

MicroRNA-1301-3p promotes the progression of non-small cell lung cancer by targeting Thy-1 and predicts poor prognosis of patients

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Received July 14, 2020; Accepted November 26, 2020

DOI: 10.3892/ol.2021.12589

Abstract. The role of microRNA (miR)-1301-3p has been investigated in breast cancer and colorectal cancer. Dysregulation of miR-1301-3p expression in non-small cell lung cancer (NSCLC) is speculated to be associated with tumor progression, which was systemically investigated in the present study. Reverse transcription-quantitative PCR analysis was performed to detect miR-1301-3p expression in 124 paired tissue samples and cultured cell lines. The results demonstrated that miR-1301-3p expression was regulated by transfection with miR-1301-3p mimic or inhibitor, and the proliferation, migration and invasion of the transfected cells were assessed via the Cell Counting Kit-8 and Transwell assays. In addition, miR-1301-3p expression was significantly upregulated in NSCLC tissues and cells compared with normal tissues and normal cells, respectively. Notably, upregulated miR-1301-3p expression in NSCLC tissues was significantly associated with the TNM stage, lymph node metastasis and poor prognosis of patients with NSCLC. Furthermore, upregulated miR-1301-3p expression in NSCLC cells promoted cell proliferation, migration and invasion, the effects of which were reversed following miR-1301-3p knockdown. Thy-1 was identified as a direct target of miR-1301-3p, which serves as a tumor promoter in the progression of NSCLC. Taken together, the results of the present study suggest that upregulated miR-1301-3p expression in NSCLC acts as an independent prognostic factor and a tumor promoter by targeting thy-1, thus provides a potential therapeutic target for NSCLC.

Introduction

Non-small cell lung cancer (NSCLC) is one of the most common types of lung cancer, which accounts for the majority of cancer-associated mortalities with the incidence rate of 1.3 million cases per year according to the statistical data until 2018 (1,2). Advancements in molecular biology and therapeutic strategies for NSCLC have allowed identification of novel drugs and treatments; however, the 5-year survival rate (<15%) of patients with NSCLC remains poor (3,4). Current therapies in the clinic, such as surgical resection, chemotherapy and radiotherapy, have been established with limited positive effects on the long-term survival of patients (5). Poor patients prognosis is predominantly due to advanced local invasion and distant metastasis at diagnosis (6). Recently, a number of studies have focused on investigating novel therapeutic strategies for patients with NSCLC, and identifying effective therapeutic targets (7-9).

MicroRNAs (miRNAs/miRs) are a class of short, highly conserved non-coding RNAs that regulate gene expression by binding to the 3'-untranslated region (UTR) of target mRNAs (10,11). miRNAs have the ability to predict clinical outcomes, detect cancer and monitor disease conditions (10). Previous studies have reported that miRNAs participate in the progression and development of different types of cancer, including NSCLC (12,13). A previous study demonstrated that miR-1301-3p inhibits cell proliferation, and induces cell cycle arrest and apoptosis of breast cancer (14). In prostate cancer, miR-1301-3p promotes the expansion of prostate cancer stem cells by targeting GSK3 β and SFRP1, and activating the Wnt pathway (15). In addition, miR-1301-3p expression is associated with the pathological stages of colorectal cancer (16). A recent miRNA expression profile revealed that miR-1301-3p expression is significantly upregulated in NSCLC tissues compared with normal clinical samples, suggesting that miR-1301-3p participates in the development of NSCLC (17), which has not yet been fully investigated. Thy-1 is a glycosylphosphatidylinositol-linked outer membrane leaflet glycoprotein that is closely associated with idiopathic pulmonary fibrosis, which can increase the risk of lung cancer (18). Thus, it is speculated that Thy-1 may play a vital role in the development of NSCLC.

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Key words: non-small cell lung cancer, microRNA-1301-3p, thy-1, progression, development

The present study aimed to investigate the role of miR-1301-3p in NSCLC to determine its association with the prognosis and progression of NSCLC. In addition, the effects of the underlying molecular mechanisms of miR-1301-3p were also investigated.

