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MiR-608 exerts tumor suppressive function in lung adenocarcinoma by directly targeting MIF

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Abstract. – OBJECTIVE: Lung adenocarcinoma (LA) is considered as a highly aggressive disease with heterogeneous prognosis. The molecular mechanisms of LA progression remain elusive. Recent studies have shown that dysregulation of microRNAs (miRNAs) is prevalent in LA, playing a significant role in tumor progression. The present work aims to analyze the expression and function of miR-608 in LA.

PATIENTS AND METHODS: Quantitative Real-time polymerase chain reaction (qRT-PCR) assay and Western blot were performed to detect expressions of miR-608 and migration inhibitory factor (MIF). Luciferase reporter assays were carried out to investigate the regulatory effect of miR-608 on MIF. The cell invasion and the migration capabilities were detected by transwell assay.

RESULTS: QRT-PCR indicated that miR-608 expressions in LA tissues were markedly reduced than that of the normal tissues. Moreover, the expression of MIF, a potential target gene of miR-608, was inversely associated with miR-608 expression in LA. Furthermore, miR-608 overexpression could inhibit LA invasion and migration, which was reversed by MIF knockdown.

CONCLUSIONS: Our study revealed the mechanisms that miR-608 suppressed LA invasion and migration by targeting MIF, suggesting that miR-608/MIF axis could be used as a potential prognostic biomarker and therapeutic target for LA.

Key Words:
Lung adenocarcinoma (LA), Invasion, Migration, MiR-608, MIF.

Introduction

Lung adenocarcinoma (LA) is the most common form of lung cancer and belongs to the non-small cell lung cancer[1]. Lung cancer is the most common cause of tumor death worldwide, and its morbidity is increasing annually[2]. Non-small cell lung cancer (NSCLC), including squamous cell carcinoma, adenocarcinoma and large cell carcinoma, accounts for over 85% of all lung cancer types[3]. Lung cancers have a high metastatic potential, which is the major cause of treatment failure. The prognosis of LA patients principally correlates with tumor metastasis[4]. More than 90% of LA patients die of metastasis rather than from their primary tumors, suggesting that metastasis is a key prognostic factor[5]. In recent years, no significant improvement has been made on the 5-year overall survival of NSCLC since the majority of patients are diagnosed in advanced stage[6]. Effective diagnostic methods of NSCLC are still lacked. Therefore, exploring novel and specific biomarkers for the earlier detection of LA should be emphasized.

MicroRNAs (miRNAs) are a group of short, non-coding RNAs that negatively regulate expressions of the target genes by binding to their 3'-UTR, thereby controlling a variety of cellular functions such as proliferation, differentiation and apoptosis[7]. Emerging evidence has indicated that aberrant expressions of miRNAs are closely associated with tumorigenesis and cancer progression via the regulation of key oncogenes or tumor suppressors[8]. For example, Lu et al[9] found that miR-186 inhibited prostate cancer cell proliferation and tumor growth by targeting YY1 and CDK6. Li et al[10] reported that miR-223 promoted gastric cancer invasion and metastasis by targeting tumor suppressor EPB41L3. MiR-370 acted as a tumor suppressor via the downregulation of

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PIM1 in hepatocellular carcinoma[11]. Therefore, relative reports have indicated that miRNA may serve as a prognostic indicator and therapeutic target. A number of investigations have demonstrated that aberrant miRNA expression contributes to lung cancer progression, such as miR-204[12], miR-148a[13] and miR-125b[14]. However, the functions of miR-608 in the regulation of LA progression are limited.

Macrophage migration inhibitory factor (MIF), a multifunctional cytokine generally involving in both innate and adaptive immunity[15], displays pleiotropic functions such as cytokine, chemokine and cell proliferation[16,17]. Although inflammation is an integral component of tumor biology, it is generally appreciated that MIF represents an important link between chronic inflammation and tumorigenesis[18]. Accumulating evidence indicated that MIF is overexpressed in many neoplasms such as brain[19], colon[20] and pancreas[21]. Moreover, relative literatures showed that MIF upregulation correlates with the aggressive and metastatic potential of diverse cancers[22]. MIF knockdown reduces disease severity, malignant aggressiveness, angiogenesis, cell proliferation, anti-tumor immunity, but induces apoptosis[23]. MIF exerts a tumorigenic role in oncogenic transformation and malignant progression.

