Inhibition mechanism of lung cancer cell metastasis through targeted regulation of Smad3 by miR-15a

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Abstract. Effect of targeted regulation of mothers against deca-pentaplegic homolog 3 (Smad3) by microRNA-15a (miR-15a) on the proliferation, invasion and metastasis of non-small cell lung cancer (NSCLC) cells and its related mechanisms were investigated. Fifty pairs of NSCLC and para-cancerous tissues were collected to identify the expression level of miR-15a in NSCLC, para-cancerous tissue, and cell lines A549, H1299, H1975 and BEAS-2B by real-time fluorescence quantitative PCR (RT-PCR); A549 cells were transfected with miR-15a mimic; the MTT assay was performed to detect the role of miR-15a transfection in proliferation of A549 cells, the wound healing assay was carried out to identify the role of miR-15a in migration of A549 cells; Transwell invasion assay was conducted to analyze the role of miR-15a in invasion of A549 cells; western blotting was carried out to find the effect of miR-15a on Smad3 expression, and Spearman's rank correlation was used to analyze the correlation between miR-15a and Smad3 expression. NSCLC tissues and cells showed significantly lower miR-15a expression, compared with para-cancerous tissues and normal cell lines (P=0.023). miR-15a was significantly more expressed in A549 cells transfected with miR-15a mimic (P=0.043). Overexpression of miR-15a can significantly inhibit A549 cell proliferation (P=0.038), migration (P=0.033) and invasion (P=0.025), and significantly reduced the expression level of Smad3 (P=0.031) in A549 cells. Spearman's rank correlation showed negative correlation of miR-15a expression with Smad3, which may indicate negative regulation (r=-0.34, P<0.0001). Inhibition of proliferation, migration and invasion of NSCLC cells can be achieved with targeted regulation of Smad3 by miR-15a.

Introduction

Lung cancer is a leading contributor to cancer-related death worldwide. Non-small cell lung cancer (NSCLC) accounts for ~80% of all lung cancer cases, including squamous cell carcinoma and adenocarcinoma (1,2). Although great progress has been made in chemotherapy and surgery, NSCLC patients still have poor prognosis and a 5-year survival rate of <15% due to latent symptoms at the early stage and the high malignant potential of NSCLC (3). Therefore, to improve clinical efficacy of NSCLC therapies, it is necessary to seek biomarkers involved in the occurrence and development of NSCLC and clarify the pathogenesis of NSCLC.

MicroRNA (miRNA) is an endogenous non-coding RNA (4) that regulates gene expression at the post-transcriptional level by binding to the 3'- untranslated region (3'-UTR) of target mRNA (5). miRNA participates in a variety of biological processes, including cell proliferation, differentiation, invasion, angiogenesis and apoptosis (6). Abnormal expression of miRNA has been reported to play a critical role in the occurrence and development of tumors. Wan *et al* (7) suggested that miR-27b expression is notably downregulated in NSCLC tissues and cells, and expression of LIMK1 is upregulated to inhibit the proliferation and invasion of tumors. miR-205 can be used as a new therapeutic target due to its downregulation in glioma and its inhibition of the migration and invasion of tumor cells through targeting YAP1. The latest evidence links many miRNAs to the regulation of the occurrence and progression of NSCLC (8), and the abnormally expressed miRNA is involved in tumor progression in NSCLC as an oncogene or tumor inhibiting factor (9). Despite the headway in research on miRNA in NSCLC, the relationship between them has not been well-established and requires further efforts. miR-15a-3p is found downregulated in cervical cancer while it inhibits tumor cell proliferation, induces cell apoptosis, and raises the sensitivity of tumor cells to radiotherapy by regulating TPD (10). Jin *et al* (11) found that miR-15a may be a molecular therapeutic target for thyroid cancer, which inhibits RET/AKT signaling pathways to inhibit metastasis

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and invasion of thyroid cancer However, the effect of miR-15a on the biological function of NSCLC and its mechanism of action in NSCLC are still unclear.

The current study focused on the role of miR-15a in NSCLC metastasis and in the proliferation, metastasis and invasion of NSCLC by targeted-regulating of mothers against decapita-plegic homolog3 (Smad3) expression, providing fundamental theoretical basis for further understanding the occurrence and development mechanism of NSCLC and prognosis evaluation of NSCLC patients.

