Original Paper

MiR-424 Promotes Non-Small Cell Lung Cancer Progression and Metastasis through Regulating the Tumor Suppressor Gene TNFAIP1

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Key Words

Non-small cell lung cancer (NSCLC) • miR-424 • TNFAIP1 • Cell migration and invasion

Abstract

Background/Aims: This study aimed to investigate the potential roles of miR-424 expression in non-small cell lung cancer (NSCLC) metastasis and growth and its underlying mechanism. **Methods:** The expression of miR-424 in two NSCLC cell lines (A549 and H1975) was altered by transfection with miR-424 mimic and inhibitor. Effects of miR-424 overexpression and suppression on cell migration, invasion and colony formation were analyzed. Target genes for miR-424 were predicted using bioinformatics method and then verified using luciferase assay. Effects of miR-424 expression on cell migration, invasion and proliferation were reanalyzed on the condition of TNFAIP1 was silenced. Moreover, TNFAIP1 silencing and miR-424 modified A549 cells were subcutaneous injected into nude BALB/c mice to confirm the regulation of miR-424 on TNFAIP1 in regulating tumor growth. **Results:** Compared with the control, miR-424 overexpression significantly increased the migrated and invaded cells, as well as the proliferation colonies. TNFAIP1 was a predicted target gene for miR-424, and was negatively regulated by miR-424. TNFAIP1 silence significantly increased the migrated and invaded cells compared to that in control, while these increases were abolished by miR-424 suppression. Animal experiment further evidenced miR-424 affected tumor growth by regulating TNFAIP1. **Conclusions:** These data demonstrate that miR-424 may be a contributor for NSCLC progression and metastasis through involving in cell migration, invasion and proliferation via inhibiting TNFAIP1. This study may provide theoretical basis for miR-424 in NSCLC target therapeutic treatment.

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Introduction

Lung cancer is a worldwide common malignancy which arises from lung tissue and has become one of the most common factor leading to human deaths [1]. Statistics show that morbidity for lung cancer is high and the 5-year survival rate for lung cancer is approximately 14% [2]. Non-small cell lung cancer (NSCLC) and small cell lung cancer are the two types for lung cancer based on the histological characteristics, in which NSCLC accounts for about 80% of lung cancer [3]. Although many treatment methods including immune or medication therapy, surgery and genetic therapy play crucial roles in NSCLC treatment [4–5], the results still remain unsatisfactory due to the unknown complicate pathogenesis of NSCLC. Therefore, to explore several useful target genes for NSCLC treatment will be necessary.

microRNAs (miRNAs) are endogenous 20–22 nt in length and are highly conserved non-coding RNAs that function in varieties of biological processes at the transcriptional or post-transcriptional level by targeting the 3'UTR of genes [6]. Accordingly, increasing evidence has demonstrated that various miRNAs are involved in the progression and biological of NSCLC [7–9]. For example, miR-21 promotes NSCLC invasion and chemo-sensitivity through targeting SMAD7 [10]. miR-509-5p decreases NSCLC cells proliferation, migration and invasive capability by attenuating FOXM1 [11]. Dividing miRNAs has become the biomarkers in lung cancer diagnosis, which is benefit from the easy detection in serum [12, 13]. In recent studies, miR-424 has been underlined in several kinds of tumors progression and development including bladder cancer and ovarian cancer [14, 15]. Additionally, Donnem et al. refers that several miRNAs including miR-424 are correlated significantly with NSCLC [16]. But although several studies have investigated the significance of miR-424 in NSCLC, few have reported the mechanism of miR-424 in NSCLC development and progression.

Tumor necrosis factor, alpha-induced protein 1 (TNFAIP1) expression can be induced by the tumor necrosis factor alpha in umbilical vein endothelial cells [17]. Pivotal tumor suppressor roles of TNFAIP1 have been implicated in variety of diseases, including anorexia nervosa, and paclitaxel resistance [18, 19]. In addition, roles of TNFAIP1 mutation in tumor progression and development have been demonstrated, such as in breast cancer [20]. Also, Zhou et al. reports that miR-31 functions as an oncogene in gastric carcinoma via targeting TNFAIP1 [21]. Similarly, Cai et al. demonstrates that miR-224 promotes NSCLC tumor progression through targeting TNFAIP1 and Smad4 [22].

