MicroRNA-370 inhibits the progression of non-small cell lung cancer by downregulating oncogene TRAF4

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Abstract. Lung cancer is the leading cause of cancer-related deaths, of which most can be attributed to non-small cell lung cancer (NSCLC). microRNAs (miRNAs) are a group of small non-coding RNAs that focus on post-transcriptional modification. The present study aimed to investigate the role and function of microRNA-370 (miR-370) in NSCLC and explore the underlying functional mechanisms. We found that miR-370 was significantly downregulated in the tumor tissues of NSCLC patients as well as in NSCLC cell lines. Overexpression of miR-370 by infection of recombinant lentivirus markedly inhibited cell proliferation and promoted cell apoptosis of NSCLC cells. In addition, in vivo tumor formation of NSCLC cells was decreased by miR-370 overexpression. Through bioinformatic analysis, we found that tumor necrosis factor receptor-associated factor 4 (TRAF4), an oncogene as previously reported, was predicted as a putative target gene of miR-370. The direct targeting relationship between miR-370 and the 3’-untranslated region was validated by dual-luciferase reporter assay. Furthermore, overexpression of miR-370 downregulated the protein expression of TRAF4 in the NSCLC cells. Moreover, the growth inhibitory effect of miR-370 overexpression on NSCLC cells was abrogated by TRAF4 overexpression. In conclusion, our results suggest that miR-370 plays an important role in NSCLC by regulating TRAF4 and may be a potential target for the treatment of NSCLC.

Introduction

Lung cancer is the leading cause of cancer-related deaths, and non-small cell lung cancer (NSCLC) accounts for most cases of lung cancer (1). It has been estimated that NSCLC may occupy the leading position for the next decade (2). Obviously, early treatment is extremely important as the 5-year survival rate is ~80% in stage I/II but declines significantly to about 14% in stage III/IV (1). In fact, only 25% of NSCLC patients are treated in early stages and this leads to a high mortality rate (2). In spite of the development in the fields of oncology and surgery, the prognosis of lung cancer remains unsatisfactory, and lung cancer often recurrences within one year (3).

microRNAs (miRNAs) are characterized by endogenous, single-stranded, non-coding RNA consisting of fewer than 22 nucleotides (4). As known, miRNAs play a role in negatively regulating targeted gene translation post-transcriptionally and specifically without changing DNA sequences by pairing with the 3’-untranslated region of the target mRNAs, leading to translation inhibition or mRNA degradation (5,6), consequently controlling cell proliferation and apoptosis, and stem cell differentiation (4,7,8). Moreover, accumulating evidence has shown that miRNAs act as oncogenes or inhibit cancer development and progression according to the roles of their target genes (9). To date, miRNAs are believed to participate in the regulation of the expression of more than half of the human genes (7,10,11) and a considerable number have been found to be deregulated in cancer cells compared with normal cells (12,13). In other words, miRNAs play a key role in regulating tumorigenesis, which is characterized by its aberrant expression (14,15).

There are relevant studies concerning both miRNAs and NSCLC. For example, it has been reported that miR-449a expression is decreased in NSCLC, which could promote cell proliferation and inhibit apoptosis (16). Similarly, clinical data analysis has shown that decreased miR-224 and increased miRNA-96 expression in NSCLC tissues are closely related to clinicopathologic stage and a poor prognosis (17,18). Furthermore, other miRNAs, including miR-30b/c, miR-99a, miR-493 and miR-27b, have all exhibited inhibitory effects on both the proliferation and invasion of NSCLC cells (19-22). These previous studies and results indicate that miRNAs may act as potential therapeutic targets of human NSCLC.

Another cancer-related miRNA is microRNA-370 (miR-370). As reported, Lo et al demonstrated that miR-370 was overexpressed in gastric carcinoma, which indicated a more advanced nodal metastasis and a higher clinical stage (23). Notably, miR-370 demonstrated opposite effects in laryngeal squamous cell carcinoma. The results reported by Yungang et al showed that miR-370 was downregulated in human laryngeal squamous cell carcinoma tissues, suggesting...
that miR-370 may function as a tumor suppressor (24). We focused on lung cancer research and aimed to ascertain whether, how and in which manner miR-370 functions in NSCLC. However, studies on the expression and functions of miR-370 in NSCLC are sparse. Therefore, we compared the expression of miR-370 in NSCLC and normal tissues. Next, with the aid of two cell models, we evaluated the effects of high expression of miR-370 on the proliferation and apoptosis of NSCLC cells. Furthermore, we assessed the tumor formation ability in vivo under conditions of high and low expression of miR-370.

