

LIMD2 targeted by miR-34a promotes the proliferation and invasion of non-small cell lung cancer cells

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Abstract. A previous study indicated that LIM domain containing 2 (LIMD2) is an oncogene in a variety of human cancers, including breast, bladder and thyroid cancers, and melanoma; however, the role of LIMD2 in non-small cell lung cancer (NSCLC) remains largely unknown. In the present study, by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis, it was demonstrated that LIMD2 was significantly upregulated in NSCLC tissues compared with adjacent normal tissues. Consistently, LIMD2 was also upregulated in NSCLC cell lines. Furthermore, the present study reported that knockdown of LIMD2 significantly inhibited the proliferation and invasion of H1299 and A549 cells by Cell Counting Kit-8 and Transwell assays. In addition, the expression of LIMD2 was determined to be regulated by microRNA (miR)-34a in the present study. RT-qPCR and western blot analysis indicated that overexpression of miR-34a notably reduced the mRNA and protein expression levels of LIMD2 in H1299 and H549 cells. Additionally, the present study reported an inverse correlation between the expression of LIMD2 and miR-34a in NSCLC tissues. A luciferase reporter assay also demonstrated that miR-34a directly targeted the mRNA expression of LIMD2 in NSCLC cells. Finally, miR-34a was revealed to possess a tumor suppressive role in NSCLC cells. Collectively, the results of the present study revealed that LIMD2 promoted NSCLC progression and was regulated by miR-34a.

Introduction

Lung cancer is one of the most common and aggressive types of cancer, and the leading cause of cancer-associated mortality worldwide (1). Among all patients with lung cancer, non-small-cell lung cancer (NSCLC) accounts for 75-80% of all cases (2). Surgery and radiotherapy remain the predominant therapeutic approaches for treating NSCLC (3); however, the majority of patients with NSCLC are diagnosed at advanced stages accompanied with metastasis, which leads to highly unsatisfactory outcomes of therapy (4). Thus, to develop novel strategies for patients with NSCLC, it is necessary to investigate the mechanism underlying the development and progression of NSCLC.

LIM domain containing 2 (LIMD2) belongs to the LIMD protein family (5). In the human genome, 135 identifiable LIM-encoding sequences have been located within 58 genes (5). LIMD2 has been demonstrated to serve as a biomarker for papillary thyroid carcinoma lymph node metastasis (6); however, knowledge regarding role of LIMD2 in other types of cancer and its functional mechanism remain very limited. Thus, investigation is required to determine whether LIMD2 regulates the aggressive behaviors associated with NSCLC, including proliferation and invasion.

MicroRNAs (miRNAs) are a class of noncoding RNAs with a length of ~22 nucleotides (7). MiRNAs can regulate gene expression by binding to the complementary sequence of 3'-untranslated region (UTR) of target mRNAs (8). Accumulating evidence has indicated that miRNAs exert notably important functions in various biological processes, including cell survival, proliferation, migration and invasion (9,10). For instance, Xie *et al* (11) reported that Let-7c inhibits the growth of cholangiocarcinoma. Wang *et al* (12) revealed that miR-598 inhibits the cell proliferation and invasion of glioblastoma by directly targeting MACC1 (12). At present, the importance of miRNAs in a variety of cancers is widely acknowledged (11,12).

In the present study, the function of LIMD2 and its expression mechanism in NSCLC was investigated. The expression of LIMD2 was significantly upregulated in NSCLC tissues and cell lines; knockdown of LIMD2 suppressed the proliferation

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and invasion of NSCLC cells as reported by the present study. Additionally, LIMD2 expression was regulated by miR-34a. Collectively, the results of the present study demonstrated the important roles of LIMD2 in the progression of NSCLC.

Materials and methods

Patient samples. NSCLC tissues and adjacent non-cancerous tissues were collected from 42 patients at The Second Affiliated Hospital of Harbin Medical University (Harbin, China) between May 2014 and September 2016. Tissue samples were frozen and stored at -80°C . The samples were divided into LIMD2 low expression group and LIMD2 high expression group (the median value of LIMD2 expression was used as the cutoff value). The associations between the expression of LIMD2 and the clinicopathological features of patients with non-small-cell lung cancer is given in Table I. The present study was approved by the Research Ethics Committee of the Second Affiliated Hospital of Harbin Medical University. Informed consent was obtained from patients for the collection of samples in accordance with the Declaration of Helsinki. All patients had not undergone radical prostatectomy and/or any other treatment prior to the present study.