Patients and Methods

Patients

All human LA tissues and their corresponding adjacent lung samples were obtained from 52 patients who underwent lung resection surgery in our hospital from March 2016 to January 2018. LA tissues were pathologically confirmed, and all specimens were stored at -80°C for further analysis. Written informed consent was obtained from all of the participants prior to tumor sample collection. The study was approved by the Ethics Committee of The Affiliated Yantai Yuhuangding Hospital of Qingdao University.

Cell Lines

The lung adenocarcinoma A549 cells and the normal lung epithelial cells BEAS-2B were purchased from the Cell Bank of China Academy of Sciences (Shanghai, China). A549 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Inc. Waltham, MA, USA) and 100 U/mL penicillin and 100 μg/mL streptomycin (Thermo Fisher Scientific, Inc. Waltham, MA, USA) in a 37°C humidified incubator containing 5% CO_2.

Cell Transfection

MiR-608 mimics, miR-608 inhibitor, MIF siRNA and the corresponding controls were purchased from Gene Pharma (Shanghai, China). The plasmid was transfected into LA cell line A549 by Lipofectamine® 2000 (Thermo Fisher Scientific, Inc. Waltham, MA, USA) in strict accordance with the manufacturer's instructions.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

To quantitatively confirm the expression levels of miR-608 and MIF in LA tissues and cells, qRT-PCR was performed. Firstly, TRIzol reagent (Thermo Fisher Scientific, Inc. Waltham, MA, USA) was used to isolate the total RNA from the LA tissues and cells according to the manufacturer's protocol. Reverse transcription was carried out using PrimeScript™ RT reagent kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). Subsequently, complementary Deoxyribose Nucleic Acid (cDNA) amplification was performed using the SYBR Green Master Mix kit (TaKaRa, Dalian, China) on the system of ABI 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene and U6 were used as endogenous controls. The relative expression levels of genes were calculated by the $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study were as follows: MIF, F: 5'-GAACCGCTCCTACAGCAAGCT-3', R: 5'-GCGAAGGTGGAGTTGTTCCA-3'; microRNA-608, F: 5'-AGGGGTGGTGTTGGGACAGCTCCGT-3', R: 5'-ACGGAGCTGTCCCAACACCACCCCT-3'.

Western Blot

Cells were lysed in ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer containing protease inhibitor cocktail (Roche, Basel, Switzerland). Protein concentration was determined using a bicinchoninic acid (BCA) Kit (Pierce, Rockford, IL, USA). After being separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), protein sample was electro transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) in transfer buffer. The membrane was blocked in 5% non-fat milk for 2 h and