The tumor necrosis factor receptor-associated factor (TRAF) family consists of 7 members (TRAF1-7) (25), which have been demonstrated to participate in human diseases, including tumors and cancer, immune system disorders and neurodiseases (26,27). Therefore, targeting these molecules may contribute to the treatment of TRAF-mediated human diseases. Among the seven TRAF family members, TRAF4 is the first member that has been found to be overexpressed in cancer and is now considered as an oncogene (28,29). TRAF4 has been reported to promote breast cancer progression (30); however, the underlying mechanism of TRAF4 in NSCLC has not been fully investigated or determined.

Based on the present experiments, we confirmed a close connection between TRAF4 and miR-370 in NSCLC cell lines, demonstrating that TRAF4 may be a negative downstream effector of miR-370. In contrast, TRAF4 overexpression had a promotive effect on NSCLC cells. In conclusion, our results showed a growth inhibitory effect of miR-370 on NSCLC and provides an experimental basis for its potential therapeutic use.

Materials and methods

Sample collection and animals. All of the samples were collected from the First Affiliated Hospital of Xi’an Jiaotong University. We obtained informed consents from all of the participants following the approval of the Ethics Committee of Xi’an Jiaotong University. The samples were collected before any other therapeutic methods were employed on the patients. In total, 25 early-stage NSCLC and 23 adjacent non-tumor samples were collected. The samples were immediately stored in liquid nitrogen for further analysis. Female BALB/c mice were purchased from the Animal Experimental Center of the College of Medicine, Xi’an Jiaotong University and raised in a specific pathogen-free environment. Female BALB/c mice were purchased from GenePharma Co., Ltd. (Shanghai, China). Scramble lentivirus vectors served as negative controls. A549 and H358 cells were plated in 6-well plates (5x10⁴ cells/well) in preparation for use. The lentiviruses were diluted in 0.2 ml complete medium containing Polybrene (8 mg/ml) and added to the cells for 12 h of incubation at 37˚C, followed by incubation in 0.3 ml of freshly prepared Polybrene-DMEM for another 24 h, which was replaced with fresh DMEM and the cells were cultured for another 3 days. Subsequently, miR-370 and TRAF4 expression was measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), respectively.

RT-qPCR. Total RNA was isolated from the samples and cells using TRIzol reagent (Invitrogen). The reverse transcription reaction was performed with 5 µg of RNA using the Transcriptor First Strand cDNA Synthesis kit (Roche, Basel, Switzerland) following the manufacturer’s instructions. RT-qPCR was conducted using FastStart Universal Real-Time PCR Master Mix (Roche) on the PikoReal Real-Time PCR Detection system (Thermo Fisher Scientific, Waltham, MA, USA) with cycling conditions of 95˚C for 10 min, followed by 45 cycles of 95˚C for 15 sec and 60˚C for 60 sec. β-actin served as an internal control for TRAF4 and U6 small nuclear RNA (snRNA) for miR-370. The primer sequences were as follows: miR-370 forward, 5'-GTA GCC GAT ATC TTC TGC TGC TAC-3' and reverse, 5'-TAG TAG AAG GTA CCA CCC GAC G-3'; U6 forward, 5'-GTA GAT ACT GCA GTA CG-3' and reverse, 5'-ATC GCA TGT ACC TAC TG-3'; TRAF4 forward, 5'-CGT GAC ATT TGA TCA TGC AG-3' and reverse, 5'-ATT GGA AGT AGT TGC AG-3'; and β-actin forward, 5'-CCA AGT TGC ATT TGA CCT GG-3' and reverse, 5'-GAC CTG CGA GAT GCC TCA CC-3'. The threshold cycle (Ct) value was recorded. Each sample was measured in triplicate, and the relative expression of miR-370 to U6 and TRAF4 to β-actin was calculated using the 2^-ΔΔCt method.