Cell culture and transfection. Human NSCLC cell lines H1299, A549, H358 and H460 were purchased from the American Type Culture Collection (Manassas, VA, USA). Human bronchial epithelial cells (HBEC) were obtained from the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in a humidified 5% CO_2 incubator.

Small interfering (si)RNA against LIMD2 (5'-GACCCA CCACTTACCATA-3') or negative control (si-NC; 5'-AAT TCTCCGAACGTGTAC-3') and miR-34a (5'-UGGCAG UGUCUAGCUGGUUGU-3') mimic or negative control (NC mimic; 5'-ACAUCUGCGUAAGAUUCGAGUCUA-3') were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.) and 50 nM per plasmid was transfected into H1299 and A549 cells, in which LIMD2 expression levels were higher compared with the other cell lines, using Lipofectamine[®] 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). The LIMD2 coding sequence was cloned into the pcDNA3.1 vector (Invitrogen) between Not I and EcoR I, and 1 mg plasmid was transfected into H1299 and A549 cells as well as 50 nM miR-34a or NC mimics using Lipofectamine[®] 3000. Then, 48 h after transfection, the transfection efficiency was determined using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) as described below.

Cell proliferation. Each well of a 96-well plate was seeded with $\sim 5,000$ transfected cells. Cell proliferation was assessed using a Cell Counting Kit-8 assay (CCK-8, Shanghai U-Sea biotech, Shanghai, China). Following incubation for 72 h at 37°C , 10 μl CCK-8 was added to each well and incubated for 2 h in an incubator at 37°C . The absorbance was determined using a multimode microplate reader (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany) at 450 nm.

Transwell assay. Cell invasion assays were performed using Transwell chambers (Corning Incorporated, Corning, NY, USA). Transfected cells (2×10^4) were seeded in the upper chamber (pre-coated with Matrigel) and incubated in serum-free RPMI-1640 media, and the lower chamber was coated with complete RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). After 24 h at 37°C , migrated cells on the bottom surface of the filters were fixed with 4% paraformaldehyde for 1 h at 25°C , and stained with 0.1% crystal violet solution (MedChem Express, Shanghai, China) for 30 min at 25°C , and counted under an inverted microscope at $\times 100$ magnifications; 3 fields per view were analyzed. The experiments were performed in triplicate.

RT-qPCR. Total RNA was extracted from tumor tissues or cultured cells with TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. Then, cDNA was synthesized with the M-MLV reverse transcriptase (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. Subsequently, transcripts were analyzed via qPCR using a TaqMan MicroRNA Assay kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) on an ABI 7300 qPCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Each experiment was repeated 3 times. The thermocycling conditions were: Denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec and elongation at 60°C for 1 min. The relative expression levels of LIMD2 and miR-34a were calculated and normalized to endogenous that of GAPDH and U6, respectively. The primer sequences were: miR-34a forward, 5'-AACGAGACGACG ACAGAC-3' and reverse, 5'-UGGCAGUGUCUAGCUGG UUGU-3'; U6 forward, 5'-AACGAGACGACGACAGAC-3' and reverse, 5'-GCAAATTCGTGAAGCGTTCCATA-3'; LIMD2 forward, 5'-GCCCATCCTGTTGTGACCAA-3' and reverse, 5'-ATGGTGAGTCTGCACCCTTC-3'; GAPDH forward, 5'-ATGTTGCAACCGGGAAGGAA-3' and reverse, 5'-AGGAAAAGCATCACCCGGAG-3'. Expression fold was calculated based on the $2^{-\Delta\Delta\text{Ct}}$ method (13).