Materials and methods

Patients. The present study recruited 124 patients with NSCLC at Binzhou Medical University Hospital, between January 2013 and December 2015. The inclusion criteria was as following: i) 18 years old or above; ii) histologically diagnosed with NSCLC and were amenable to surgery; iii) had never undergone any kinds of anti-cancer therapy, such as chemotherapy and radiotherapy prior to surgery; iv) clinical data were completed. Patients diagnosed with other cancers were excluded. NSCLC tissues and adjacent normal tissues (about 2 cm from the lesion) were collected via surgical resection. Collected tissues were immediately frozen in liquid nitrogen and stored at -80°C until subsequent experimentation. The clinicopathological characteristics of the patients are presented in Table I. Survival analysis was performed via a 5-year follow-up survey at 6, 9, 12, 15, 18, 21, 24, 30, 36, 42, 48, and 60 months after surgery by telephone. The present study was approved by the Ethics Committee of Binzhou Medical University Hospital (Binzhou, China; approval no. 201212), and written informed consent was provided by all patients prior to tissue collection.

Cell culture and transfection. The NSCLC cell lines, A549, H1299, MRC5 and SK-LU-1, and the lung epithelial cell line, BEAS-2B, were purchased from the American Typical Culture Collection. Cells were maintained in DMEM medium (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc.), at 37°C with 5% CO_2 .

miR-1301-3p mimic, miR-1301-3p inhibitor or the corresponding negative controls (25 nM; Guangzhou RiboBio Co., Ltd.) were transfected into A549 and H1299 cells using Lipofectamine[®] 3000 transfection reagent (cat. no. L3000-015, Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for the overexpression or knockdown of miR-1301-3p. Transfected cells were available for following experiments after 24 h of the transfection. The following sequences were used: miR-1301-3p mimic, 5'-UUGCAGCUGCCUGGGAGU GACUUC-3'; and miR-1301-3p inhibitor, 5'-GAAGUCACU CCCGGCAAGCUGCAA-3'.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from tissues and cultured cells using TRIzol[®] reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Total RNA was reverse transcribed into cDNA using the TaqMan microRNA reverse transcription kit (cat. no. 4366596, Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. qPCR was subsequently performed using the SYBR Green I Master Mix kit (cat. no. 12223012, Invitrogen; Thermo Fisher Scientific, Inc.) and the 7300 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following primer sequences were used for qPCR: miR-1301-3p forward, 5'-TTACAGCTGCCTGAGAGTACTTA-3' and reverse,

5'-CTCTACAGCTATATTGCCAGCCA-3'; and U6 forward 5'-CGCTTCGGCAGGCATTATATAC-3' and reverse 5'-AAG GGGCCATGCTAATCTT-3'. The following thermocycling conditions were used for qPCR: 10 sec at 95°C , followed by 40 cycles of 5 sec at 95°C and 20 sec at 60°C . Relative expression levels were calculated using the $2^{-\Delta\Delta\text{C}_q}$ method (19) and normalized to the internal reference gene U6. All experiments were performed in triplicate.

Cell proliferation assay. The proliferative ability of transfected cells was assessed via the Cell Counting Kit-8 (CCK-8) assay. Briefly, A549 and H1299 cells were seeded into 96-well plates at a density of 5×10^3 and incubated at 37°C with 5% CO_2 for 0, 24, 48 and 72 h. Subsequently, CCK-8 reagent (Dojindo Molecular Technologies, Inc.) was added to each well and incubated for 1 h at 37°C . Cell proliferation was measured at a wavelength of 450 nm using a microplate reader (Synergy 4, BioTek Instruments, Inc.).

Migration and invasion assays. A total of 1×10^5 A549 and H1299 cells were plated in the upper chambers of 24-well Transwell plates in DMEM culture medium without FBS, whereas culture medium supplemented with 10% FBS was plated into the lower chambers as the chemoattractant. For the invasion assay, Transwell membranes were precoated with Matrigel (BD Biosciences) at 37°C for 1 h. Following incubation at 37°C for 48 h, the migratory/invasive cells were stained with 0.1% crystal violet at 37°C for 5 min and counted under a light microscope (magnification, $\times 400$).

