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MicroRNA-149 inhibits the progression of lung adenocarcinoma through targeting RAP1B and inactivating Wnt/β-catenin pathway

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Abstract. – OBJECTIVE: MicroRNAs (miRNAs) are important regulators in the progression of lung adenocarcinoma (LAD). Moreover, microRNA-149 (miR-149) exhibits different roles in human cancers. Hence, this study mainly focused on the function of miR-149 in LAD.

PATIENTS AND METHODS: Western blot analysis and Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) were used to quantify expression levels. The regulatory mechanism of miR-149/RAP1B was explored by methyl thiazolyl tetrazolium (MTT), transwell, and Dual-Luciferase reporter assays.

RESULTS: Downregulation of miR-149 was detected in LAD and predicted worse prognosis in patients with LAD. Functionally, overexpression of miR-149 inhibited cell viability and metastasis in LAD. In addition, miR-149 directly targets RAP1B and restrained its expression in LAD. Furthermore, upregulation of RAP1B attenuated the inhibitory effect of miR-149 on LAD. Besides that, miR-149 blocked epithelial-mesenchymal transition (EMT) and Wnt/β-catenin pathway in LAD.

CONCLUSIONS: MiR-149 inhibited the progression of LAD by restraining RAP1B/EMT and inactivating Wnt/β-catenin pathway.

Key Words:
MiR-149, Lung adenocarcinoma, RAP1B, Cell viability, Metastasis.

Introduction

Today, lung cancer has the highest mortality rate in malignant tumors. About 300,000 new patients suffering lung cancer occur in China each year[1]. Among them, lung adenocarcinoma (LAD) patients account for about 40% of lung cancer patients[2]. In addition, the prognosis of many LAD patients is not optimistic due to the high invasiveness of lung cancer cells[3]. However, although the diagnosis and treatment of LAD have been improved, the cure rate and survival rate are still very poor[4]. Therefore, investigating and developing new methods is meaningful for the diagnosis and treatment of LAD patients.

As a popular target, microRNAs (miRNAs) are widely recognized as important regulatory factors in the development of cancers[5]. In the biological progression of LAD, some important miRNAs have also been discovered. Among them, miR-145 was found to suppress cell invasion and migration in LAD via downregulating N-cadherin[6]. In contrast, miR-222 accelerated LAD cell migration, invasion, and proliferation by modulating ETS1[7]. In this study, the different roles of miR-149 in human cancers have caught our attention. It has been reported that miR-149 was correlated with lung cancer risk in Chinese non-smoking female[8]. Downregulation of miR-149 was found in renal cell carcinoma and colonic carcinoma[9]. Notably, miR-149 restrained cell migration and invasion in colorectal cancer by inhibiting FOXM1 expression[10]. However, upregulation of miR-149 was detected in T-cell acute lymphoblastic leukemia[11]. Furthermore, miR-149 promoted human osteosarcoma progression *via* targeting bone morphogenetic protein 9 (BMP9)[12]. These studies indicate that the role of miR-149 is dependent on the type of cancers. Moreover, the specific role of miR-149 remains unclear in LAD. Therefore, the dysregulation of miR-149 was investigated in LAD.

As a member of the RAS oncogene family, RAP1B was found to be involved in the regulation of human cancers. Lin et al[13] found that downregulation of RAP1B inhibited the

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metastasis of ovarian cancer cell. Moreover, RAP1B promoted cell migration by regulating isoproterenol in glioma[14]. Recently, RAP1B has been reported to regulate cell invasion and proliferation by targeting RAP1B in colorectal cancer[15]. However, the interaction between miR-149 and RAP1B has not been elucidated. Hence, the regulatory mechanism of miR-149/RAP1B was investigated in the current study. In addition, it was explored in LAD that how miR-149 regulates epithelial-mesenchymal transition (EMT) and Wnt/β-catenin pathway. The EMT and Wnt/β-catenin pathways are important processes involved in LAD development. Mou et al[16] proposed that miR-485 inhibited LAD metastasis and EMT *via* targeting Flot2. Moreover, PRC1 contributed to the tumorigenesis of LAD associated with Wnt/β-catenin pathway[17]. Through a series of studies on the function of miR-149, this study will help us better understand the pathogenesis of LAD.