Western blotting. Following transfection, total protein was extracted from tumor tissues or cultured cells by radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.). All the protein lysates were separated using 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Thermo Fisher Scientific, Inc.). The membrane was blocked using 5% non-fat milk in PBS (Thermo Fisher Scientific, Inc.) containing 0.1% Tween-20 (Sigma-Aldrich; Merck KGaA) at room temperature for 3 h. Then, the membrane was incubated for 2 h at 25°C with specific primary anti-human antibodies: GAPDH (1:5,000; cat. no. SAB2701826; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and LIMD2 (1:2,000; cat. no. SAB1307182; Sigma-Aldrich; Merck KGaA), followed by incubation for 1 h at 25°C with a goat horseradish peroxidase-conjugated secondary antibody (1:2,000; sc-2005; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Membranes were then washed with PBS for 10 min, and the protein bands were visualized using an Enhanced Chemiluminescence Western Blotting kit (Pierce; Thermo Fisher Scientific, Inc.), in accordance with the manufacturer's protocol. Protein densitometry was performed

Table I. Association between the expression of LIMD2 with the clinicopathological features of patients with non-small-cell lung cancer.

Features	Low LIMD2 expression (n=19)	High LIMD2 expression (n=23)	P-value
Age (years)			0.366
≤50	12	11	
>50	7	12	
Sex			0.750
Male	13	14	
Female	6	9	
TNM stage			0.016 ^a
I-II	14	8	
III-IV	5	15	
Tumor size (cm)			0.026 ^a
≤4	11	5	
>4	8	18	
Lymph node metastasis			0.049 ^a
Negative	9	4	
Positive	10	19	

^aP<0.05 as determined by a χ^2 test. LIMD2, LIM domain containing 2; TNM, tumor, node and metastasis.

using ImageJ software (version 1.41; National Institutes of Health, Bethesda, MD, USA). GAPDH was used as a control. The experiment was repeated 3 times.

Luciferase reporter assay. The target gene LIMD2 and potential binding site were predicted using TargetScan7 (http://www.targetscan.org/vert_71/). A luciferase reporter assay was performed to determine the direct binding of miR-34a to the target gene LIMD2. The wild type (WT) and mutant (Mut) 3'-UTR sequence of LIMD2 was directly synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China) and then inserted into pGL3 plasmid (Ambion; Thermo Fisher Scientific, Inc.) between Mlu I and Bgl II. Cells (2×10^4) were cultured in 24-well plates, and each well was transfected with 0.2 μ g firefly luciferase reporter plasmid, and equal amounts of miR-34a (50 nM) and NC mimics (50 nM) using Lipofectamine[®] 2000. After 24 h, Firefly and *Renilla* luciferase activities were measured using the Luc-Pair miRNA Luciferase Assay kit (GeneCopoeia, Inc., Rockville, MD, USA) according to the manufacturer's protocols. Luciferase activities were normalized to that of *Renilla* luciferase.

Statistical analysis. All experiments were performed 3 times and results expressed as mean \pm standard deviation. All statistical analyses were performed using SPSS software version 20.0 (IBM Corp., Armonk, NY, USA). For comparisons between multiple groups, one-way analysis of variance followed by a Tukey's post-hoc test was applied and a two-tailed Student's t-test was performed for comparisons between two groups. Pearson's correlation coefficient analysis was used to determine the correlation between LIMD2 and miR-34a expression.

The association between the expression of LIMD2 with the clinicopathological features of patients with non-small-cell lung cancer was analysis using a χ^2 test. P<0.05 was considered to indicate a statistically significant difference.

Results

LIMD2 is upregulated in NSCLC tissues. To investigate the function of LIMD2 in NSCLC, the expression levels of LIMD2 were determined by RT-qPCR. The present study reported that LIMD2 expression levels were significantly upregulated in NSCLC tissues (n=41) compared with in adjacent normal tissues (n=41) (Fig. 1A). Additionally, the expression levels of LIMD2 in NSCLC cell lines were determined. The results indicated that the expression levels of LIMD2 were significantly upregulated in NSCLC cell lines, including H1299, A549, H358 and H522 cells, compared with human bronchial epithelial cells (HBECs) (Fig. 1B). Consistently, the protein expression levels of LIMD2 in NSCLC tissues and cell lines were notably upregulated compared with in normal tissue and HBECs, respectively (Fig. 1C and D). In addition, the present study reported that LIMD2 expression levels were significantly higher in NSCLC tissues with metastasis than tissues without (Fig. 1E). These data suggest LIMD2 may be involved in NSCLC progression.