Materials and methods

Main reagents, instruments and cell lines. Annexin V-FITC, MTT kits, and HRP-labeled Goat Anti-Rabbit IgG (A0208) were from Beyotime Biotechnology; RPMI-1640 medium, fetal bovine serum, penicillin-streptomycin and trypsin from Gibco; Thermo Fisher Scientific, Inc.; TRIzol reagent and Transwell cell culture plates from Corning Inc.; Promega M-MLV reverse transcription kits from Promega Corporation; YBR Premix Ex Taq from Takara Biotechnology Co., Ltd.; miR-15a overexpression plasmid was synthesized by Guangzhou RiboBio Co., Ltd.; Lipofectamine® 3000 Transfection kit was from Invitrogen; Thermo Fisher Scientific, Inc.; Smad3 protein (rabbit anti-human Smad3 monoclonal antibody, ab40854) from Abcam; GAPDH antibody (mouse anti-human GAPDH monoclonal antibody, SC-32233) from Santa Cruz Biotechnology, Inc.; Immobilon Western HRP from Thermo Fisher Scientific, Inc.

Human NSCLC cell lines (A549, H1299, and H1975) and the normal lung cells (BEAS-2B) were all from Shanghai Institute of Biochemistry and Cell Biology, CAS. The cells were cultured in DMEM (Corning Inc.) medium containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Inc.) and 1% streptomycin (Corning Inc.) at 37°C with the concentration of 5% CO_2.

Clinical specimens. Fifty patients with NSCLC who underwent surgical treatment in the thoracic surgery department of Shandong Provincial Chest Hospital (Jinan, China) between January 2016 and December 2018 were enrolled. Inclusion criteria: The patients received surgical treatment in the above hospital and had primary lesions, and all specimens were pathologically confirmed as NSCLC. Exclusion criteria: those who received radiotherapy, chemotherapy or interventional therapies before treatment, and those who had other metastases before treatment. Tumor tissues and para-cancerous tissue of NSCLC patients were collected (2 cm away from tumor). The specimens were stored in a liquid nitrogen container 10 min after surgery *in vitro* for subsequent steps. The study was examined and approved by the Ethics Committee of Shandong Provincial Chest Hospital. Signed informed consents were obtained from the patients and/or the guardians.

Cell culture and transfection. FBS, penicillin-streptomycin, and RPMI-1640 basal medium were prepared into RMPI-1640 complete medium with 10% FBS and 1% penicillin-strepto-mycin. The cells were cultured at 37°C with the concentration of 5% CO_2. The cells were inoculated in a 6-well plate with an inoculation density of ~$2.5x10^6$ cells/well, and incubated in a constant temperature incubator. Logarithmically growing cells were chosen and inoculated in a culture plate, the cell confluence was ~80% before transfection. The cells were divided into the empty plasmid group (miR-NC group), and the transfection simulation sequence group (5'-CTCAA CTGGTGTCGTGGAGTC-3') (miR-15a mimic group). After 36 h of transfection, the cells were trypsinized and then collected for subsequent steps.

Detection of the expression level of miR-15a mRNA before and after transfection via RT-PCR. Total RNA was extracted from tissues and cells using TRIzol reagent, quantitatively detected in terms of content with an ultraviolet spectropho-tometer, and then reverse transcribed to obtain cDNA and the transcribed cDNA was amplified by RT-PCR. The primer sequences are shown in Table I. RT-PCR reactions were performed with 10 μl SYBR Premix Ex Taq, 0.4 μl forward primer, 0.4 μl reverse primer, 2 μl cDNA, and 7.2 μl sterilized distilled water. Pre-denaturation lasted for 10 min at 95°C, denaturation for 30 sec at 95°C, annealing for 30 sec at 60°C and extension for 30 sec at 74°C. The circle was repeated 40 times.

Detection of the proliferation of tumor cells via the MTT assay. Trypsinization was carried out 36 h post transfection to collect cells of each group, and then the cells were inoculated in 96-well plates with $5x10^3$ cells/well, respectively. OD value was measured at 490 nm 4 h after 20 μl of MTT solution was added to each well at days 2, 3, 4 and 5 of inoculation, and a cell growth curve was plotted. The trial was repeated 3 times taking the average OD value. The cell growth inhibitory concentration (IC) = (1 - average OD value of miR-15a mimic group / average OD value of miR-NC group) x 100%.