Target prediction and dual-luciferase reporter assay. The TargetScan database (http://www.targetscan.org/vert_71) was used to predict the target of miR-1301-3p and verify the 3'-UTR binding region of miR-1301-3p.

The dual-luciferase reporter assay was performed using a dual-luciferase reporter assay system (Promega Corporation), according to the manufacturer's protocol. The recombinant vectors, pGL3-thy-1-wt and pGL3-thy-1-mut were generated using the pmirGLO vector (Promega Corporation). A549 cells were seeded into 96-well plates and co-transfected with miR-1301-3p mimic or miR-1301-3p inhibitor, and pGL-thy-1-3'UTR-wt or pGL-thy-1-3'UTR-mut using Lipofectamine[®] 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and incubated at 37°C . Luciferase activities were detected 24 h post-transfection with reference to *Renilla* luciferase activity.

Statistical analysis. Statistical analysis was performed using SPSS 20.0 software (IBM Corp.) and GraphPad Prism 7.0 software (GraphPad Software, Inc.). Data are presented as the mean \pm standard deviation obtained from at least triplicate experiments or determination. Paired Student's t-test was used to compare differences between two groups, while one-way ANOVA followed by Tukey's post hoc test were used to compare differences between multiple groups. The average expression level of miR-1301-3p (3.63) in NSCLC tissues was used as the cut-off value to divide 124 patients with NSCLC into high ($n=73$) and low ($n=51$) miR-1301-3p expression groups. The association between miR-1301-3p expression and the clinicopathological characteristics of patients with NSCLC was

Table I. Association between miR-1301-3p expression and the clinicopathological characteristics of patients with non-small cell lung cancer (n=124).

Characteristic	Patient, n	Low miR-1301-3p expression (n=51)	High miR-1301-3p expression (n=73)	P-value
Age, years				0.704
<60	59	30	29	
≥60	65	21	44	
Sex				0.453
Male	70	31	39	
Female	54	20	34	
TNM stage				0.025 ^a
I-II	86	39	47	
III-IV	38	12	26	
Lymph node metastasis				0.027 ^a
Negative	84	34	50	
Positive	40	17	23	
Differentiation				0.136
Well-moderate	79	39	40	
Poor	45	12	33	
Smoking				0.270
No	61	23	38	
Yes	63	28	35	
Tumor size, cm				0.262
<4	67	35	32	
≥4	57	16	41	

^aP<0.05. miR, microRNA; TNM, tumor-node-metastasis.

estimated by the Pearson's χ^2 test. The Kaplan-Meier method followed by log-rank test, and Cox regression analyses were performed to assess survival and determine the prognostic value of miR-1301-3p, respectively.

Results

miR-1301-3p expression is significantly upregulated in NSCLC tissues and cells. miR-1301-3p expression was significantly higher in NSCLC tissues compared with adjacent normal tissues (P<0.001; Fig. 1A). Similarly, miR-1301-3p expression was significantly upregulated in NSCLC cells compared with BEAS-2B cells (P<0.001; Fig. 1B).

miR-1301-3p expression is significantly associated with TNM stage and lymph node metastasis. The average expression level of miR-1301-3p (3.63) in NSCLC tissues was used as the cut-off value to divide 124 patients with NSCLC into high (n=73) and low (n=51) miR-1301-3p expression groups. As presented in Table I, patients with high miR-1301-3p expression were significantly associated with positive lymph node metastasis (P=0.027) and an advanced clinical stage (P=0.025).

Overexpression of miR-1301-3p is associated with a poor prognosis of patients with NSCLC. Survival information was obtained via a 5-year follow-up survey and plotted using the Kaplan-Meier method. As presented in Fig. 2, patients with

Table II. Cox regression analysis of miR-1301-3p and survival of patients with NSCLC.