LIMD2 knockdown suppresses the proliferation and invasion of NSCLC cells. To determine the effects of LIMD2 on NSCLC cells, LIMD2 expression was downregulated in H1299 and A549 cells via transfection with specific siRNAs. RT-qPCR analysis revealed that the expression levels of

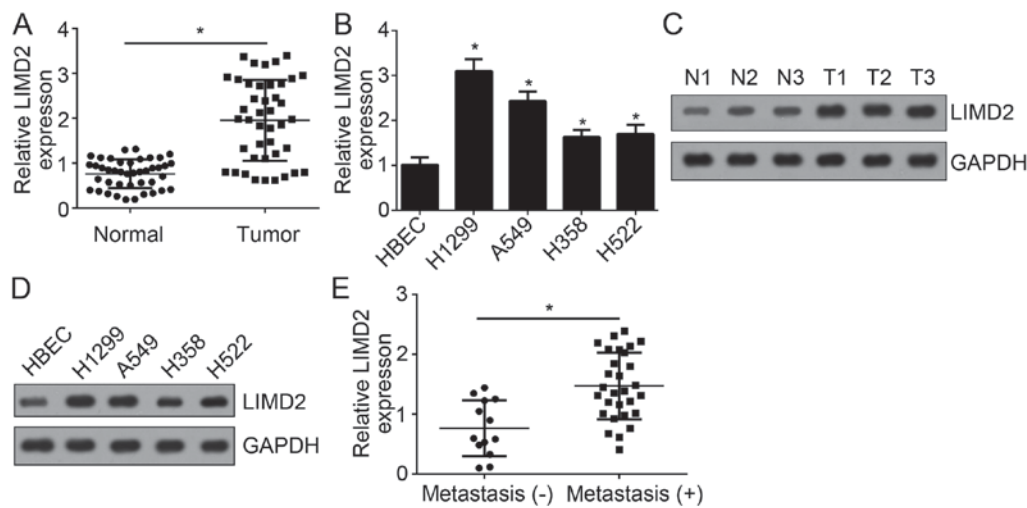


Figure 1. LIMD2 is upregulated in NSCLC tissues. (A) Expression levels of LIMD2 were downregulated in NSCLC tissues compared with in adjacent normal tissues as determined by RT-qPCR. (B) Expression levels of LIMD2 in NSCLC cell lines as determined by RT-qPCR. (C) Protein expression levels of LIMD2 in three pairs of NSCLC samples and adjacent normal tissues. (D) Protein expression levels of LIMD2 in NSCLC cell lines were determined by western blotting. (E) Relative expression levels of LIMD2 in NSCLC tissues with or NSCLC without metastasis. * $P < 0.05$ vs. Normal tissues, HBEC cells or NSCLC without metastasis. All data presented are presented as the means \pm standard deviation collected from three independent experiments. LIMD2, LIM domain containing 2; N, normal tissue; NSCLC, non-small-cell lung cancer; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; T, NSCLC tissues.

LIMD2 were significantly downregulated in H1299 and A549 cells transfected with siLIMD2 compared with in the control group (Fig. 2A). The present study also performed CCK-8 and Transwell assays to determine the effects of LIMD2 on cell proliferation and invasion, respectively. The CCK-8 assay indicated that knockdown of LIMD2 significantly suppressed the proliferation of NSCLC cells at 72 h compared with in the control group at 72 h (Fig. 2B). Furthermore, the present study reported that LIMD2 silencing significantly reduced the number of migrated cells compared with in the control group (Fig. 2C). The above data indicated that LIMD2 contributes to NSCLC progression.