Detection of the migration of tumor cells via wound healing assay. The cells in the logarithmic growth phase were cultured until confluence of 80%, and gently pushed to generate wounds on the surface. Then PBS was used to wash the cells 3 times. Complete medium was replaced, recording the wounds and the cell culture was continued. After 24 h of culture, the wounds were photographed and recorded to compare their width, and to statistically analyze the cell migration of each group.

Detection of the invasion of tumor cells via Transwell invasion assay. Trypsinization was carried out 36 h post transfection to collect cells of each group, and then the cells were inoculated in 24-well plates with $5x10^3$ cells/well, respectively. Serum-free medium (200 μl) containing penicillin-streptomycin was added to the upper layer of Transwell cell culture insert, and 400 μl of complete culture medium containing 10% FBS and 1% penicillin-streptomycin to the lower layer, to culture the cells for 12 h at 37°C with the concentration of 5% CO_2. The cell culture insert was then washed 3 times with PBS to remove non-migrated cells. The cells were fixed with 4% para-formaldehyde solution for 10 min, and then washed with PBS 3 times. Subsequently, the cells were dyed with 0.5% crystal violet solution for 10-15 min and rinsed with PBS 3 times. The final step was to count migrated cells. The trial was repeated 3 times to average the values.
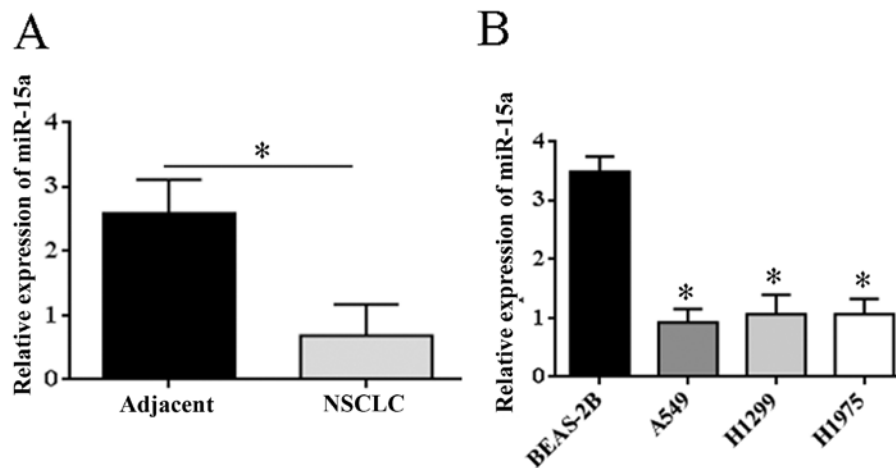
Figure 1. Relative expression of miR-15a mRNA in NSCLC tissues and cells. (A) RT-PCR results showed that miR-15a mRNA expression was significantly lower in NSCLC tissues (n=50). (B) RT-PCR results showed that expression of miR-15a mRNA was significantly lower in NSCLC cells. *P<0.05. miR, microRNA; NSCLC, non-small cell lung cancer; RT-PCR, real-time fluorescence quantitative PCR.

Table I. Primer sequences.

Primer	Primer sequence
GAPDH	F, 5'-GTGGACCTCATGCTACAT-3'
	R, 5'-TGTGAGGGAGATGCTCAGTG-3'
miR-15a	F, 5'-TCCAGCTGGCAGCATG-3'
	R, 5'-GTCGTGGAGTCACTCG-3'

F, forward; R, reverse.

Prediction of the miR-15a target gene. Prediction of the human miR-15a target genes on TargetScan (http://www.Targetscan.org) showed that the higher the score of the binding of mRNA to miR-15a seed region, the greater the possibility of the binding.

Detection of the expression level of Smad3 protein via western blotting. Precooled 1X PBS was used to collect and wash the cells twice; the cells were centrifuged at 1,200 x g at 4°C for 5 min, precipitated, and lysed with 100 RIPA lysate. After centrifugation, the cells were isolated by adding 10 μl of protein to 10% polyacrylamide gel. The isolated protein was transferred onto the PVDF membrane by a wet transfer method (current 300 mA, 1.5 h) and then sealed at room temperature for 1 h before western blotting was carried out. Primary Smad3 protein and GAPDH antibody were diluted at 1:2,000 with 5% fat-free milk, and hybridized overnight at 4°C, and the PVDF membrane was washed 3 times with 1X PBST for 5 min each time. The second antibody was diluted at 1:5,000 with 5% fat-free milk powder, and incubated for 2 h at room temperature. The PVDF membrane was washed 3 times with 1X PBST for 5 min each time. ECL luminescent solution was prepared, developed and exposed. Then, the strip quantitative analysis was performed (Gelpro Analyzer, Media Cybernetics, Inc.).