Western blotting. Proteins were extracted using RIPA buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) (both from Sigma-Aldrich, St. Louis, MO, USA). The protein concentration was measured by the BCA protein assay kit (CWBio, Beijing, China). A total of 20 µg of protein was isolated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane, followed by the removal of non-specific binding in the membrane with 2.5% skimmed milk. Subsequently, the membrane was incubated with primary antibodies for 4 h at 37˚C, including mouse anti-TRAF4, -caspase-3 and -Bax (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by washing 3 times with Tris-buffered saline and Tween-20 (TBST) and incubation with a secondary antibody (Santa Cruz Biotechnology) conjugated with horseradish peroxidase (HRP) for 2 h at room temperature. Finally, the protein band was detected by an enhanced chemiluminescence (ECL) detection system (Amersham, Little Chalfont, UK).

MTT and BrdU assays. For the MTT assay, A549 and H358 cells were seeded in 96-well plates and reached a confluency of 80%. Subsequently, the cells were transfected with the lentivirus gene transfer vectors of hsa-miR-370 and TRAF4,
as previously described. Next, 20 µl of MTT was added to each well and incubated for 4 h at 37˚C with 5% CO₂. Dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added to dissolve the formazan crystals and the absorbance was measured at 490 nm.

For the BrdU assay, when the A549 and H358 cells grew to a confluency of 50% in 96-well plates, the medium was replaced with fresh brdU medium and the cells were incubated in 5% CO₂ at 37˚C for 1 h. After being washed with phosphate-buffered saline (PBS) and fixed with 70% ethanol, the cell slides were incubated with the primary human anti-BrdU antibody and secondary FITC-conjugated-anti-human antibody. The cells were observed and counted in randomly selected fields under a fluorescence microscope (Olympus). Data are presented as percentages of BrdU-positive cells vs. total cells.

**Tumor formation assay.** A549 and H358 cells (2x10⁶ cells) transfected with the lentivirus vectors overexpressing miR-370 and scramble lentivirus vectors were diluted in 200 µl of PBS followed by injection subcutaneously into the right flank of BALB/c mice. The tumor volume was measured every 2 days with a Vernier caliper and presented as Volume = length x width² x π/6. Five weeks later, the mice were sacrificed by subcutaneous injection with excessive sodium pentobarbital (40 mg/kg), and the tumors were removed and measured.

**Dual-luciferase reporter assay.** The possible miR-370 binding sites in the TRAF4 gene 3'-untranslated regions (3'-UTR) were predicted via microRNA.org. The cDNA fragments containing the predicted miR-370 binding sites in TRAF4 were amplified and subcloned into pmirGLO Dual-Luciferase miRNA Target Expression Vector (named pmirGLO-TRAF4; Promega, Madison, WI, USA). As a control, the pmirGLO-mutTRAF4 plasmids were also constructed using cDNA fragments containing corresponding mutated nucleotides for miR-370. The pre-miR-370 and pre-miR-scramble plasmids were constructed and amplified in preparation for use. Subsequently, HEK-293T cells, cultured in 6-well plates with a confluency of 80%, were co-transfected with 100 ng of pmirGLO-TRAF4 or pmirGLO-mutTRAF4 vectors in the presence of 40 nM pre-miR-370 or pre-miR-scramble using Lipofectamine transfection reagent (Invitrogen) and incubated for 48 h. The cells were harvested and the luciferase activities were measured using the Dual-Luciferase Reporter Assay kit (Promega) according to the manufacturer's instructions.

**Statistical analysis.** Data are presented as mean ± standard deviation (SD). The differences were analyzed by the Student's t-test or one-way ANOVA analysis. A p<0.05 was considered to be statistically significant and a p<0.01 extremely significant.
**Results**

miR-370 expression is downregulated in NSCLC samples and cells. To investigate the expression of miR-370 in the NSCLC patient tissues and cell lines and compare its expression with normal samples or cells, RT-qPCR was performed. As shown in Fig. 1A, miR-370 expression was significantly inhibited in the NSCLC tissues compared with that in the normal tissues. Furthermore, miR-370 expression was significantly downregulated in the NSCLC cell lines (Fig. 1B), including A549 and H358 cells, in contrast to the normal lung epithelium BEAS-2B cells. These results suggest that miR-370 may play a role in the tumorigenesis of NSCLC.