Patients and Methods

Experimental Sample

Ninety-one patients with LAD in Chengyang People's Hospital participated in this study. Before the start of the experiment, we received informed consents from all LAD patients. Experimental LAD and normal tissues were obtained from these patients. Ninety-one LAD patients (male=59; female=32; average age, 44.55±6.02; range 38-65 years; smoked=72) did not receive any treatment except surgery. The investigation was approved by the Institutional Ethics Committee of Chengyang People's Hospital.

Cell Culture and Transfection

Normal lung epithelial cells BEAS-2B and A549 LAD cell line were purchased from the American type culture collection (ATCC; Manassas, VA, USA). The growth conditions of these cells were 5% CO_2, 37°C and culture solution [90% Dulbecco's Modified Eagle's Medium (DMEM, Hyclone, South Logan, UT, USA) +10% fetal bovine serum (FBS, Hyclone, South Logan, UT, USA)]. Next, A549 cells were transfected with miR-149 mimics and inhibitor or RAP1B siRNA and vector (RiboBio, Guangzhou, China) for further experiment.

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

Total RNA extraction was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The complementary deoxyribonucleic acids (cDNAs) solution was synthesized using the First-Strand cDNA Synthesis kit (Promega, Madison, WI, USA). RT-qPCR assay was performed using the miScript SYBR®Green PCR kit (Qiagen, Duesseldorf, Germany). MiR-149 or RAP1B was normalized to U6 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) internal reference using the $2^{-\Delta\Delta ct}$ method. The primers used in our work were as follows: miR-149, forward primer: 5'-GCA GTT TCA GCC AGA ATG TG-3', reverse primer: 5'-AGG GTG ACT AGA GTA CTG TGG-3'; U6, forward primer: 5'-CTC GCT TCG GCA GCA CA-3', reverse primer: 5'-AAC GCT TCA CGA ATT TGC GT-3'; RAP1B forward primer: 5'-GTG AAT CCC TTG CTT GCT CAT-3', reverse primer: 5'-AAT ACT GTG GCT CCC TGT TGG-3'; GAPDH forward, 5'-ACA TCG CTC AGA CAC CAT G-3', reverse, 5'-TGT AGT TGA GGT CAA TGA AGG G-3'.

Methyl Thiazolyl Tetrazolium (MTT) Assay

Transfected A549 cells (5×10^3 cells/well) were inoculated in DMEM (10% FBS) for 24, 48, 72 or 96 h. Next, A549 cell suspension was incubated with 20 μL MTT for 4 h. After removing MTT solution, 150 μL of dimethyl sulfoxide (DMSO) was added. Finally, cell viability was evaluated using a microscope (Olympus Corp., Tokyo, Japan) to determine the optical density at 490 nm.

Transwell Assay

First, upper chamber with Matrigel was prepared to detect cell invasion. Next, transfected A549 cells (2×10^4 cells/well) were added in the upper chamber. The lower chamber containing 10% FBS was used to induce cell invasion. Next, A549 cells were incubated for 24 h at 37°C with 5% CO_2. The moving cells were fixed and stained for 30 min. The moving cells were then observed in a light microscope (Olympus Corporation, Shanghai, China).

Western Blot Analysis

Proteins were extracted using Radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). Next, 25 μg protein was separated using 10% dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were transferred to polyvinylidene difluoride (PVDF) membranes

(Roche, Basel, Switzerland). Blocked with 5% non-fat milk, protein samples were incubated overnight at 4°C with E-cadherin, N-cadherin, Vimentin, RAP1B, β-cateni, and GAPDH primary antibodies (Abcam, Cambridge, MA, USA). Then, horseradish peroxidase (HRP)-conjugated secondary antibodies (Abcam, Cambridge, MA, USA) were added to incubate the protein samples for 1 h. Enhanced chemiluminescence (ECL, Pierce, Rockford, IL, USA) was used to assess protein expression.