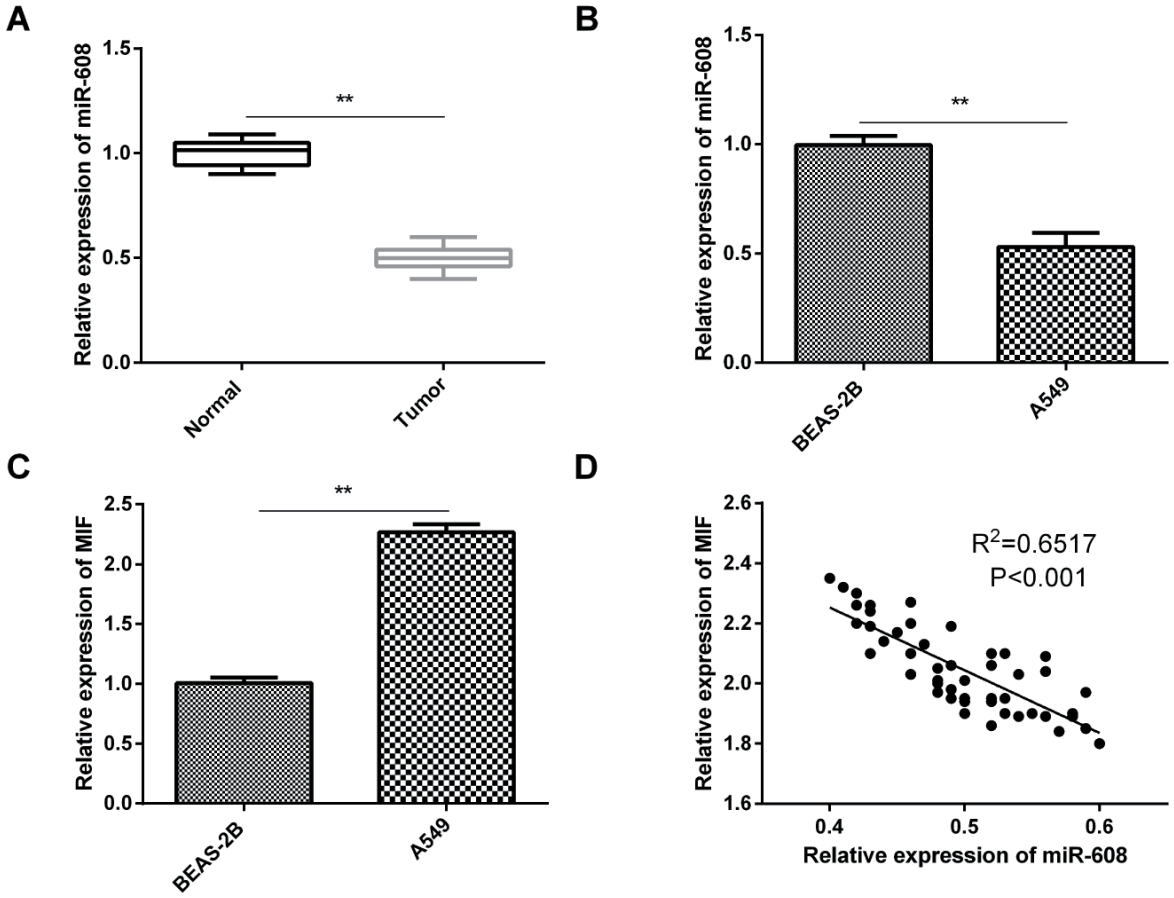

Figure 1. Analysis of miR-608 and MIF expressions in LA tissue samples and cells using qRT-PCR. ***A-B,*** The relative expressions of miR-608 in LA tissues and cells ($^{**}p<0.01$). ***C,*** Expressions of MIF in LA cells ($^{**}p<0.01$). ***D,*** Regression analysis of correlation between miR-608 and MIF expressions.

incubated overnight at 4°C with anti-MIF rabbit antibody (1:4000, ab175189, Abcam, Cambridge, MA, USA). Subsequently, the membrane was washed with Tris-buffered saline and Tween-20 (TBST-20) buffer (Beyotime, Shanghai, China) and incubated for 1 h at room temperature with secondary goat anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (1:3000, ab191866; Abcam, Cambridge, MA, USA). Rabbit anti-human GAPDH antibody (1:2000; ab128915; Abcam, Cambridge, MA, USA) was used as an internal control. The Western blotting results were analyzed using the enhanced chemiluminescence system (Pierce, Rockford, IL, USA).

Transwell Assays

Transwell assays were carried out to determine the invasion and migration capacities of transfected LA cells. For invasion analysis, the transwell chambers (Corning, Corning, NY, USA) were treated with Matrigel (Clontech, Mountain View, CA, USA), which was used as the extracellular matrix. Transfected LA cells were added into the upper transwell chamber and cultivated for 48 h in serum-free medium. The lower chamber was supplied with medium containing 10% fetal bovine serum (FBS). After cell incubation, we removed the nonadherent cells on the upper surface with cotton swab carefully. Subsequently, adherent cells in the lower surface were fixed and stained. Migration assay was performed as the same as the invasion assay except for pre-coating matrigel. The invasion or migration results were observed using an inverted microscope (Olympus, Tokyo, Japan) in five randomly selected fields.