In this study, we analyzed the potential effects of miR-424 expression on NSCLC cells proliferation and metastasis in A549 and H1975 cells, and siRNA-mediated gene silencing. Further deep experimental methods were used to assess the effects of miR-424 suppression on NSCLC cell invasion, migration and proliferation through targeting TNFAIP1. This study aimed to investigate the possible roles of miR-424 in NSCLC metastasis and to illustrate its potential mechanism.

Material and Methods

Cell Culture

Human lung adenocarcinoma cell lines A549 and H1975 were both purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and were cultured in RPMI-1640 media (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL), 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma-Aldrich, St. Louis, MO) and 1% L-glutamine (Invitrogen, Gaithersburg, MD) at 37°C with 5% CO₂.

Treatment with hsa-miR-424 mimic or inhibitor

Cells were planted on 60-mm dish and after 24 h of incubation, cells were treated with Lipofectamine® RNAiMAX Transfection Reagent (3 µL per 1 mL media, Life Technologies, Gaithersburg, MD) and mimic or inhibitor in Opti-MEM® I Reduced Serum Medium (Life Technologies) [22]. After 48 h transfection, the cells were prepared for further analysis.

MTT assay

Cells were planted onto 96-well plates at 5×10^3 cells/well in 0.1 mL of culture medium. After 24 h, cells were transfected with mimic or inhibitor of miR-424 or control for another 48 h. Following treatment, 20 μ L of MTT solution (0.5 mg/mL; Sigma-Aldrich) were added to each well and incubated for 4 h at 37°C. Then, 150 μ L dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added and incubated for 4 h at room temperature. The OD was measured using a Synergy plate reader (Biotech, USA).

Cell migration and invasion

Cell migration and invasion were evaluated using Transwell migration chambers (8- μ m pore size; Corning, Cambridge, MA). The membranes for the invasion assay were coated with a diluted ECM solution (Sigma-Aldrich). Cells at a density of 5×10^4 cells/well were seeded in the upper portion of a chamber with serum-free medium after transfection. Medium contained 10% FBS served as a chemoattractant in the lower chamber. After 48 h of incubation at 37°C, non-migrated and non-invasive cells on the top of the membrane were scraped and removed by cotton swabs, and the migrated and invasive cells were stained with Diff-Quik staining (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and then counted using light microscopy. The migration and invasion assay was repeated 3 times with duplicate wells.

Colony formation assay

Cells were seeded onto 6 well-plates at a density of 1×10^3 cells per well. Subsequently, A549 cells were treated and allowed to grow for 10 days until visible clones appeared. Colony formation was stained by 0.1% crystal violet solution (Sigma-Aldrich), air dried, and counted.

siRNA transfection

Cells were planted on 60-mm dish. After 24 h of incubation, cells were treated with Lipofectamine® RNAiMAX Transfection Reagent (3 μ L per 1 mL medium; Life Technologies) and siRNAs (100 nM) in Opti-MEM® I Reduced Serum Medium (Life Technologies). After 48 h of transfection, the cells were prepared for further gene expression. To investigate the effect of TNFAIP1 siRNA treatment on cell proliferation, migration and invasion, the cell lysates were collected from each dish.