Luciferase Reporter Assay

A luciferase vector plasmid complementary deoxyribonucleic acid 3.1 (pcDNA3.1) harboring the Wt-RAP1B-3'UTR or Mut-RAP1B-3'UTR and miR-149 mimics was transfected into A549 cells. After 48 h, we discarded the medium and washed it with PBS. Finally, Luciferase activity was assessed using a Dual-Luciferase assay system (Promega, Madison, WI, USA).

Statistical Analysis

Data are shown as mean ± SD (standard deviation), which was analyzed by Statistical Product and Service Solutions (SPSS) 18.0 (SPSS, Chicago, IL, USA) or GraphPad Prism 6 (La Jolla, CA, USA). Comparisons between multiple groups were performed using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). These methods include Chi-squared test, one-way analysis of variance (ANOVA) with Bonferroni post-hoc test and Univariate Kaplan-Meier method with log-rank test. Statistical difference was defined as $p<0.05$.

Results

MiR-149 Was Downregulated in LAD

First, RT-qPCR was used to observe the alternation of miR-149 expression in LAD. The results showed that miR-149 was downregulated in LAD tissues compared to normal tissues ($p<0.01$, Figure 1A). Similarly, downregulation of miR-149 was found in A549 cells compared to BEAS-2B cells ($p<0.01$, Figure 1B). In addition, the prognosis of LAD patients was detected in this study. We found that LAD patients with low miR-149 expression showed shorter overall survival ($p=0.029$, Figure 1C). Based on these results, we conclude that miR-149 may regulate tumorigenesis of LAD.

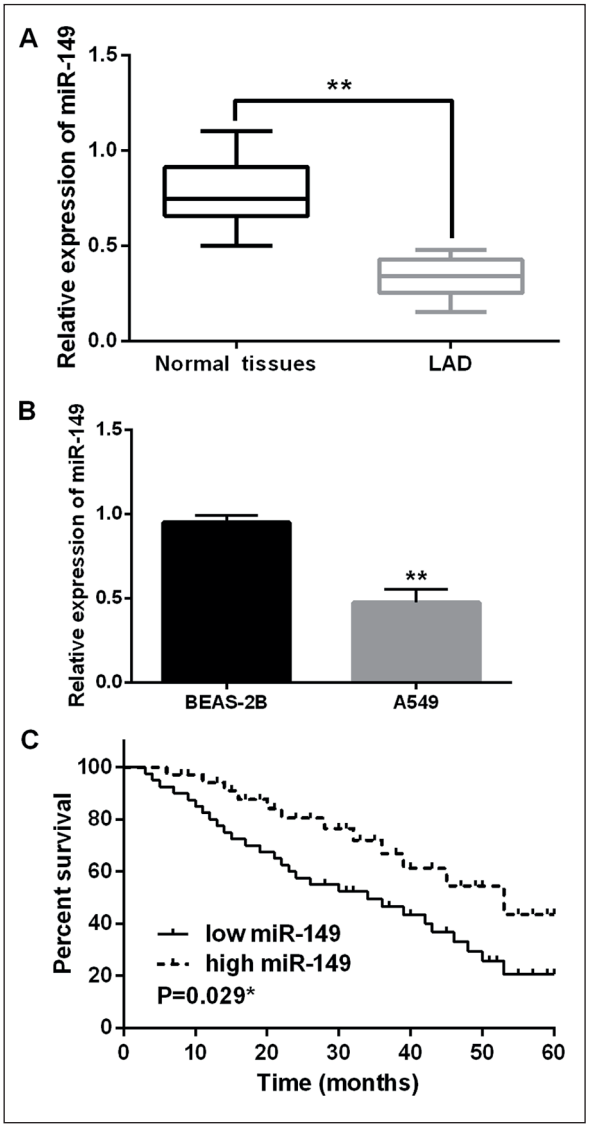

Figure 1. MiR-149 expression was reduced in LAD tissues. **A,** MiR-149 expressions in LAD tissues. **B,** MiR-149 expressions in A549 and BEAS-2B cells. **C,** Shorter overall survival was found in LAD patients with low miR-149 expression **$p<0.01$.