Statistical analysis. IBM SPSS Statistics 20.0 was used to make statistical analysis of the collected data, with GraphPad Prism 8 to draw statistical charts. All data were obtained by 3 independent trials. The measurement data were expressed as the mean ± standard deviation (mean ± SD), whereas the count data were represented as a percentage (%). The t-test was used to analyze the differences between the two groups, variance analysis for differences among groups, and Pearson's correlation coefficient for the correlation between variables. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression level of miR-15a mRNA in NSCLC tissues and cells. RT-PCR results showed that expression of miR-15a mRNA was significantly lower in NSCLC tissue (P=0.023) compared with para-carcinoma tissues (Fig. 1A). Expression level of miR-15a mRNA was detected in three NSCL cell lines (H1975, A549, and H1299) and in normal lung cells (BEAS-2B), and it was found that expression of miR-15a mRNA was significantly lower in NSCLC cell lines than that in the normal lung cells (P<0.05) (Fig. 1B).

Expression level of miR-15a mRNA in A549 cells after transfection. A549 cells with the lowest relative expression of miR-15a were transfected with miR-15a mimic. RT-PCR indicated significantly higher expression level of miR-15a mRNA in A549 cells relative to miR-NC group after transfection (Fig. 2). The difference was statistically significant (P=0.043). The results showed that the tumor cell models were successfully transfected with miR-15a and could be used in subsequent trials.

Overexpression of miR-15a significantly inhibits the proliferation of NSCLC cells. Via the MTT assay, it was found that compared with miR-NC group, A549 cells which were transfected with miR-15a mimic had significantly reduced cell viability and proliferation significantly slowed down on the 2nd, 3rd, 4th and 5th days after adding MTT solution (P=0.038). This indicated that miR-15a could significantly inhibit the proliferation of NSCLC cell lines (Fig. 3).
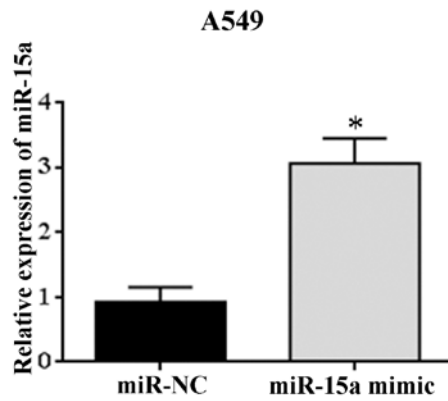
Figure 2. Relative expression of miR-15a mRNA in A549 cells. RT-PCR results showed that A549 cells that underwent transfection of miR-15a mimic showed significantly higher expression level of miR-15a mRNA, compared with miR-NC group. *P<0.05 in comparison with miR-NC group. miR, microRNA; RT-PCR, real-time fluorescence quantitative PCR.
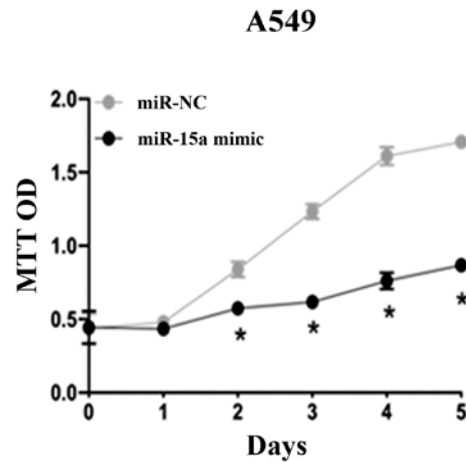

Figure 3. Effect of miR-15a overexpression on proliferation of A549 cells. MTT assay results showed that overexpression of miR-15a significantly inhibited the proliferation of NSCLC cells. *P<0.05 in comparison with miR-NC group. miR, microRNA; NSCLC, non-small cell lung cancer.