Luciferase Reporter Assay

Reporter vector containing MIF-3'-UTR-WT or corresponding MIF-3'-UTR-MUT was transfected into LA cells along with miR-608 mimics

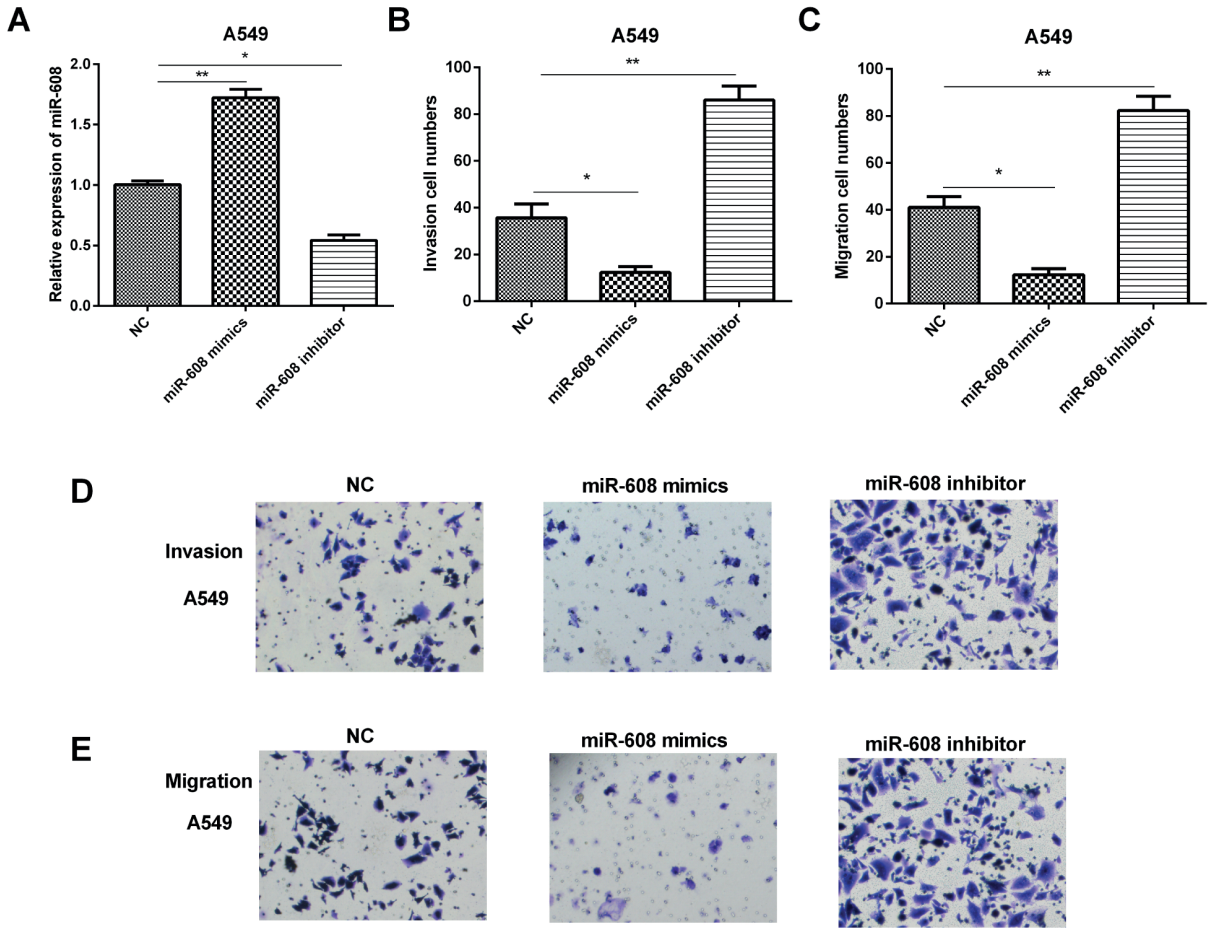

Figure 2. MiR-608 inhibited cell invasion and migration in LA cells. ***A,*** The miR-608 expressions in transfected LA cell lines were evaluated using qRT-PCR ($^{*}p<0.05$, $^{**}p<0.01$). ***B-C***, The invasion or migration cell numbers of LA cells were counted ($^{*}p<0.05$, $^{**}p<0.01$). ***D-E***, Transwell assays were carried out to detect cell invasion and migration in transfected LA cells.