MiR-149 Inhibited LAD Cell Proliferation and Metastasis

A549 cells with miR-149 mimics or inhibitor were prepared to explore the role of miR-149 in LAD. RT-qPCR showed that miR-149 expression was enhanced by its mimics but was reduced by its inhibitor ($p<0.01$, Figure 2A). MTT assay indicated that overexpression of miR-149 restrained

proliferation of A549 cells. In contrast, downregulation of miR-149 enhanced the ability of cell proliferation in A549 cells (p<0.05 or 0.01, Figure 2B). Besides that, miR-149 overexpression was found to inhibit cell migration. But knockdown of miR-149 promoted migration of A549 cells (p<0.01, Figure 2C). Consistently, overexpression of miR-149 restrained A549 cell invasion. And miR-149 inhibitor promoted cell invasion in LAD cells (p<0.01, Figure 2D). These results reveal that miR-149 serves as a tumor suppressive miRNA in LAD.

MiR-149 Directly Targets RAP1B

In addition, RAP1B is predicted to have a

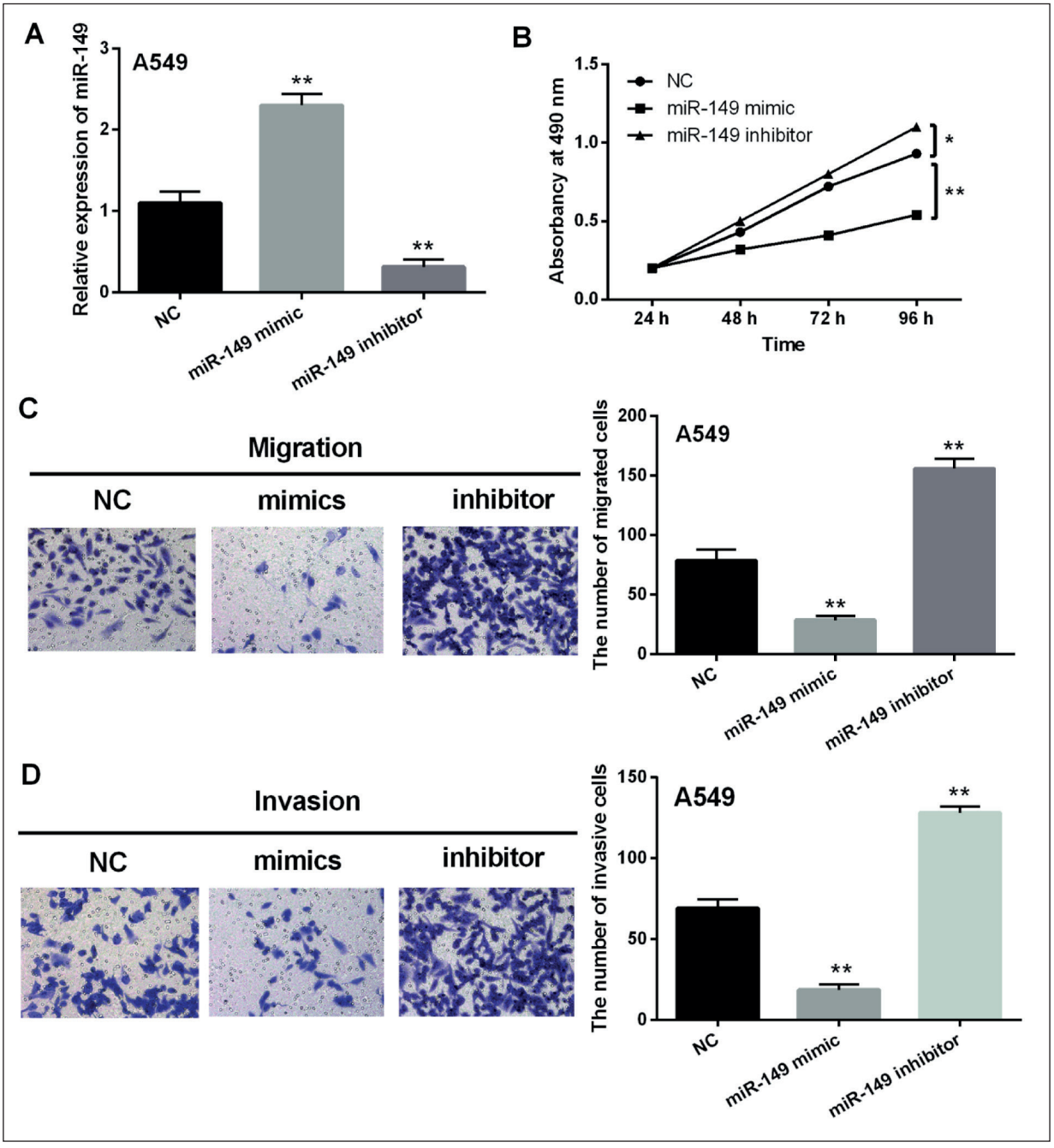

Figure 2. MiR-149 inhibited cell viability and metastasis in LAD. **A,** MiR-149 mimics or inhibitor regulated its expression in A549 cells. **B, C, D,** miR-149 mimics or inhibitor regulated A549 cell proliferation, migration and invasion (magnification, 200×) *p<0.05, **p<0.01.