qRT-PCR

Total mRNA was isolated from cells with Trizol (Invitrogen) according to the manufacturer's protocol. The isolated RNA was treated with RNase-free DNase I (Promega Biotech, Madison, WI) to remove the mixed DNA. Consequently, concentration and purity of isolated RNA were measured with SMA 400 UV-VIS (Merinton, Shanghai, China). RNA was used for cDNA synthesis with the PrimerScript 1st Strand cDNA Synthesis Kit (Takara, Dalian, China). Expressions of targets were detected in an Eppendorf Mastercycler (Brinkman Instrument, Greiner, Frick, Germany, NY) using the SYBR ExScript RT-qPCR Kit (Takara, Dalian, China). The total reaction system of 20 μ L volume was as follows: 1 μ L cDNA from the above PCR, 10 μ L SYBR Premix EX Taq, 1 μ L each of primers (10 μ M), and 7 μ L ddH₂O. The PCR program was as follows: denaturation at 50°C for 10 min; 95°C for 10 min; followed by 45 cycles of 95°C for 10 s, and 60°C for 1 min. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one product was amplified and detected. Phosphoglycerate dehydrogenase (GAPDH) was chosen as the internal control. Primers used for targets amplification were as follows: miR-424 sense: 5'-AGC AGC AAT TCA TGT GAA G-3', antisense: 5'-GCA GCG CCT CAC GTT TT-3', and TNFAIP1 sense: 5'-ATA GAC ACG GAT TAC CCA GC-3', antisense: 5'-CAG GCT TTG CTC AAT GC-3'.

Western blotting

A total of 50 μ g protein (per lane) were separated onto a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and blotted onto polyvinylidene fluoride (PVDF) membranes. The membrane was blocked in PBST (0.1% Tween in PBS) and probed with primary antibodies (1:1,000 for TNFAIP1 and 1:5,000 for GAPDH, Sigma-Aldrich) at 4°C overnight. The membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (1:1,000 dilution). The immunoreactive protein bands were developed by enhanced chemiluminescence. The immunoreactive bands were analyzed by a densitometer.

Luciferase assay

Cells in 24-well plates were transfected with TNFAIP1-luc (SABiosciences) along with various expression plasmids, using the Lipofectamine transfection reagent (Invitrogen). Transfected cells were lysed, and the luciferase activities in the cell lysates were analyzed using a Luciferase Reporter assay kit (Promega). Luciferase activity was measured as relative light units using a luminometer (Turner Design Sunnyvale, CA).

Tumor growth in nude mice

This experiment was approved by the Animal Care Committee of Tumor Hospital of Yunnan Province. Twenty nude BALB/c mice aging 4 to 6 weeks (obtained from the Animal center of Shanghai Laboratory, Shanghai, China) were subcutaneous injected with the tumor cells (5 repeat in each group). Each subcutaneous injection consisted of 1×10^5 A549 cells, and cells were transfected with different kinds of vectors, namely, control group (no vector), si-TNFAIP1 group (cells transfected with silenced TNFAIP1 vector), si-TNFAIP1+mimic group (cells transfected with silenced TNFAIP1 vector and miR-424 mimic), and si-TNFAIP1+inhibitor group (cells transfected with silenced TNFAIP1 vector and miR-424 inhibitor). The injected mice were kept in a specific pathogen-free environment and the day when tumor was firstly grossly visible was recorded, and the tumor size was measured and recorded every 2 weeks after. When tumor was grown at about 3 months, the tumor was taken out and measured for the comparison of tumor size. Tumor volume was calculated as $\text{length} \times \text{width}^2 / 2$, where the length and width are the longest and shortest axes in millimeters [23].

Statistical analysis

All experiments were repeated three times. The results of multiple experiments are presented as the mean $\pm$ SD. Data were analyzed by SPSS 19.0 statistical software (SPSS Inc., Chicago, IL, USA) using Student's *t* test or one-way analysis of variance (ANOVA) as appropriate. $P < 0.05$ was considered to indicate a statistically significant result.

Results

miR-424 expression promotes A549 cells migration and invasion

Compared to the controls, the migrated cells were significantly increased when cells were transfected with miR-424 mimic ($P < 0.05$), but the migrated cells in inhibitor group were approximately to control group and there was no significant difference between two groups (Fig. 1A and B). Similarly, miR-424 mimic transfection significantly increased invasive A549 cells compared to control and in inhibitor group ($P < 0.05$, Fig. 1C and D).

Fig. 1. Influence of miR-424 expression on A549 cells migration and invasion. A: Transwell assay showed that the migrated cells were drastically increased when cells were transfected with miR-424 than that in control, however, when cells were transfected with miR-424 inhibitor, there was no significant difference of migrated cells between control and Inhibitor group; C-D: miR-424 overexpression significantly enhanced A549 cells invasion compared to the control, while this promoting effect was decreased in cells transfected with miR-424 inhibitor. *: $P < 0.05$ compared to the control.