by the Lipofectamine 2000 (Thermo Fisher Scientific, Inc. Waltham, MA, USA) according to the manufacturer's manual. 48 h later, the Dual Luciferase Reporter Assay kit (Promega, Madison, WI, USA) was used to detect the relative luciferase activities 48 hours after the transfection.

Statistical Analysis

All above experiments were performed for 3 times. The statistical analysis was evaluated by the GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) and statistical product and service solutions (SPSS) 18.0 version (SPSS Inc. Chicago, IL, USA). Student's *t*-test was applied to evaluate the differences between two groups. The data was indicated as means ± SD. The differences were identified as statistically significant when $p<0.05$.

Results

Down-Regulation of miR-608 Expression and Up-Regulation of MIF Expression in LA

Firstly, we performed qRT-PCR to assess the miR-608 expressions in LA tissues. The results indicated that miR-608 expressions were significantly reduced in LA tissues compared with those of adjacent normal tissues (Figure 1A). Besides, compared to the normal lung epithelial cells, the miR-608 expression in LA cells was found to be significantly upregulated (Figure 1B). Subsequently, we examined the MIF expression in LA cells and the results demonstrated a significant increase in LA cells in contrast with the normal lung epithelial cells (Figure 1C). Moreover, we investigated the correlation between miR-608

expression and MIF expression in LA. MiR-608 expression was found to be negatively related to the MIF expression in LA (Figure 1D).

Effects of miR-608 on LA Cell Invasion and Migration

To further explore the effects of miR-608 on LA progression, miR-608 mimics or inhibitor was transfected into A549 cells for miR-608 overexpression or downregulation. Transfection efficacies of miR-608 mimics and miR-608 inhibitor were shown in Figure 2A. Then, we investigated the functions of miR-608 in LA cell invasion by performing transwell assays. Results demonstrated that miR-608 mimics could suppress cell invasion while miR-608 inhibitor markedly promoted the invasion ability of LA cells (Figure 2B and 2D). We continued to study the functions of miR-608 in LA cell migration. Similarly, transwell assays indicated that miR-608 mimics inhibited cell migration and miR-608 inhibitor markedly enhanced the migration ability of LA cells (Figure 2C and 2E).

MiR-608 Overexpression Downregulated the MIF Expression by Biding to its 3'-UTR in LA

To further determine the tumor-suppressive mechanisms of miR-608, TargetScan was searched and MIF was confirmed to be a potential target for miR-608. To verify whether MIF was directly targeted by miR-608, the wild-type or mutant-type MIF 3'-UTR was cloned into a luciferase reporter gene to generate MIF 3'-UTR-WT and MIF 3'-UTR-MUT (Figure 3A). Sub-