LIMD2 is downregulated by miR-34a. The present study investigated the mechanism underlying the regulation of LIMD2 expression in NSCLC cells. The results demonstrated that overexpression of miR-34a significantly suppressed the mRNA expression levels of LIMD2 in H1299 and A549 cells compared with in the control (Fig. 3A). Additionally, western blot analysis revealed that ectopic expression of miR-34a markedly decreased the protein expression levels of LIMD2 in H1299 and A549 cells compared with in the control (Fig. 3B). Furthermore, an inverse correlation between the expression of LIMD2 and miR-34a was observed in NSCLC tissues (Fig. 3C).

miRNAs have been demonstrated to regulate the degradation of target mRNAs (12). To investigate whether LIMD2 is a direct target of miR-34a, bioinformatics analysis was conducted using TargetScan. The results indicated that LIMD2 is a potential target of miR-34a. To verify this prediction, luciferase reporter assays with the WT and Mut 3'-UTR sequence of LIMD2 mRNA were performed. The results demonstrated that overexpression of miR-34a significantly suppressed the luciferase activity in H1299 and A549 cells transfected with WT-LIMD2 3'-UTR reporter plasmid compared with in the control group; no notable differences were observed within

cells overexpressing miR-34a containing the Mut-3'UTR compared with in the controls (Fig. 3D). These results demonstrated that miR-34a directly targets LIMD2.

MiR-34a is downregulated in NSCLC tissues. LIMD2 was proposed to act as an oncogene in NSCLC in the present study; however, miR-34a inhibited the expression of LIMD2 in NSCLC cells. To determine the role of miR-34a in NSCLC, the expression levels of miR-34a were determined by RT-qPCR. The results demonstrated that miR-34a expression levels were significantly downregulated in NSCLC tissues compared with in adjacent normal tissues (Fig. 4A). Additionally, lower expression levels of miR-34a were observed in NSCLC cell lines compared with in HBEC cells (Fig. 4B). Furthermore, the expression levels of miR-34a were significantly lower in NSCLC tissues with metastasis compared with in those without metastasis (Fig. 4C). These data implied that miR-34a may be a tumor suppressor in NSCLC.

MiR-34a suppresses NSCLC cell proliferation and invasion. The present study investigated whether the effects of miR-34a on NSCLC cells are associated with LIMD2. RT-qPCR analysis indicated that the overexpression of LIMD2 significantly increased the mRNA expression levels of LIMD2 in H1299 and A549 cells compared with in the vector-transfected group (Fig. 5A). CCK-8 and Transwell assays revealed that the overexpression of miR-34a significantly suppressed the proliferation and invasion, of NSCLC cells respectively, compared with in the control group (Fig. 5B). Of note, restoration of LIMD2 expression significantly reversed the inhibitory effects of miR-34a on H1299 and A549 cell proliferation and invasion compared with in the miR-34a overexpression group (Fig. 5B and C). Taken together, the data from the present study demonstrated that miR-34a suppressed NSCLC progression through targeting LIMD2.