binding site with miR-149 (TargetScan, http://www.targetscan.org/, Figure 3A). Next, Luciferase reporter assay was designed to verify the relationship between them. MiR-149 mimics were found to lessen the luciferase activity of Wt-RAP1B, but had no effect on Mut-RAP1B (p<0.01, Figure 3B). Next, RAP1B expression was observed to be affected by miR-149 mimics or inhibitor. We found that miR-149 mimics reduced RAP1B expression, but miR-149 inhibitor increased it (p<0.01, Figure 3C). To investigate whether RAP1B is involved in LAD progression, abnormal expression of RAP1B was detected in LAD tissues. Upregulation of RAP1B was observed in LAD tissues (p<0.01, Figure 3D). Furthermore, a negative correlation between miR-149 and RAP1B expression was found in LAD tissues (p<0.0001, R^2=0.5013; Figure 3E). Collectively, miR-149 directly targets RAP1B and negatively regulates its expression in LAD.

MiR-149 Regulated LAD Progression through Regulating RAP1B

The RAP1B vector was transfected into A549 cells with miR-149 mimics to explore their interaction in LAD. First, it was observed that the decreased expression of RAP1B induced by miR-149 mimics was restored by RAP1B vector (Figure 4A). Functionally, miR-149-mediated inhibition of cell proliferation was attenuated by RAP1B overexpression (p<0.01, Figure 4B). Furthermore, upregulation of RAP1B