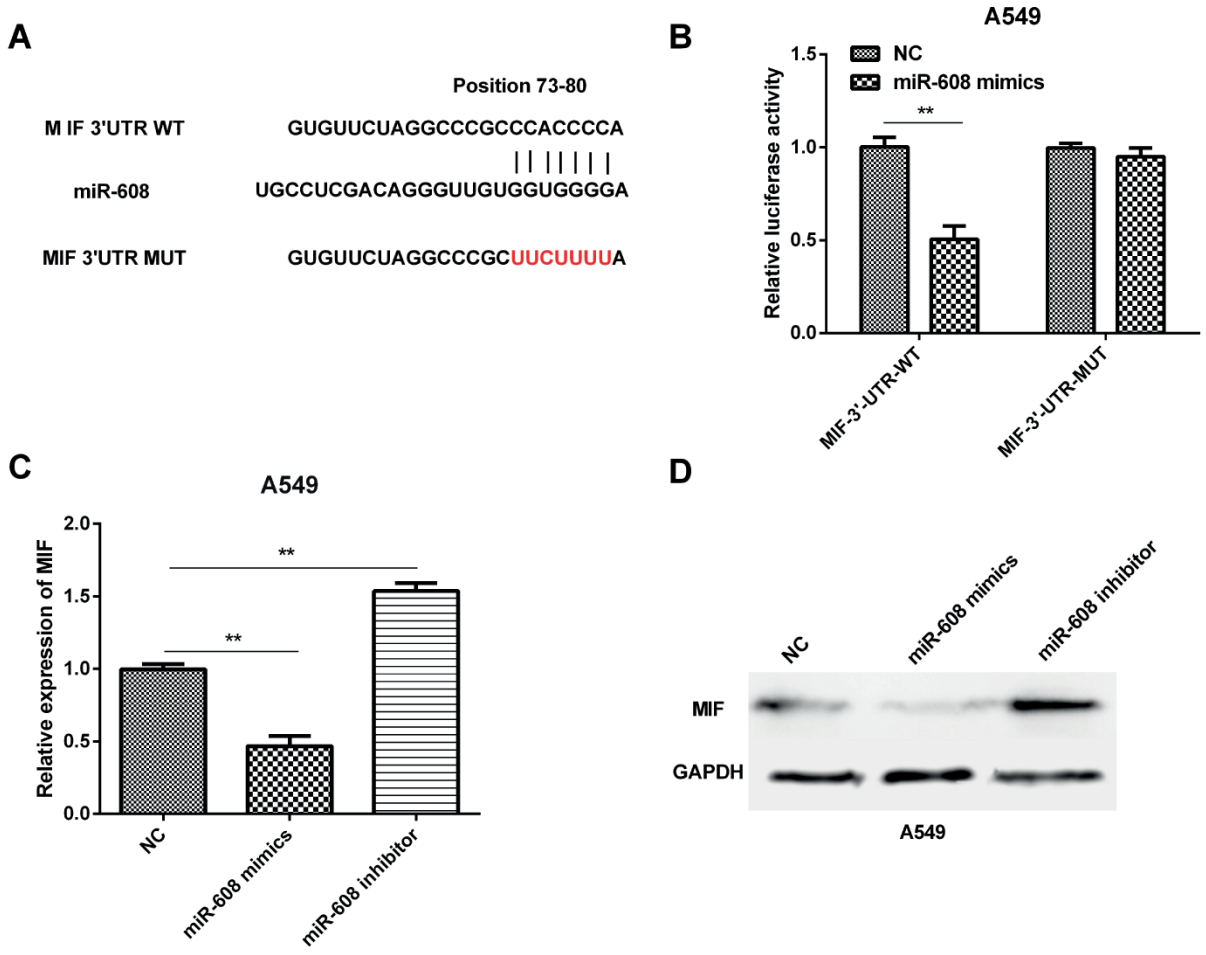

Figure 3. MiR-608 de-regulated MIF expression via binding to the MIF 3'-UTR directly. ***A,*** The binding sequences of miR-608 in the MIF 3'-UTR. ***B,*** The luciferase reporter gene assays were carried out to measure the fluorescence activities of the MIF 3'UTR in LA cells co-transfected with MIF-3'UTR-WT or MIF-3'UTR-MUT and miR-608 mimics, respectively ($^{**}p<0.01$). ***C,*** QRT-PCR results showed the mRNA level of MIF in LA cells with different transfections ($^{**}p<0.01$). ***D,*** Western blots results showed the protein level of MIF in LA cells with different transfections.

sequently, the luciferase reporter assays were carried out by co-transfecting the luciferase vector and miR-608 mimics into A549 cells. The luciferase activities of each group were quantified. Results revealed that the MIF 3'-UTR-WT luciferase activity was significantly declined in contrast with the control group. However, there was no remarkable difference between the MIF 3'-UTR-MUT luciferase activity and the corresponding control (Figure 3B). Subsequently, we investigated whether miR-608 could modulate the mRNA and protein expressions of MIF. Both qRT-PCR and Western blot analysis revealed that MF expression was negatively regulated by miR-608 (Figure 3C and 3D). The data suggested that miR-608 repressed MIF expressions at both mRNA level and protein level through binding to its 3'-UTR directly.