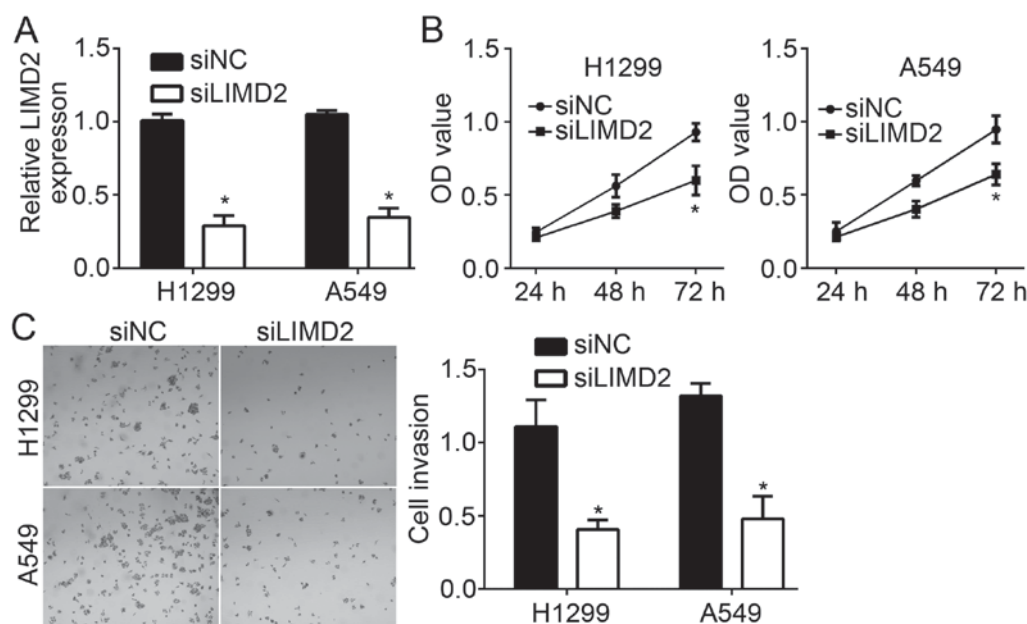


Figure 2. LIMD2 knockdown suppresses the proliferation and invasion of non-small-cell lung cancer cells. (A) Relative expression levels of LIMD2 in H1299 and A549 cells transfected with siLIMD2 or siNC. (B) Cell Counting Kit-8 assay was used to analyze cell proliferation. (C) Transwell assays were utilized for the determination of cell invasion (magnification, x100). * $P < 0.05$ vs. siNC group. All data were collected from three independent experiments. LIMD2, LIM domain containing 2; siRNA, small interfering RNA; siNC, negative control siRNA; siLIMD2, LIMD2 siRNA.

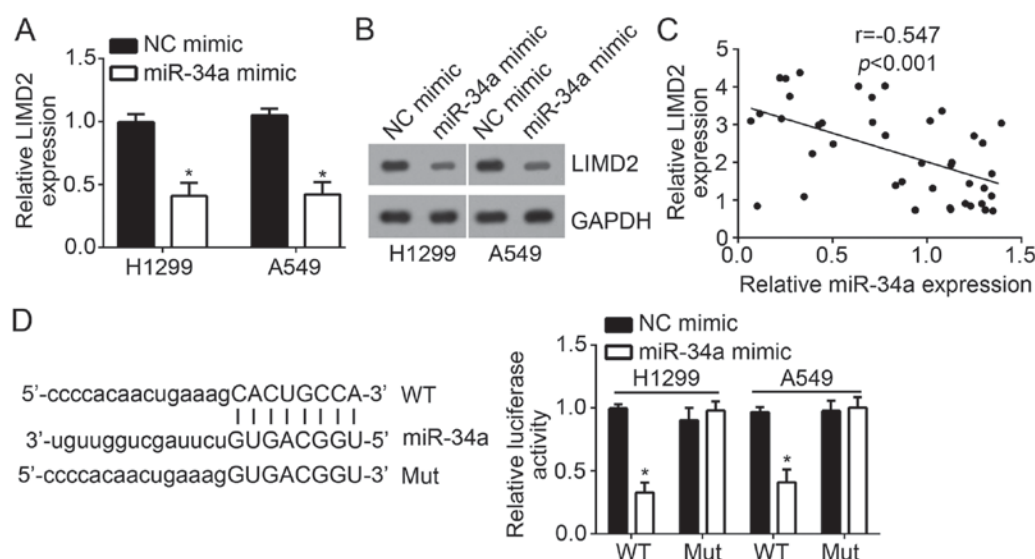


Figure 3. LIMD2 is downregulated by miR-34a. (A) Overexpression of miR-34a suppressed the mRNA expression levels of LIMD2 in H1299 and A549 cells. (B) miR-34a overexpression reduced the protein expression levels of LIMD2 in H1299 and A549 cells. (C) Reverse transcription-quantitative polymerase chain reaction analysis for the correlation between miR-34a and LIMD2 expression in non-small-cell lung cancer tissues. (D) Luciferase reporter assays revealed that miR-34a overexpression reduced luciferase activity in H1299 and A549 cells transfected with WT reporter plasmid. * $P < 0.05$ vs. NC group. All data presented are presented as the mean \pm standard deviation collected from three independent experiments. LIMD2, LIM domain containing 2; miR, microRNA; Mut, mutant; NC, negative control; WT, wild type.

Discussion

As one of the most prevalent and malignant types of tumors worldwide, lung cancer accounts for numerous cancer-associated mortalities (1). NSCLC contributes to 80% of all lung cancer cases (12). The study of lung cancer has received increasing attention. The mechanism underlying the development and progression of lung cancer remains largely unknown; effective therapeutic strategies for the treatment of lung cancer

are urgently required. In the present study, it was proposed that LIMD2 promoted the proliferation and invasion of NSCLC cells. In addition, the regulatory mechanism of LIMD2 expression was investigated in the present study, in which miR-34a was demonstrated to directly target LIMD2 in NSCLC cells.