Effect of miR-15a overexpression on migration and invasion of NSCLC. The wound healing assay showed that A549 cells had significantly reduced migration than miR-NC group after transfection of miR-15a mimic, and the difference was statistically significant (P=0.033). It suggested that overexpression of miR-15a can significantly inhibit the migration of NSCLC cell lines (Fig. 4A). According to the Transwell invasion assay, the cell invasion of miR-15a mimic group was significantly reduced in comparison with that of miR-NC group (P=0.025), indicating significant inhibition of the invasion ability of NSCLC cell line by miR-15a overexpression (Fig. 4B). The above showed that overexpression of miR-15a may weaken the cell migration and invasion of A549 cells, and inhibit tumor cell metastasis.

miR-15a regulates Smad3 protein expression. Bioinformatics analysis predicted the binding sites of miR-15a on Smad3 (Fig. 5A). Western blotting results showed that miR-15a was overexpressed in A549 cells, significantly reducing the expression level of Smad3 protein (P=0.031) (Fig. 5B and C). Pearson correlation analysis showed that miR-15a mRNA level was significantly negatively correlated with Smad3 expression level, suggesting that miR-15a and Smad3 may have negative regulatory relationships (r=-0.34, P<0.0001) (Fig. 5D).

Discussion

In this study, NSCLC cell lines were transfected with miR-15a to investigate the function of miR-15a in the occurrence and development of NSCLC. It was found that miR-15a served as a tumor inhibiting factor in NSCLC. Overexpression of miR-15a inhibited cell proliferation, migration and invasion of NSCLC. The findings showed that Smad3 is a target gene of miR-15a in NSCLC.

miRNA is an endogenous non-coding small RNA, which can bind to 3'-UTR of target mRNA to inhibit transcription of target genes or degrade target mRNA fragments, and regulate its expression at a post-transcription level (9,12). Increasing evidence links miRNA to occurrence and development of cancers (13). Due to organ specificity, miRNA differs in different organs in terms of types and proportions. miRNAs are related to the functional regulation of organs, so miRNAs

can be used as specific biological markers for many different diseases. Therefore, research on the role of NSCLC-specific miRNAs in its occurrence and development process can provide new insights into the study of the occurrence and development of NSCLC as well as new schemes for clinical treatment of NSCLC.