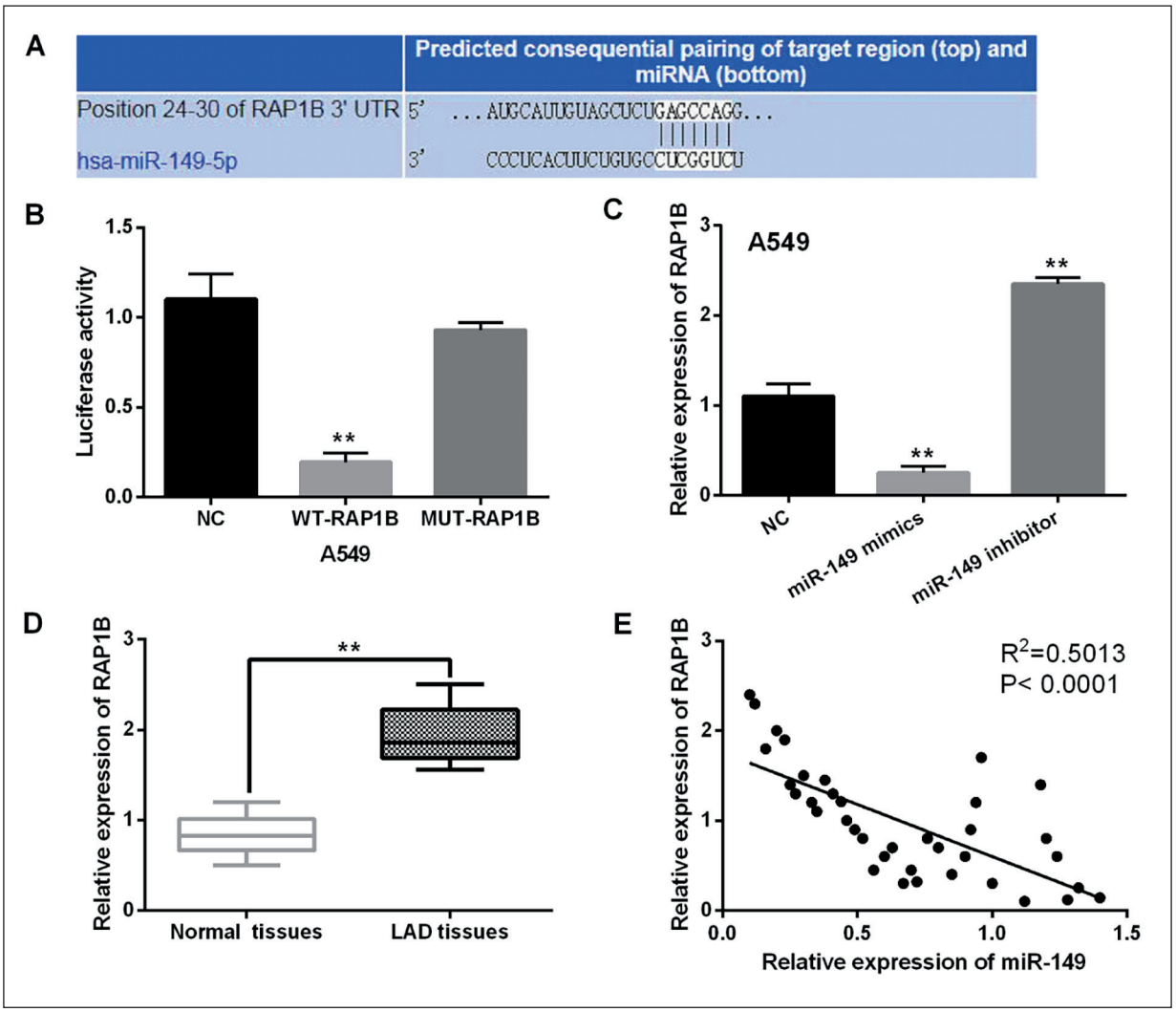

Figure 3. MiR-149 directly targeted RAP1B. **A,** The binding sites between miR-149 and RAP1B. **B,** Luciferase reporter assay. **C,** RAP1B expression in A549 cells with miR-149 mimics or inhibitor. **D,** RAP1B expression in LAD tissues. **E,** A negative correlation between miR-149 and RAP1B. **p<0.01.