Previous studies have indicated that LIMD2 is a novel metastasis-associated cytoplasmic protein, whose endogenous expression levels are correlated with malignant behaviors in breast cancer, bladder and thyroid cancers, and

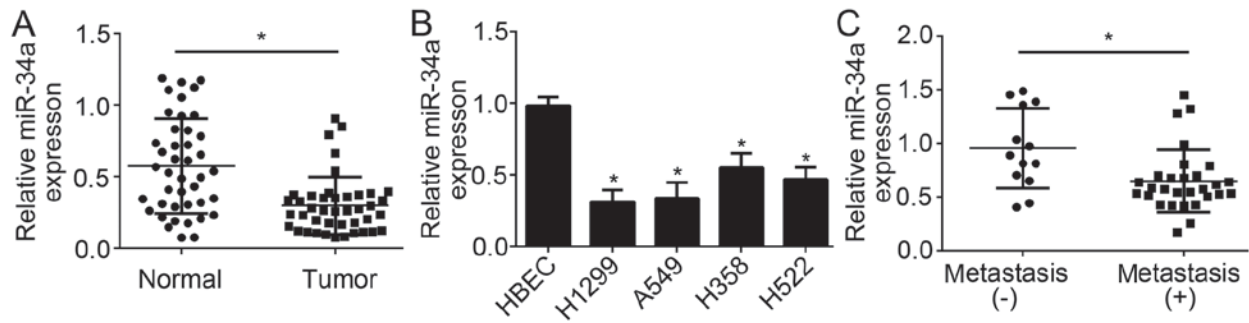


Figure 4. MiR-34a is downregulated in NSCLC tissues. (A) Relative expression levels of miR-34a in NSCLC tissues and adjacent normal tissues. (B) Relative expression levels of miR-34a in NSCLC cell lines were determined by reverse transcription-quantitative polymerase chain reaction. (C) Relative expression levels of LIMD2 in NSCLC tissues with or without metastasis. * $P < 0.05$ vs. normal tissues, HBECs or NSCLC without metastasis. All data presented are presented as means \pm standard deviation collected from three independent experiments. HBEC, human bronchial epithelial cell; miR, microRNA; NSCLC, non-small-cell lung cancer.