MIF Was Implicated in the Regulation of miR-608 on LA Invasion and Migration

To determine whether MIF downregulation was associated with the inhibited invasion and migration of LA cells regulated by miR-608, MIF siRNA was first constructed. Briefly, miR-608 inhibitor and MIF siRNA were co-transfected into A549 cells and the transfection efficiencies were evaluated by qRT-PCR and Western blots analysis. Results revealed that both mRNA and protein expression levels of MIF in A549 cells co-transfected with miR-608 inhibitor and MIF siRNA were significantly lower than those only transfected with miR-608 inhibitor (Figure 4A and 4B). Subsequently, transwell assays were performed to determine whether MIF downregulation was related to invasion and migration of LA cells. Data showed that knockdown of MIF significantly reversed the effects of miR-608 on cell migration and invasion capacities in LA cells (Figure 4C and 4D). These findings indicated that the anti-tumor effect of miR-608 is partly dependent on the regulation of target gene MIF.

Discussion

The incidence and mortality of lung cancer have steadily increased worldwide in the past several decades. Lung cancer is a fatal disease that is tightly associated with cigarette smoking. Apart from other histological subtypes, LA is the most common histological subtype among non-smokers[24]. Despite recent advances in the treatment of LA including surgical resection, chemotherapy, radiation therapy or a combination of targeted therapy, the survival and prognosis of advanced LA patients remain unsatisfactory with 5-year survival rate less than 20%[25]. Therefore, identification of new prognostic biomarkers related to LA tumorigenesis and progression is urgently needed. MiRNAs have been constantly determined to play a crucial role in carcinogenesis, cell invasion and metastasis[26].

MiR-608 has been reported to be a prognostic factor for cancer patients in different cancer types, such as colorectal cancer[27], nasopharyngeal cancer[28] and breast cancer[29], but its role in LA still needs to be fully elaborated. In our study, we found that miR-608 expression was notably decreased in LA. MiR-608 overexpression was confirmed to inhibit invasion and migration of LA cells by downregulating MIF expression. The data suggested that miR-608 played a suppressive role in LA. Our results are consistent with published reports of decreased expression of miR-608 and its suppressive function in tumor progression. For instance, Wang et al[30] found that miR-608 inhibited the migration and invasion of glioma stem cells by targeting macrophage migration inhibitory factor; Yang et al[31] reported that miR-608 inhibited colon cancer tumorigenesis by targeting NAA10.

Recently, the possible role of MIF in the treatment of various cancers has been well studied. MIF/CD74 axis was a target for novel therapies in colon carcinomatosis[32]. MIF was a diagnostic marker for HCV/HBV induced hepatocellular carcinoma[33]. Basement membrane protein ladinin-1 and the MIF-CD44-beta1 integrin-signaling axis are implicated in laryngeal cancer metastasis[34]. Here, we identified MIF as a direct target of miR-608 in LA. We also found that knockdown of MIF reversed the effects of miR-608 inhibitor on cell invasion and migration in LA cells. These results suggested that miR-608 exerts tumor suppressor roles in LA by targeting MIF.

Conclusions

We found that miR-608 was decreased in LA. We also showed that the downregulated miR-608 expression was inversely related to the MIF expression in LA. MiR-608 overexpression could suppress invasion and migration of LA by