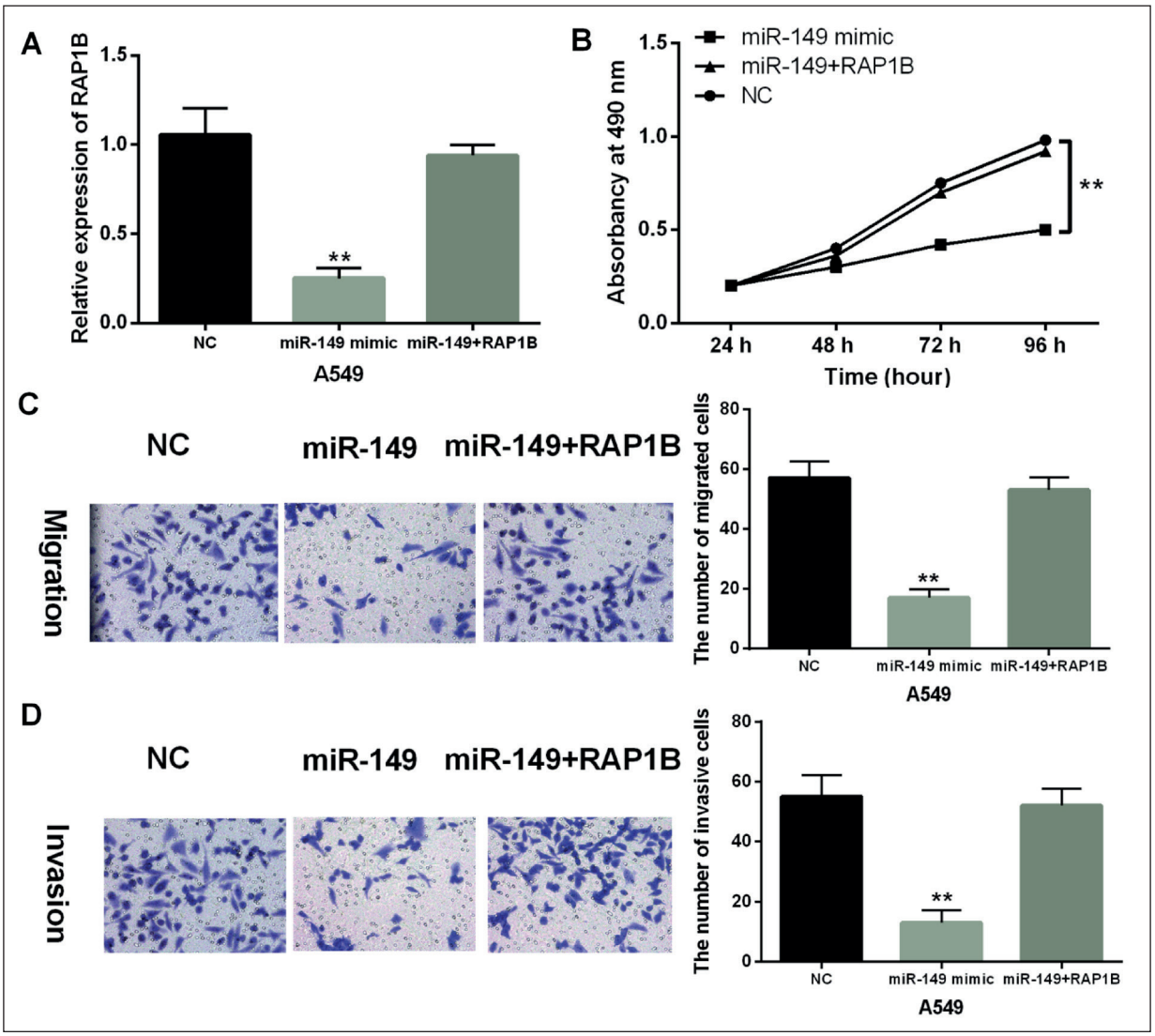

Figure 4. MiR-149 regulated LAD progression through regulating RAP1B. **A,** RAP1B expression in A549 cells with miR-149 mimics and RAP1B vector. **B, C, D,** RAP1B vector regulated cell proliferation, migration and invasion in A549 cells with miR-149 mimics (magnification, 200×) $^{**}p<0.01$.

attenuated the inhibitory effect of miR-149 on cell invasion and migration in A549 cells ($p<0.01$, Figure 4C, 4D). Taken together, miR-149 restrained LAD progression by suppressing RAP1B expression.

MiR-149 Blocked EMT and Wnt/β-Catenin Pathway in LAD

Finally, we explored how miR-149 regulates the EMT and Wnt/β-catenin pathways to further explain its molecular mechanism in LAD. As for EMT, we detected that the expression of N-cadherin and Vimentin was restrained by miR-149 overexpression. Furthermore, overexpression of miR-149 promoted expression of E-cadherin (Figure 5). In addition, knockdown of miR-149 inhibited E-cadherin expression and promoted N-cadherin and Vimentin expressions in A549 cells (Figure 5). Next, we analyzed the expression of β-catenin in A549 cells. MiR-149 mimics was demonstrated to restrain β-catenin expression, when miR-149 inhibitor promoted its expression (Figure 5). Therefore, we believe that miR-149 exerts an inhibitory effect in LAD through blocking EMT and Wnt/β-catenin pathways in LAD.