Fig. 2. Influence of miR-424 expression on A549 cells viability and colony formation. A: the relative expression of miR-424 in cells transfected with miR-424 mimic or inhibitor; B: A549 cells viability was significantly increased when cells were transfected with miR-424; C-D: when cells were transfected with miR-424, the colonies were more than that in control, but colony number in Inhibitor group was almost similar to the control group. *: $P < 0.05$; **: $P < 0.01$ compared to the control.

miR-424 promotes A549 cells proliferation and colony formation

When A549 cells were transfected with miR-424 mimic, the expression level of miR-424 was assessed, and the results revealed that miR-424 was highly expressed while it was lowly expressed by the inhibitor transfection ($P < 0.05$, Fig. 2A). The cell viability was significantly increased compared to the control group ($P < 0.05$, Fig. 2B), indicating miR-424 overexpression increased A549 cells viability. Colony formation assay was conducted to further test the effects of miR-424 expression on A549 cells proliferation. Overexpression of miR-424 significantly increased the colonies compared to the control group ($P < 0.05$, Fig. 2C and 2D).

miR-424 regulates the target gene *TNFAIP1* expression in A549 cells

By bioinformatics analysis we found that *TNFAIP1* was the target gene for miR-424 and there were two regulatory sites on *TNFAIP1* 3'-UTR (Fig. 3A). Luciferase assay was conducted to further verify whether *TNFAIP1* gene was the target for miR-424. When miR-424 and target gene promoter sequence were co-transfected into A549 cells, the relative luciferase activity and expression level of *TNFAIP1* were both significantly decreased compared to the control group ($P < 0.05$). In contrast, when cells were added with miR-424 inhibitor, the two indexes including relative luciferase activity and expression level were significantly increased compared with the control group ($P < 0.05$ or $P < 0.01$) (Fig. 3B and 3C), indicating that miR-424 could negatively regulate *TNFAIP1* expression in A549 cells.

miR-424 promotes cell migration and invasion in *TNFAIP1* silenced A549 cells

The target gene *TNFAIP1* was silenced in A549 cells to further investigate the effects of miR-424 on A549 cells migration and proliferation (Fig. 4A-4C). When target gene *TNFAIP1* was silenced in A549 cells, the migrated and invasive cells, as well as the colony number were all significantly increased compared to the control ($P < 0.05$), indicating *TNFAIP1* was the tumor suppressor for lung cancer. Accordingly, when *TNFAIP1* silenced A549 cells

Fig. 3. Prediction of target gene for miR-424 in A549 cells A: bioinformatics analysis showed that there were two regulatory sites on TNFAIP1 3'-UTR for miR-424; B: Luciferase assay performed that TNFAIP1 was negatively regulated by miR-424 in A549 cells, but this negative regulation was reversed by miR-424 inhibitor; C: qRT-PCR analysis and western blotting showed that TNFAIP1 level was down-regulated by miR-424. *: $P < 0.05$; **: $P < 0.01$ compared to the control.

3. 4. Influence of miR-424 on cell proliferation, migration and invasion when TNFAIP1 was silenced in A549 cells. A-C: TNFAIP1 silence significantly increased the number of migrated and invasive cells, as well as the number of colony for A549 cells. On the other side, the number of migrated cells, invasive cells and colony for A549 cells were significantly increased with miR-424 mimic addition compared to the control, however, the three kinds of cells were all declined approximately to that in control when cells were treated with miR-424 inhibitor. *: $P < 0.05$ compared to the control.

were transfected with miR-424 mimic, cell migration, invasion and proliferation abilities were all highly increased compared to the control ($P < 0.05$), implying miR-424 plays some contribute roles in A549 cells migration, invasion and proliferation. Besides, miR-

Fig. 5. Influence of miR-424 expression on H1975 cells migration and invasion. A-B: Transwell assay showed that the migrated and invasive cells number were both increased when cells were treated with miR-424 mimic than that in control, however, they were both reduced when cells were treated with miR-424 inhibitor. *: $P < 0.05$ compared to the control.