Characteristic	HR factor	95% CI	P-value
miR-1301-3p	2.450	1.166-5.150	0.018
Age	1.223	0.657-2.276	0.525
Sex	1.290	0.671-2.481	0.445
TNM stage	2.162	1.093-4.276	0.027
Lymph node metastasis	2.181	1.087-4.374	0.028
Differentiation	1.548	0.824-2.907	0.175
Smoking	1.502	1.785-2.873	0.219
Tumor size	1.445	0.758-2.756	0.263

miR, microRNA; TNM, tumor-node-metastasis; HR, hazard ratio; CI, confidence interval.

high miR-1301-3p expression had a significantly shorter overall survival time than those with low miR-1301-3p expression (Log-rank P=0.022).

Cox regression analysis was performed to determine the prognostic value of miR-1301-3p. The results demonstrated that miR-1301-3p expression [hazard ratio (HR), 2.450; 95% confidence interval (CI), 1.166-5.150; P=0.018], TNM stage (HR, 2.162; 95% CI, 1.093-4.276; P=0.027) and lymph node

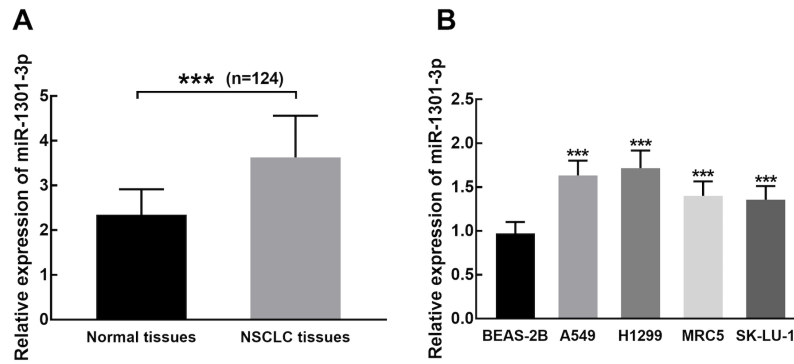


Figure 1. miR-1301-3p expression is upregulated in NSCLC tissues and cells. (A) miR-1301-3p expression was significantly higher in NSCLC tissues compared with adjacent normal tissues. (B) miR-1301-3p expression was significantly upregulated in the NSCLC cell lines, A549, H1299, MRC5 and SK-LU-1, compared with normal BEAS-2B epithelial cells. *** $P < 0.001$ vs. normal tissues and BEAS-2B cells. miR, microRNA; NSCLC, non-small cell lung cancer.

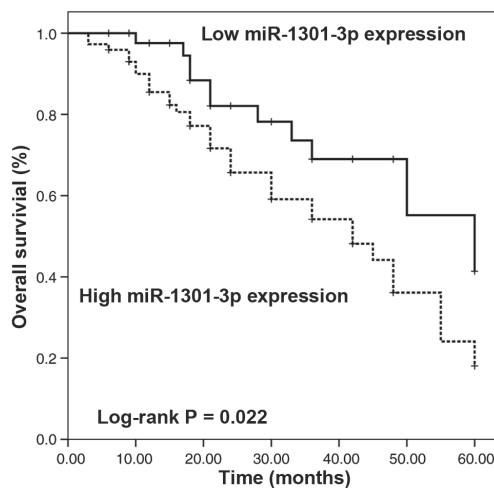


Figure 2. Kaplan-Meier curve summarizing the survival information of patients based on miR-1301-3p expression. Patients with high miR-1301-3p expression had a significantly shorter overall survival time than those with low miR-1301-3p expression. Log-rank $P = 0.022$. miR, microRNA.

metastasis (HR, 2.181; 95% CI, 1.087-4.374; $P = 0.028$) were independent factors for the clinical prognosis of patients with NSCLC (Table II).