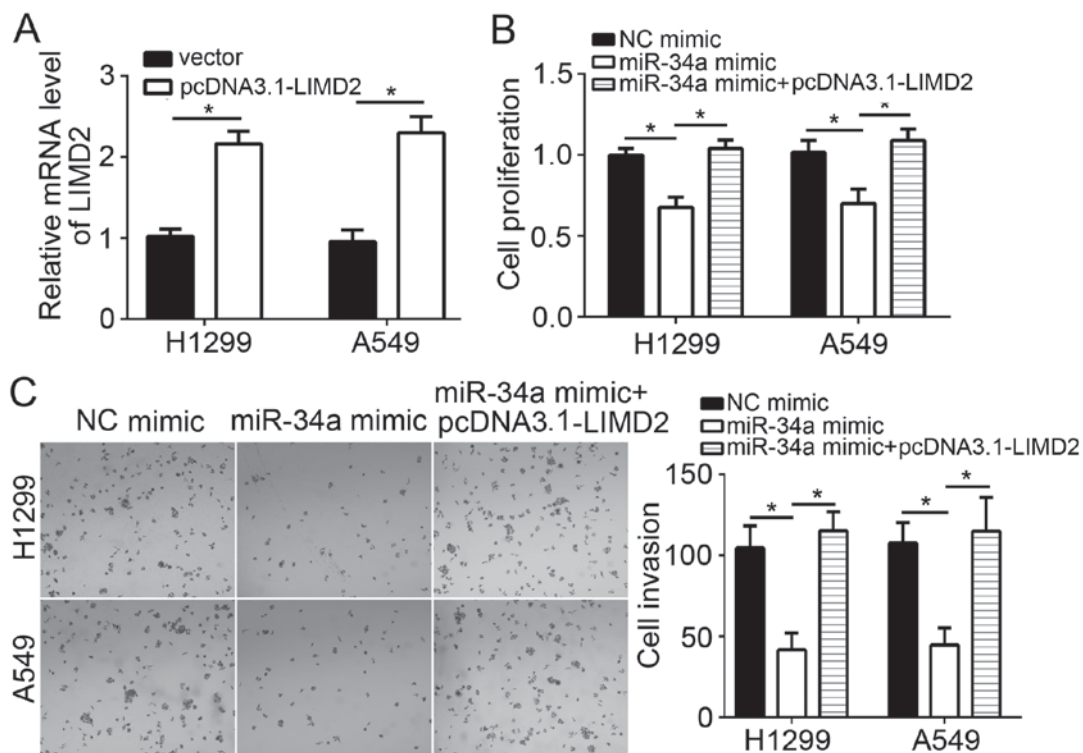


Figure 5. MiR-34a suppresses NSCLC cell proliferation and invasion. (A) Relative mRNA expression levels of LIMD2 in H1299 and A549 cells transfected with pcDNA3.1-LIMD2 expressing vector or empty vector. (B) Cell Counting Kit-8 assays were used to analyze cell proliferation. (C) Transwell assays were used to analyze the invasion of H1299 and A549 cells (magnification, $\times 100$). * $P < 0.05$. All data presented are presented as the mean \pm standard deviation collected from three independent experiments. LIMD2, LIM domain containing 2; miR, microRNA.

melanoma (5,14). Knockdown of LIMD2 has been revealed to inhibit cell invasive ability, motility and cell morphology, which was consistent with tumor grade in human thyroid cancer (14); however, whether LIMD2 serves a similar role in other cancers remains unknown. To determine the correlation between LIMD2 and NSCLC, the expression levels of LIMD2 in NSCLC tissues were analyzed by RT-qPCR in the present study; LIMD2 expression levels were higher in NSCLC cell lines than in normal cells. Additionally, the present study reported that LIMD2 expression was positively correlated with tumor metastasis, and tumor, node and metastasis stages in clinical samples (Table I). Furthermore, the expression levels of LIMD2 were higher in the metastatic cell line H1299

than within A549 cells. This further indicated that LIMD2 is associated with tumor metastasis. These results suggested that LIMD2 is involved in the progression of NSCLC. Functional experiments demonstrated that LIMD2 knockdown significantly inhibited the proliferation and invasion potentials of NSCLC cells in the present study.

MiRNAs are a group of short noncoding RNAs of ~22 nucleotides in length (12). MiRNAs have been widely demonstrated to regulate gene expression via binding to the mRNA of target genes (15-17); miRNAs are often aberrantly expressed in cancer cells (18). As miRNAs have been observed to regulate the proliferation, migration, invasion and other biological processes, a close association between miRNA

expression and tumor progression has been reported (19). For instance, Qiao *et al* (20) reported that miR-154 inhibited the growth and metastasis of gastric cancer cells by directly targeting metadherin (20). Cao *et al* (17) revealed that miR-552 promoted tumor cell proliferation and migration by directly targeting dachshund family transcription factor 1 via the Wnt/ β -catenin signaling pathway in colorectal cancer (17). The mechanism underlying the regulation of LIMD2 expression by miRNAs is not well studied. As of the important role of LIMD2 in NSCLC, it is necessary to determine the regulatory mechanism of LIMD2 expression. In the present study, miR-34a was reported to directly target LIMD2 mRNA; overexpression of miR-34a notably inhibited the mRNA and protein expression levels of LIMD2 in NSCLC cells. A recent report reported that miR-34a serves as a tumor suppressor in lung cancer (21). The present study also indicated that miR-34a inhibited the proliferation and invasion of NSCLC cells.

In conclusion, the present study demonstrated the essential role of LIMD2 in NSCLC cells; LIMD2 suppressed the proliferation and invasion of NSCLC cells, which suggested that LIMD2 may be a promising therapeutic target for the treatment of NSCLC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

FW and ZL performed the majority of the experiments and analyzed the data. LX, YiL, YL, XZ and YW collected sample tissues and performed some experiments. DL made substantial contributions to the concept and design of the present study and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

For the use of human samples, the present study was approved by the Institutional Ethics Committee of the Second Affiliated Hospital of Harbin Medical University (Harbin, China) and all enrolled patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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