miR-370 is overexpressed in A549 and H358 cells via lentivirus transfection. Next, miR-370 was overexpressed via lentivirus transfection in the A549 and H358 cells. The miR-370 expression had a 5.5-fold and 5.6-fold increase in the A549 cells, when compared with the level in the control group and scramble vector group, respectively (Fig. 2A). Similarly, in the H358 cells, the data were 6.5-fold and 5.4-fold, respectively (Fig. 2B). These results confirmed the overexpression of miR-370 in the A549 and H358 cells via lentivirus transfection.

miR-370 overexpression inhibits proliferation and induces the apoptosis of A549 and H358 cells. Since we know that miR-370 is a unique molecule in NSCLC tissues, we next
investigated its effects on NSCLC cell lines. Cell proliferation was evaluated by MTT and BrdU assays. As shown in Fig. 3A, there was a sharp decrease in cell proliferation following miR-370 overexpression; in A549 cells, cell proliferation had a 66% decrease and in H358 cells, cell proliferation had a 78% decrease compared with the control group. The BrdU results also showed a reduced percentage of BrdU incorporation after miR-370 overexpression (Fig. 3B), suggesting that miR-370 could inhibit the proliferation of NSCLC cells. Cell apoptosis was evaluated by western blotting. Upregulated expression of apoptosis-related genes, including caspase-3 and Bax-2, at the protein level was noted (Fig. 3C) The densitometry analysis demonstrated a 2.1-fold and 2.6-fold increase in caspase-3 in the A549 and H358 cells, respectively, and a 5.2-fold and 4.7-fold increase in Bax-2 compared with the control group, respectively (Fig. 3D and E). These results confirmed that miR-370 had a positive effect on NSCLC cell apoptosis.

**miR-370 overexpression inhibits tumor formation in vivo.** To evaluate the effects of miR-370 on NSCLC cell growth in vivo, A549 and H358 cells transfected with hsa-miR-370 vectors were injected into BALB/c mice. As shown in Fig. 4A, tumor formation was significantly inhibited 15 days after injection with the A549 hsa-miR-370 vector-transfected cells. The H358 hsa-miR-370 vector-transfected cells also had a lower decrease in tumor formation ability (Fig. 4B). At 35 days post-injection, the tumor volume was 20.00±3.05 and 17.97±0.85 mm³ in the A549 and H358 cells transfected with hsa-miR-370, respectively, compared with 216.23±3.51 and 157.80±5.55 mm³ in the scramble vector groups (Fig. 4A and B).

**TRAF4 is a target gene of miR-370.** To explore the potential mechanism of miR-370 in regulating NSCLC, the predicted target genes of miR-370 were screened by bioinformatic analysis. As shown in Fig. 5A, TRAF4, which is an important oncogene, was shown to bind with hsa-miR-370 within the 3'-UTR, but mut-TRAF4 had no binding sites with hsa-miR-370 within the 3'-UTR. To confirm whether the predicted binding sites for miR-370 within the 3'-UTR of TRAF4 were correct, dual-luciferase reporter assay was performed. The results demonstrated that hsa-miR-370 significantly reduced the luciferase activity in the presence of pmirGLO-TRAF4, whereas the luciferase activity was not affected by hsa-miR-370 in the presence of pmirGLO-mutTRAF4 compared with the scramble group (Fig. 5B). The above results were consistent with the predicted analysis shown in Fig. 5A. The data imply that miR-370 directly binds to the 3'-UTR of TRAF4, in other words, TRAF4 is a target gene of miR-370.

**TRAF4 expression is regulated by miR-370 at the protein level but not at the mRNA level.** To identify the effects of miR-370 on TRAF4 expression, RT-qPCR and western blotting were performed. Notably, transfection of A549 and H358 cells with hsa-miR-370 had no effect on the expression of TRAF4 at the mRNA level (Fig. 6A), whereas at the protein level TRAF4 was significantly inhibited (Fig. 6B and C), suggesting that miR-370 prevented TRAF4 expression at the post-transcriptional translation level.