Overexpression of miR-1301-3p promotes proliferation, migration and invasion of NSCLC cells. To determine the biological function of miR-1301-3p in NSCLC, miR-1301-3p expression was regulated by transfection with miR-1301-3p mimic or miR-1301-3p inhibitor. RT-qPCR analysis demonstrated that transfection with miR-1301-3p mimic significantly increased miR-1301-3p expression in A549 and H1299 cells, while miR-1301-3p inhibitor significantly decreased miR-1301-3p expression compared with the mock and negative controls ($P < 0.001$; Fig. 3A).

Proliferation of the transfected cells was detected via the CCK-8 assay. Overexpression of miR-1301-3p promoted A549 and H1299 cell proliferation, whereas miR-1301-3p knockdown significantly suppressed the proliferation of A549 and H1299 cells ($P < 0.01$; Fig. 3B). Similarly, the migratory and invasive abilities of NSCLC cells significantly enhanced

following overexpression of miR-1301-3p, the effects of which were reversed following miR-1301-3p knockdown ($P < 0.001$; Fig. 4A and B).

Thy-1 is a direct target of miR-1301-3p. To determine the molecular mechanism underlying the biological function of miR-1301-3p in NSCLC, the target of miR-1301-3p was investigated. The target of miR-1301-3p was first predicted using the TargetScan database, and the potential binding sites are presented in Fig. 5A, which was further confirmed via the dual-luciferase reporter assay.

The luciferase activity of Thy-1 was inhibited following overexpression of miR-1301-3p and increased following miR-1301-3p knockdown in the Thy-1 wild-type group ($P < 0.001$; Fig. 5B). Conversely, neither miR-1301-3p overexpression or knockdown affected the luciferase activity of Thy-1 in the Thy-1 mutant group (Fig. 5B), suggesting that Thy-1 is a direct target of miR-1301-3p.

Discussion

NSCLC is a common malignant tumor that accounts for the majority of lung cancer cases, including adenocarcinoma, squamous cell carcinoma, large cell carcinoma and several other subtypes (20). The clinical outcome of NSCLC remains unsatisfactory due to the high metastasis and recurrence rates (4). It is well-known that miRNAs play important roles in the progression and development of several diseases, including cancers (21-23). For example, miR4766-5p, miR-1915-3p and miR-615-3p are associated with tumor development in gastric cancer (24-26). Several miRNAs have been identified as effective biomarkers for the progression of NSCLC. For example, miR-330-3p has been identified as an upregulated miRNA in NSCLC, in which upregulation remarkably promotes the proliferation, invasion and migration of NSCLC cells by activating the MAPK/ERK signaling pathway (27). The prognostic value of several miRNAs has also been reported in NSCLC and different types of cancer (28,29). For example, downregulated miR-5702 expression is associated with the clinical progression and poor prognosis of patients with NSCLC (30). Furthermore, miR-552 has been identified as a prognostic predictor for patients with colorectal cancer (31).