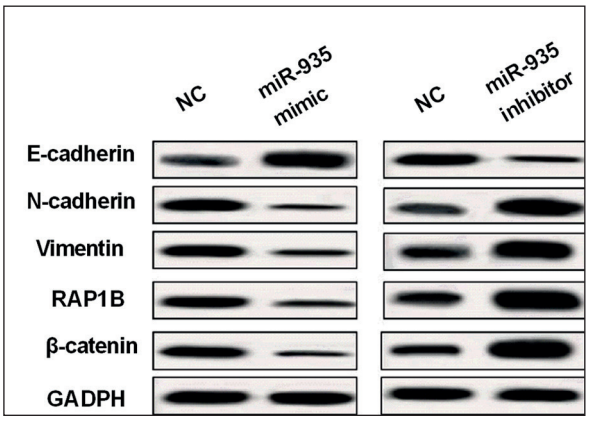

Figure 5. MiR-149 blocked EMT and Wnt/β-catenin pathway in LAD. The protein expressions of E-cadherin, N-cadherin, Vimentin, RAP1B and β-catenin were regulated by miR-149 mimics or inhibitor in A549 cells.

Discussion

Many studies have shown that dysregulation of miRNAs is involved in the pathogenesis of LAD. MiR-383 has been reported to act as a prognostic marker and inhibitor in LAD[18]. Functionally, the biological processes of LAD included proliferation, migration, invasion and EMT were restrained by miR-519d, miR-767, and miR-218[19-21]. Here, shorter overall survival in LAD patients was found to be associated with the downregulation of miR-149. We also found that miR-149 overexpression inhibited LAD cell viability and metastasis. Furthermore, miR-149 blocked EMT and Wnt/β-catenin pathway in LAD. These results suggest that miR-149 functions as a tumor inhibitor in LAD.

Consistent with our results, downregulation of miR-149 has been found in prostate cancer and glioma[22,23]. Moreover, low miR-149 expression can predict adverse prognosis in glioma patients[24]. In addition, Yang et al[25] proposed that miR-149 can induce inhibition of proliferation, migration, and invasion in bladder cancer. The same effect of miR-149 on cell proliferation, migration, and invasion was also detected in LAD. Similar to our results, overexpression of miR-149 has been demonstrated to suppress the growth and metastasis of human non-small cell lung cancer (NSCLC)[26]. Besides that, miR-149 was found to inhibit EMT by targeting FOXM1 in NSCLC[27]. Here, we also found that miR-149 restrained EMT and Wnt/β-catenin pathway in LAD. Unlike other studies, miR-149 was confirmed to directly target RAP1B and restrain its expression in LAD in this study.

Similarly, the interaction between RAP1B and other miRNAs has also been investigated in human cancers. So, miR-139 suppressed cell proliferation in human colorectal carcinoma by targeting RAP1B[28]. It was reported that miR-149 inhibited RAP1B expression to regulate glioblastoma progression[29]. Here, we also found that miR-149 suppressed the development of LAD by targeting RAP1B. Moreover, upregulation of RAP1B was observed in LAD tissues. Other authors[30,31] also reported the upregulation of RAP1B in Hirschsprung disease and esophageal squamous cell carcinoma, which was similar to our results. Besides that, upregulation of RAP1B weakened the inhibitory effect of miR-149 in LAD, reflecting that RAP1B acts as an oncogene in LAD. The carcinogenic effect of RAP1B has also been identified in renal cell carcinoma[32]. In our study, miR-149 was identified to inactivate Wnt/β-catenin pathway in LAD, which has not been reported previously. Collectively, miR-149 functions as a tumor suppressor in LAD by regulating biological processes.

Conclusions

We revealed the downregulation of miR-149 in LAD associated with poor prognosis. Moreover, miR-149 restrained cell viability and metastasis in LAD by suppressing RAP1B expression. Meanwhile, miR-149 also blocked the EMT and Wnt/β-catenin pathways in LAD. We hope our study can provide a potential therapeutic target for LAD patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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