,*** RAB23 level in MG-63 cells transfected with miR-16 mimic/inhibitor/NC. $^{**}p<0.01$, $^{*}p<0.05$.

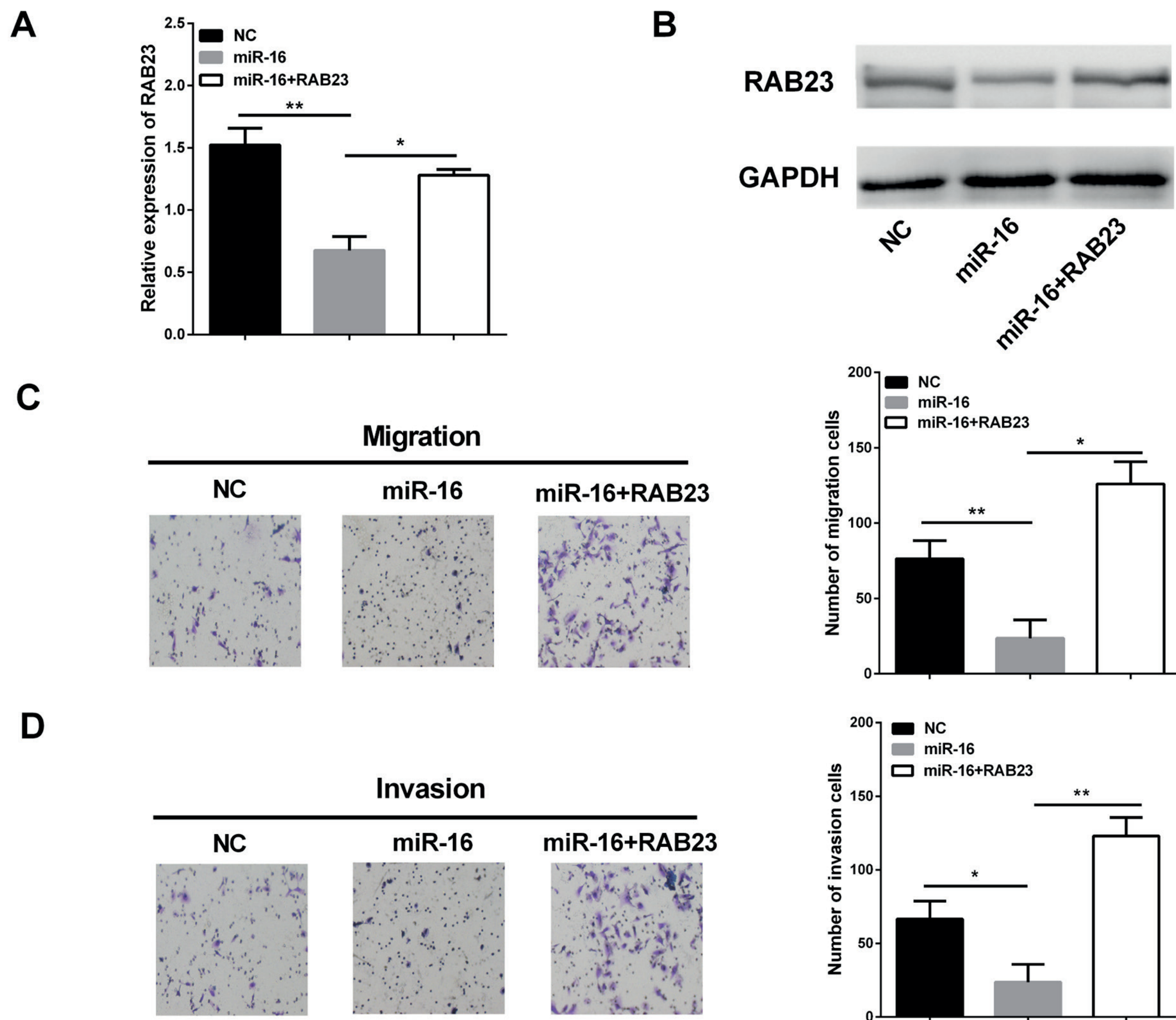

Figure 5. Over-expression of RAB23 ablates the miR-16 inhibitory effects. ***A-B***, RAB23 was abnormally expressed in MG-63 cells and RAB23 levels were determined by qRT-PCR. ***C-D***, Transwell assay was performed in MG-63 cells that were in different condition. **$p<0.01$, *$p<0.05$.

and metastasis by targeting RAB23 of osteosarcoma. We determined RAB23 as the target of miR-16 in osteosarcoma, a significantly high expression of RAB23 was observed after transfecting by miR-16 mimic. However, miR-16 inhibitor decreased RAB23 expression. Furthermore, for the first time, over-expression of RAB23 ablates the inhibitory effects of miR-16, suggesting that the interaction between miR-16 and RAB23 may have a fundamental biological role.

Conclusions

We revealed that miR-16 acts as a tumor repressor in osteosarcoma cells by reducing EMT, migration and invasion. Furthermore, we spotted that RAB23 has an inverse correlation with miR-16. This newly identified miR-16 may provide further insight into the progression and offers a promising therapeutic target for osteosarcoma. Further study to investigate the function miR-16/RAB23 axis may provide new insight into the pathogenesis and represents a potential therapeutic target for osteosarcoma.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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