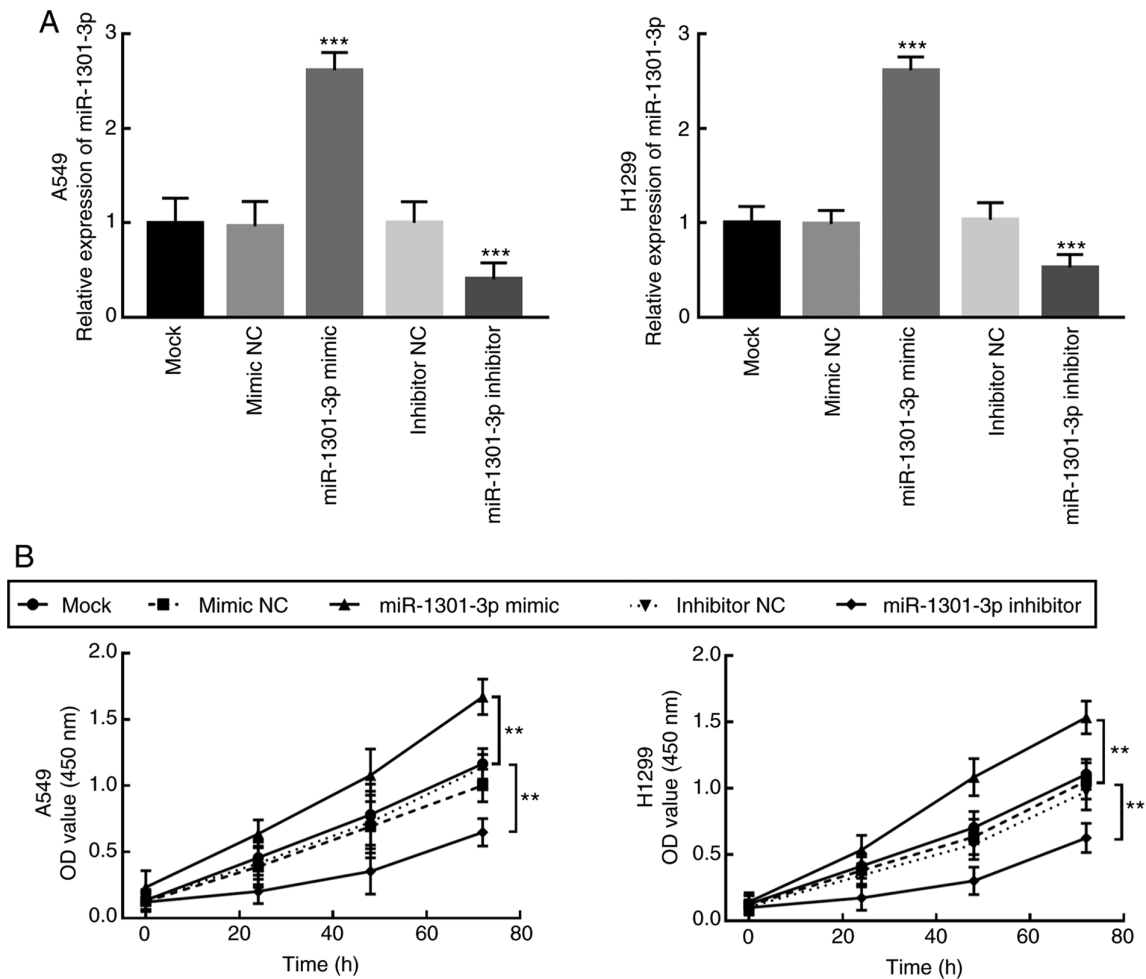


Figure 3. Overexpression of miR-1301-3p enhances the proliferation of non-small cell lung cancer cells *in vitro*. (A) miR-1301-3p expression was overexpressed in A549 and H1299 cells following transfection with miR-1301-3p mimic, and downregulated following transfection with miR-1301-3p inhibitor. (B) A549 and H1299 cell proliferation was enhanced following overexpression of miR-1301-3p and inhibited following miR-1301-3p knockdown. ** $P < 0.01$, *** $P < 0.001$ vs. mock groups. miR, microRNA; NC, negative control; OD, optical density.

In the present study, miR-1301-3p expression was significantly upregulated in NSCLC tissues and cell lines, which is consistent with the miRNA expression profile of NSCLC (17). Dysregulation of miR-1301-3p expression was significantly associated with the TNM stage and lymph node metastasis of patients, suggesting that miR-1301-3p may be involved in the development of NSCLC. In addition, overexpression of miR-1301-3p was associated with the poor prognosis of patients and was identified as an independent indicator for the clinical prognosis of NSCLC, along with advanced TNM stage and lymph node metastasis, which is consistent with a previous study on colorectal cancer (32,33).

The levels of miR-1301 and its biological functions vary in different types of cancer. For example, downregulated miR-1301-3p expression in breast cancer acts as a tumor suppressor that inhibits cell proliferation and induces cell apoptosis (14). Similarly, in colorectal cancer, overexpression of miR-1301-3p suppresses cell proliferation and invasion, induces cell apoptosis, and decreases the volume and weight of colorectal tumors (33). Conversely, miR-1301-3p promotes cell expansion of prostate cancer stem cells by inhibiting GSK3 β and SFRP1 and activating the Wnt pathway (15).