**TRAF4 overexpression abolishes the inhibitory growth effects on NSCLC cells induced by miR-370.** TRAF4 expression was
inhibited by miR-370, and we aimed to ascertain whether miR-370 suppresses the proliferation of NSCLC cells via downregulation of TRAF4. We co-transfected hsa-miR-370 with TRAF4 vectors harboring no specific-binding sequences of miR-370 in the 3′-UTR for both overexpression or single overexpression in the A549 and H358 cells. As shown in Fig. 7A–C, TRAF4 was overexpressed at both the mRNA and the protein level, and miR-370 was overexpressed at the mRNA level (Fig. 7D). Notably, TRAF4 transfection restored its expression at the protein level in the presence of miR-370 overexpression (Fig. 7C). The MTT and BrdU results clearly showed that the overexpression of TRAF4 promoted the proliferation of NSCLC cells induced by miR-370 overexpression (Fig. 7E and F).

Discussion

Lung cancer is the leading cause of cancer-related deaths worldwide (31), 80% of which are due to NSCLC (32). Great progress have been achieved in elucidating the mechanisms and in developing therapeutic methods for NSCLC in recent years, yet an effective target is urgently needed for the treatment of NSCLC. In the present study, we found that miR-370 expression was downregulated in the NSCLC tissues and cells, which indicated that miR-370 plays an important role in NSCLC. Upon this discovery, further experiments were performed. miR-370 was overexpressed in NSCLC cell lines, A549 and H358, via lentivirus transfection. The results showed that miR-370 overexpression inhibited the proliferation and promoted the apoptosis of NSCLC cells. Furthermore, miR-370 overexpression suppressed the ability of tumor formation of NSCLC cells in vivo. Next, TRAF4 was confirmed to be a target gene of miR-370 in humans, and TRAF4 negatively regulated the effects on NSCLC cells induced by miR-370, implying that miR-370 possibly functions through TRAF4. In conclusion, miR-370 may serve as an effective target for the treatment of NSCLC.

miRNAs are a group of single-stranded, non-coding molecules, participating in the regulation of their target genes at the level of translation (4,33). There are various studies on miRNAs and NSCLC. For example, Geng et al identified five miRNAs as biomarkers for NSCLC (34). Tafsiri et al investigated miRNAs and their association with clinicopathological features in NSCLC (35). miR-370 was also reported. Lo et al investigated miR-370 and its novel target TGFb-RII in the progression of gastric carcinoma (23). The authors demonstrated that miR-370 was overexpressed in gastric carcinoma. However, Yungang et al reported that miR-370 was downregulated in human laryngeal squamous cell carcinoma by targeting FoxM1 as a tumor suppressor (24). The results showed that miR-370 may have various functions in different types of cancers. Herein, we aimed to ascertain the functions of miR-370 in NSCLC and the potential mechanism.
Our data showed that miR-370 suppressed the development of NSCLC. We used bioinformatic analysis to predict the binding sequences of miR-370 and 3’-UTR of TRAF4. Next, the dual-luciferase reporter assay was employed to verify our hypothesis. The results showed that TRAF4 was indeed a target gene of miR-370 in humans, which was consistent with our hypothesis.

As reported, TRAF4 was found to be overexpressed in various cancer cells (28), implying that TRAF4 overexpression is common in the majority of cancer cells, whereas TRAF4 also has an antitumor effect (36,37). Therefore, TRAF4 has different functions in different cancer cells, dependent on the microenvironment and stimulation. Our data showed that TRAF4 was highly expressed in NSCLC cells, and miR-370 overexpression significantly inhibited the expression of TRAF4. These results suggest that TRAF4 serves as an oncogene in NSCLC. Notably, inhibition of TRAF4 expression was only shown at the protein level, namely, at the translational...
level, since miRNAs function via binding with the 3'-UTR of mRNAs. It is worth noting that the co-overexpression of miR-370 and TRAF4 demonstrated an interesting phenomenon. TRAF4 overexpression diminished the inhibitory effects on NSCLC cell proliferation caused by miR-370 overexpression, verifying their mutual crosstalk.

In conclusion, our research demonstrated that miR-370 negatively regulates oncogene TRAF4 and inhibits the proliferation of NSCLC in vitro and in vivo. Taken together, our data provide clear evidence that miR-370 functions as a tumor suppressor and may serve as an effective target for the treatment of NSCLC. Therefore, further studies are necessary to confirm the effects and investigate the underlying mechanisms of miR-370 to provide a firm foundation for the use of miR-370 in the treatment of NSCLC.

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