The miR-15a gene is located at human chromosome 13q14 and was first reported to be abnormally expressed in cancer in 2002. The deletion of miR-15a is associated with poor prognosis of patients with chronic lymphocytic leukemia (14,15). miR-15a is the first miRNA reported to be involved in tumor development, which is of great significance. Subsequent studies have reported the expression and mechanism of miR-15a in tumors. miR-15a, as a tumor inhibiting factor, is downregulated in melanoma, colorectal cancer, bladder cancer, prostate cancer and other solid tumors (16-19). MicroRNA-15 (miR-15) family, as upstream regulatory molecules, regulates different target mRNAs and plays a crucial role in the occurrence and development of tumors. Janaki Ramaiah *et al* (20) found that miR-15 inhibits the proliferation of breast cancer cells and induces apoptosis by targeting p70S6 kinase. Pouliot *et al* (21) screened miRNA using high-throughput screening to restore the sensitivity of cisplatin-resistant cells. It was found that targeted regulating of the expression of Wee1 and CHK1 by miR-15a could restore the sensitivity of cisplatin-resistant cells. Bozok *et al* (22) and Çalışkan *et al* (23) confirmed that miR-15a enhanced the anti-tumor effect of platinum chemotherapeutic drugs in drug-resistant NSCLC. In other studies, miRNA-15 family suppressed cell metastasis by regulating EMT process in malignant cancer cells (24-26). He (27) found that knockout of miR-15a may promote proliferation and invasion of lung cancer cells, inhibit cell apoptosis, and induce EMT. In this study, miR-15a was found expressed at significantly lower level in NSCLC tissues by comparison with that in para-carcinoma tissues. miR-15a expression was also relatively low in NSCLC cell lines in *in vitro* cell trials, indicating that the low expression of miR-15a may promote the occurrence of NSCLC. In addition, based on the expression of miR-15a in the three NSCLC cell lines, A549 cell line
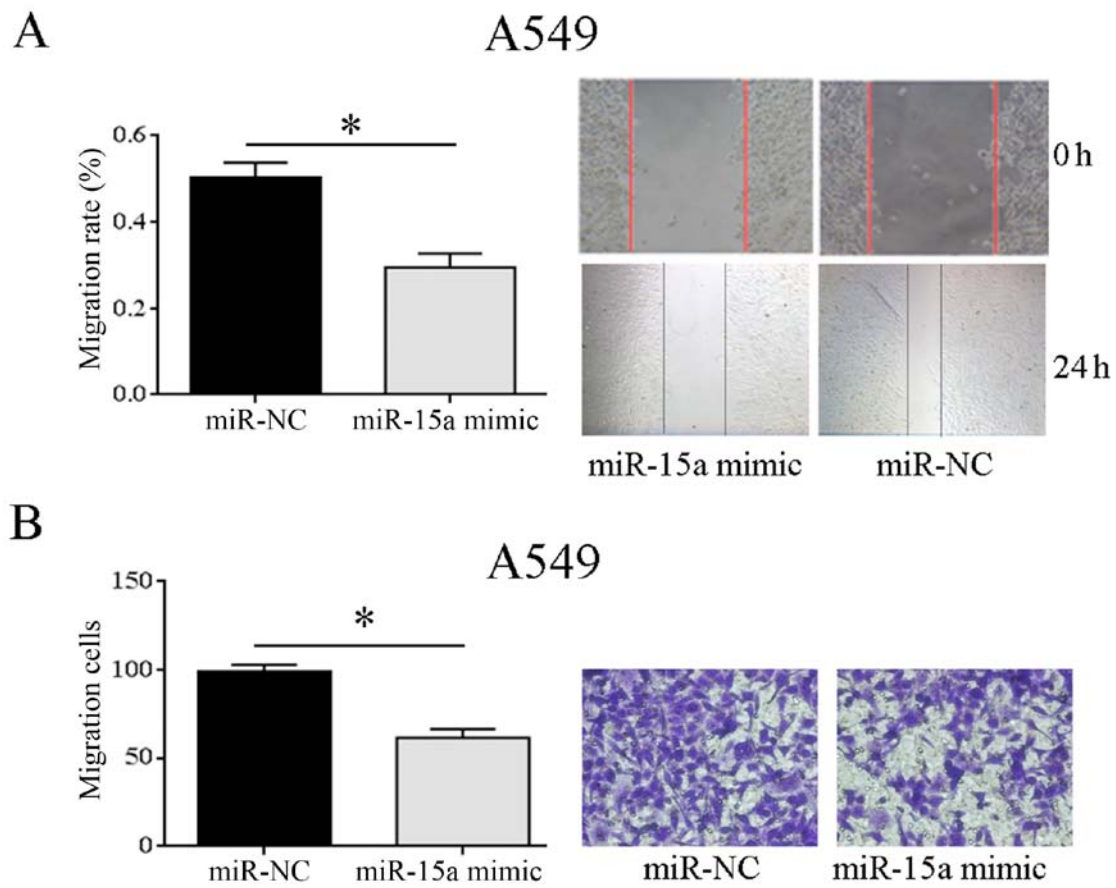
Figure 4. Effect of miR-15a overexpression on migration and invasion of A549 cells. (A) Wound healing assay results showed that miR-15a overexpression significantly inhibited the migration of NSCLC cell lines. (B) Transwell invasion assay results showed that miR-15a overexpression significantly inhibited the invasion of NSCLC cell lines. *P<0.05 in comparison with miR-NC group. miR, microRNA; NSCLC, non-small cell lung cancer.
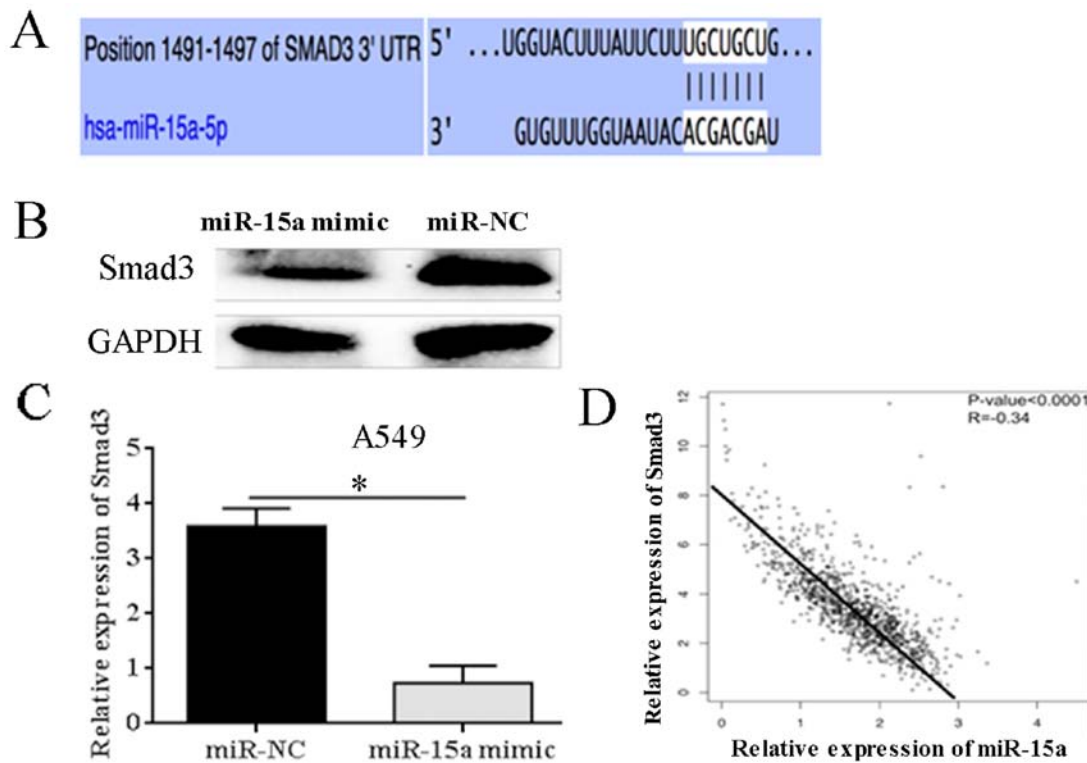