Fig. 6. Influence of miR-424 on cell migration and invasion when TNFAIP1 was silenced in H1975 cells. A: Luciferase assay confirmed that TNFAIP1 was a target of miR-424. B: qRT-PCR analysis and western blotting showed that TNFAIP1 level was negatively regulated by miR-424. C-D: TNFAIP1 silence significantly increased the number of migrated and invasive cells for H1975 cells. The number of migrated cells and invasive cells for H1975 cells were significantly increased with miR-424 mimic addition compared to the control, however, they were both reduced approximately to that in control when cells were treated with miR-424 inhibitor. *: $P < 0.05$; **: $P < 0.01$ compared to the control.

424 inhibitor application was in reverse conducted to further assess miR-424 expression on A549 cells migration and invasion. Data showed that there was no significant difference when compared to the control group.

miR-424 promotes cell migration and invasion in TNFAIP1 silenced H1975 cells

To investigate whether the migration- and invasion-promoting effects of miR-424 are not limited to A549 cells, another human lung adenocarcinoma cell line H1975 was used. As results shown in Fig. 5A and 5B, the number of migrated cells and invasive cells were both

Fig. 7. Effects of miR-424 expression on the tumor formation in rat. A-C: The size of tumor in mice injected with TNFAIP1 silencing-A549 cells was larger than that in control group, while it was even larger in mice injected with TNFAIP1 silencing- and miR-424 overexpressing-cells compared to the control group. However, the tumor size in mice injected with TNFAIP1 silencing- and miR-424 suppressing-cells was almost the same size as that in the control. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ compared to the control.

increased after miR-424 mimic transfection ($P < 0.05$), this was consistent with the findings from A549 cells. However, unlike with A549 cells, migrated and invasive cells number were reduced after miR-424 inhibitor transfection ($P < 0.05$).

Further, we asked if *TNFAIP1* also involved in miR-424-mediated H1975 cells. As results shown in Fig. 6A and 6B, in H1975 cells, *TNFAIP1* was the target for miR-424 ($P < 0.05$) and it was negatively regulated by miR-424 ($P < 0.05$, $P < 0.01$). Next, we knocked down *TNFAIP1* expression in H1975 cells by transfection with *TNFAIP1* targeted siRNA, and we found that *TNFAIP1* silence improved cell migration and invasion ($P < 0.05$, Fig. 6C and 6D). Cell migration and invasion abilities were significantly increased when cells were co-transfected with *TNFAIP1* siRNA and miR-424 mimic ($P < 0.01$). However, miR-424 suppression abolished *TNFAIP1* silence-induced increase in cell migration and invasion, as no significant difference was found between *TNFAIP1*+inhibitor group and control group.

miR-424 promotes tumor growth by regulating *TNFAIP1* in rat

To further verify the correlation between miR-424 and *TNFAIP1* on tumor cell growth, we therefore analyzed tumor size in mice by subcutaneous injection with miR-424 and *TNFAIP1* silencing-A549 cells (Fig. 7A-7C). The results revealed that tumor size in mice injected with *TNFAIP1* silencing-cells was larger than that in control mice ($P < 0.05$ or $P < 0.01$), while the tumor in mice injected with *TNFAIP1* silencing- and miR-424 overexpressing-cells was even more larger in comparison with the control group ($P < 0.001$). However, when mouse was injected with the *TNFAIP1* silencing- and miR-424 suppressing-cells, the tumor size was approximately to that in control. These results revealed that miR-424 could regulate tumor growth by targeting *TNFAIP1* at another angle.

Discussion

Increasing evidence has demonstrated that miRNAs play crucial roles in NSCLC biology, and miR-424 is up-regulated in NSCLC development [12, 16, 24]. To date, miR-424 alterations have been reported to be involved in various diseases including cancer, pulmonary arterial hypertension, and acute myeloid leukemia [16, 25, 26]. In this study, we investigated the role of miR-424 expression in NSCLC metastasis and progression. Our results showed that overexpression of miR-424 significantly increased the cell proliferation, migration and invasion, but these effects were reversed by miR-424 inhibitor application. In addition, *TNFAIP1* was the target gene for miR-424 in NSCLC progression, and was negatively regulated by miR-424. Moreover, when *TNFAIP1* was silenced, the migrated and invasive

cells were drastically increased by miR-424 overexpression.