In the present study, overexpression of miR-1301-3p promoted the proliferation, migration and invasion of NSCLC cells, suggesting that miR-1301-3p may exert a tumor promoting role in the progression of NSCLC. In addition, thy-1 was identified as a direct target of miR-1301-3p. Thy-1 is a glycosylphosphatidylinositol-linked outer membrane leaflet glycoprotein (34). In a previous study, thy-1 has been reported to suppress myofibroblastic differentiation of lung fibroblasts, which is closely associated with the occurrence of lung cancer (35). In addition, thy-1 has also been reported to exert adverse impacts on the prognosis of lung cancer (36). In melanoma, thy-1 contributes to cell metastasis by mediating the adhesion of melanoma cells (37). Thus, it was concluded that the promoting effects of miR-1301-3p were mediated by regulating thy-1.

Among the clinicopathological characteristics of patients with NSCLC, tumor size, differentiation and smoking status are important factors to assess the clinical outcome of patients (38-40). However, the small sample size limited the results of the present study, which failed to exhibit a significant association between these factors and the overall survival of patients with NSCLC. Furthermore, *in vivo* experiments are

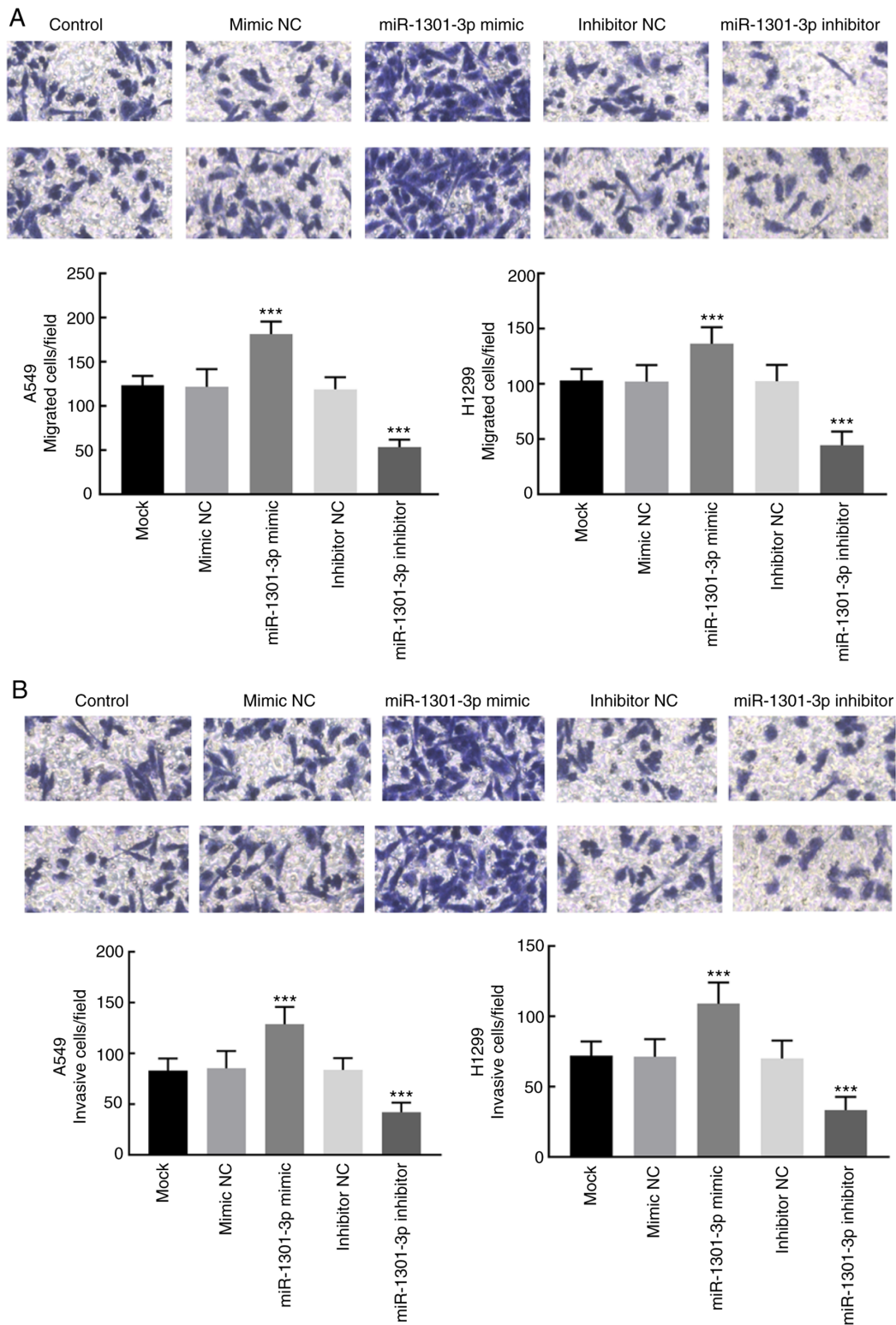


Figure 4. Overexpression of miR-1301-3p promotes the migratory and invasive abilities of non-small cell lung cancer cells *in vitro*. (A) The migration of A549 and H1299 cells was promoted following overexpression of miR-1301-3p and inhibited following miR-1301-3p knockdown. (B) The invasion of A549 and H1299 cells was promoted following overexpression of miR-1301-3p and inhibited following miR-1301-3p knockdown. *** $P < 0.001$ vs. mock groups. miR, microRNA; NC, negative control.

required in prospective studies to confirm the results presented here.