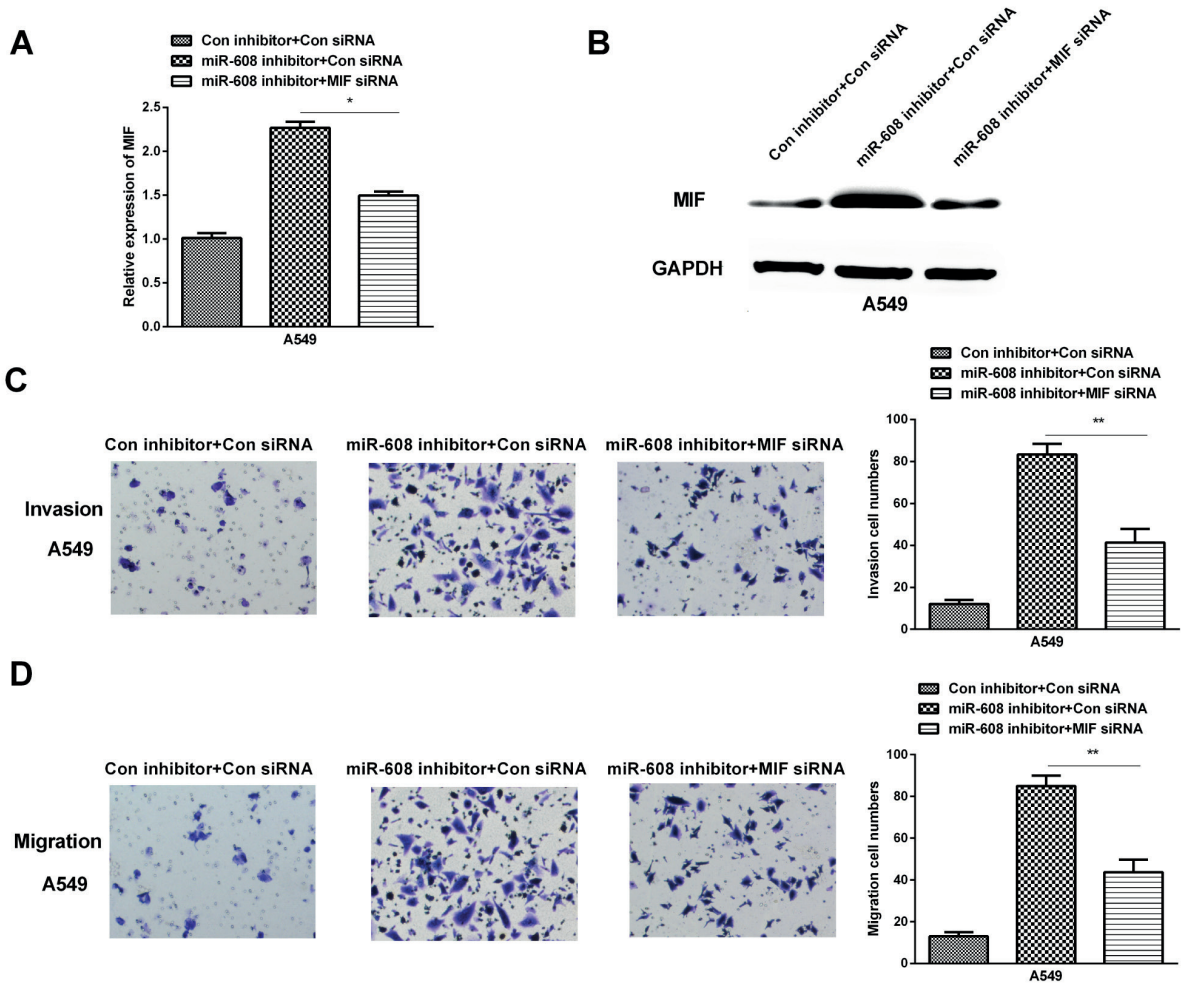

Figure 4. Knockdown of MIF abrogated the function mediated by miR-608 inhibitor in invasion and migration in LA cells. ***A-B***, The mRNA or protein expression levels of MIF were measured using qRT-PCR or Western blots in LA cells co-transfected with miR-608 inhibitor and MIF siRNA ($^{*}p<0.05$). ***C-D***, Transwell assays were conducted to observe invasion and migration capacity in LA cells co-transfected with MIF siRNA and miR-608 inhibitor ($^{**}p<0.01$).

downregulating MIF expression. These findings provide new insight into the underlying mechanisms of LA progression and highlight miR-608 as a potential prognostic biomarker and therapeutic target of LA.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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