Abundant evidences have demonstrated that cell migration and invasion are the major factors involving in tumor metastasis, as well as cell proliferation in tumor development and progression [27, 28]. Nakashima et al. demonstrated that down-regulation of miR-424 played pivotal roles in senile hemangioma therapy via promoting angiogenesis through cyclin E1 and MEK1 [29]. Donnem et al. proved that miR-424 was up-regulated in NSCLC tissue [16]. Few reports have mentioned the effects of miR-424 expression in lung cancer metastasis. However, miR-424 played crucial roles in osteosarcoma cells migration and invasion through targeting fatty acid synthase [30]. Besides, miR-424 is an contributor for prostate cancer metastasis via involving in biological including migration and invasion [31]. Our study showed that the migrated and invasive lung cancer cells, as well as the proliferation, were significantly increased when cells were transfected with miR-424 overexpression vector, indicating that miR-424 overexpression may closely correlated with NSCLC metastasis and progression.

It is well-known that miRNAs play crucial roles in diseases development through targeting the 3'-UTR of genes. Based on the reviewing work on previous studies, we confirmed that the tumor suppressor gene *TNFAIP1* was the target for miR-424 in NSCLC [22]. Rossi et al. proved that *TNFAIP1* is an ultraconserved gene which is involved in tumor progression [32]. Meanwhile, luciferase assay showed that miR-424 negatively regulated *TNFAIP1* expression in A549 and H1975 cells. Recently, Zhang and his colleagues demonstrated that miR-181a regulated pancreatic cancer growth and migration through interacting with *TNFAIP1* [33], while Zhou et al. proved that *TNFAIP1* can be directly targeted by miR-30 in gastric carcinogenesis [34]. In addition, it has been said that *TNFAIP1* is a target gene for miR-224 in NSCLC invasion and growth [22]. In addition, the *in vitro* animal study also showed the tumor size was larger by the miR-424 mimic and silence *TNFAIP1* transfection than the other groups, suggesting the promoting effect of miR-424 on tumor growth by regulating *TNFAIP1*. Based on our results, we speculated that miR-424 may be a contributor for NSCLC metastasis and growth through inhibiting the expression of tumor suppressor gene *TNFAIP1*.

Furthermore, *TNFAIP1* is a tumor suppressor gene silencing method to assess the negatively regulatory effects of miR-424 on *TNFAIP1*. *TNFAIP1* is a tumor suppressor in lung cancer through involving in cell proliferation and apoptosis [35]. Also, *TNFAIP1* was interacted with miR-181 and then involved in proliferation and migration of pancreatic cancer [33]. In this study, when *TNFAIP1* was silenced, the migrated and invasive cells were significantly increased compared to that in control group, suggesting the tumor suppressive role of *TNFAIP1* in lung cancer. Meanwhile, Nakashima and his colleagues proved that down-regulation of miR-424 enhanced the abnormal angiogenesis through cyclin E1 in senile hemangioma [29]. Additionally, *TNFAIP1* can be directly targeted by miR-224 and then participate in NSCLC migration and progression [22]. In agreement with previous data, our data showed that number of migrated and invasive cells were drastically increased when miR-424 was overexpressed, implying the *in vitro* miR-424 application would enhance NSCLC metastasis and growth at the condition of *TNFAIP1* was silenced, which in reverse demonstrated the negatively regulatory effects of miR-424 on *TNFAIP1*, as well as its effects on tumor cells metastasis and growth.

In conclusion, the data presented herein suggest that miR-424 overexpression increases lung cancer progression and metastasis through involving in cell proliferation, migration and invasion by suppressing *TNFAIP1* gene. The mechanisms revealed in our study may loan theoretical basis for the possible application of miR-424 in NSCLC target therapeutic treatment.

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Disclosure Statement

All authors claim no conflict of interest.

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