In conclusion, the results of the present study demonstrated that miR-1301-3p expression was significantly upregulated in NSCLC tissues and cells, which was

closely associated with the TNM stage and lymph node metastasis of patients. In addition, miR-1301-3p expression, advanced TNM stage and lymph node metastasis served as independent prognostic factors for NSCLC. Overexpression of miR-1301-3p significantly promoted the proliferation,

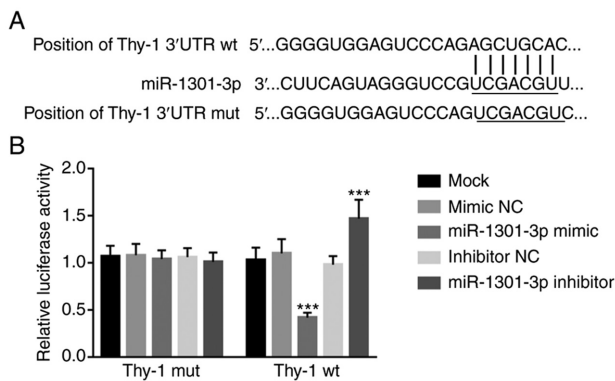


Figure 5. Thy-1 is a direct target of miR-1301-3p. (A) The predicting binding sites of miR-1301-3p in the 3'-UTR of Thy-1-wt and Thy-1-mut. (B) Overexpression of miR-1301-3p inhibited the luciferase activity of Thy-1, while miR-1301-3p knockdown enhanced the luciferase activity of Thy-1. *** $P < 0.001$ vs. mock groups. miR, microRNA; UTR, untranslated region; wt, wild-type; mut, mutant; NC, negative control.

migration and invasion of NSCLC cells by targeting thy-1. MiR-1301-3p was identified as a tumor promoter in NSCLC by regulating thy-1, which requires further *in vivo* validation.

Acknowledgements

Not applicable.

Funding

The present study was funded by Binzhou Medical University Research Program and Research Startup Fund (grant no. BY2014KJ48).

Availability of data and materials

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

Authors' contributions

All authors contributed to the conception and design of the present study. LX, NN and PH performed the experiments. LX contributed to analysis and interpretation of the data. HG revised the manuscript for important intellectual content. All authors drafted the initial manuscript, and have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Binzhou Medical University Hospital (Binzhou, China; approval no. 201212), and written informed consent was provided by all patients prior to tissue collection.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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