Figure 5. Correlation between miR-15a and Smad3. (A) Binding sites of miR-15a on Smad3. (B and C) Western blotting results showed that miR-15a overexpression inhibited Smad3 expression. *P<0.05 in comparison with miR-NC group. (D) Pearson's correlation coefficient showed that miR-15a was significantly correlated with Smad3 (P<0.0001). miR, microRNA; Smad3, mothers against decapitaplegic homolog3.

with the lowest miR-15a expression was transfected with miR-15a mimic to construct a NSCLC cell line model with overexpression of miR-15a. Metastasis is one of the most important malignant biological characteristics of tumor cells. It was found herein through wound healing assay and Transwell invasion assay that overexpression of miR-15 effectively inhibited the migration and invasion ability of NSCLC cells. Combined with previous studies, it was shown that miR-15a has universal anti-tumor effect on a variety of tumors. Its anti-tumor mechanism is not related to the origin of tumor tissue and has potential clinical development value. This drove the research team to find how miR-15a regulates the migration and invasion of NSCLC cells.

It is predicted on online bioinformatics databases (Pictar, Targetscan, miRanda) that miR-15a may be one of the genes regulating transforming growth factor-β (TGF-β) signal pathways, and may be bound to 3'UTR of Smad3. As a main transcription factor of TGF-β signal transduction, Smad3 acts as a tumor inhibiting factor and oncogene in the process of tumor occurrence and development. TGF-β signaling pathways participate in normal physiological processes such as growth and development, inflammatory responses, and immune regulation, as well as in tumor development. Jin *et al* (18) found that miR-15a/16 inhibits prostate cancer metastasis and invasion by inhibiting TGF-β signaling pathways. Underexpression or mutation of Smad3 will lead to interruption of TGF-β signaling, making cells beyond the growth inhibition of TGF-β signal pathways and eventually develop into tumor cells (28-30). Previous studies have shown that Smad3, as a negative growth signal regulator, regulates the expression of TGF-β superfamily during the occurrence and development of varying tumors, accommodates the abnormal growth cycle of cells and protects the body (31,32). In this study, western blotting and Pearson's correlation coefficient showed that overexpression of miR-15a significantly reduced the expression of Smad3, and the two had negative regulatory correlation. Combined with *in vitro* cell trial, it was shown that miR-15a can target downregulation of Smad3 to inhibit the proliferation and metastasis of NSCLC cells.

In conclusion, it was found that miR-15a is differentially expressed in NSCLC tissues and cells. miR-15a may inhibit the proliferation, migration and invasion of NSCLC cells through targeted regulation of Smad3 expression. This provides theoretical basis for the pathogenesis of NSCLC. miR-15a is expected to become a potential new target for NSCLC biotherapies.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SG detected the migration of tumor cells via wound healing assay and wrote the manuscript, ML interpreted and analyzed the data. JL designed the study and performed the experiment. YL was responsible for the analysis and discussion of the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Shandong Provincial Chest Hospital (Jinan, China). Patients who participated in this study had complete clinical data. Signed informed consents were obtained from the patients and/or the guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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