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MicroRNA-154 functions as a tumor suppressor and directly targets *HMGA2* in human non-small cell lung cancer

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ABSTRACT. MicroRNA-154 (miR-154) is dysregulated in some human malignancies and is correlated with tumor progression. However, its expression and function in non-small cell lung cancer (NSCLC) remain unclear. Therefore, we explored the effects of miR-154 on NSCLC tumorigenesis and development. Using quantitative reverse transcription-polymerase chain reaction, we detected miR-154 expression in NSCLC cell lines and primary tumor tissues. The association between miR-154 expression and clinicopathological factors was investigated, and the effects of miR-154 on the biological behavior of NSCLC cells were examined. Ultimately, the potential regulatory effect of miR-154 on high-mobility group A2 protein (HMGA2) expression was confirmed. miR-154 was significantly downregulated in NSCLC cell lines and clinical specimens. Reduced miR-154 expression was significantly associated with lymph node metastasis, advanced TNM stage, and shorter overall survival. Multivariate regression analysis confirmed that downregulation of miR-154 was an independent unfavorable prognostic factor for patients with NSCLC. Overexpression of miR-154 inhibited NSCLC cell

proliferation, invasion, and migration, and promoted cell apoptosis *in vitro*. Furthermore, a luciferase reporter assay identified *HMGA2* as a direct target of miR-154. Our findings indicate that miR-154 may act as a tumor suppressor in NSCLC and would serve as a novel therapeutic agent for miR-based therapy.

Key words: miR-154; High-mobility group A2; Proliferation; Prognosis; Non-small cell lung cancer

INTRODUCTION

Lung cancer is the leading cause of cancer death worldwide and non-small cell lung cancer (NSCLC) accounts for nearly 85% of all cases (Siegel et al., 2012). Despite recent advances in experimental and clinical oncology, NSCLC is mostly diagnosed in the late stages, and survival has not improved significantly over the past several decades. To date, the mechanisms underlying NSCLC carcinogenesis and progression remain poorly understood. Therefore, there is an urgent need to elucidate the regulatory network underlying NSCLC and develop novel biomarkers for its early diagnosis, accurate assessment, targeted therapy, and prognosis evaluation.

MicroRNAs (miRs) comprise a class of short (about 22 nucleotides long), endogenous, single-stranded, non-protein-coding RNAs that directly bind to the 3'-untranslated regions (3'-UTRs) of target messenger RNAs (mRNAs), leading to mRNA degradation or translational suppression (Bartel, 2009). It is now well known that miRs are involved in many different biological processes such as cell growth, apoptosis, development, differentiation, and endocrine homeostasis (Bartel, 2004). Accumulating evidence also suggests that miRs play an essential role in the biology of human cancers and may provide a new and promising means of dealing with cancer (Heneghan et al., 2010). Dysregulation of miR expression has been frequently reported and is closely associated with tumor initiation and development. miR-154 has been implicated in the pathogenesis of several human malignancies. It is downregulated in hepatocellular carcinoma (Wang et al., 2011a), colorectal cancer (Xin et al., 2014), and prostate cancer (Formosa et al., 2014), and acts as a candidate tumor suppressor. Decreased miR-154 expression in these tumors is associated with tumor progression and poor patient survival. However, the correlation between miR-154 expression and the clinicopathological characteristics of NSCLC has not been determined, and the biological role of miR-154 and its direct functional targets in NSCLC remain obscure.

Human high-mobility group A2 (HMGA2), an important member of the HMGA family, is a non-histone chromatin-binding protein. It contains three AT-hook domains that enable its binding to the minor groove of AT-rich DNA sequences. The level of HMGA2 expression is low, or even undetectable, in most normal adult tissues (Rogalla et al., 1996). However, re-expression of HMGA2 has been reported in many types of human cancer, including oral squamous cell carcinoma (Miyazawa et al., 2004), breast cancer (Sun et al., 2013), lung cancer (Meyer et al., 2007), pancreatic cancer (Ma et al., 2014), colorectal cancer (Wang et al., 2011b), bladder cancer (Yang et al., 2011), prostate cancer (Winkler et al., 2007), and ovarian cancer (Hetland et al., 2012). Tumors with high HMGA2 levels are prone to increased invasiveness and are associated with a poor prognosis. The mechanisms regulating HMGA2 expression are imperfectly understood, and recent studies have confirmed post-

transcriptional control of HMGA2 expression by miR-98 and miR-let-7a in lung cancer (Wang et al., 2013; Xiang et al., 2013).

In the present study, we investigated the expression and clinical significance of miR-154 in NSCLC. The effects of miR-154 on NSCLC cell phenotypes were also examined. Furthermore, a luciferase reporter assay identified *HMGA2* as a direct target of miR-154.

MATERIAL AND METHODS

Patients and clinical specimens

Paired NSCLC and adjacent non-cancerous lung tissues were obtained from 123 patients who received curative resection of NSCLC in Fuzhou General Hospital of Nanjing Military Command between January 2009 and December 2010. These tissues were flash-frozen in liquid nitrogen immediately after resection and stored at -80°C until required. None of the patients had received neoadjuvant chemo- or radio-therapy before surgery. The patient characteristics are shown in Table 1. Follow-up information was available for all patients. Overall survival was defined as the time from the day of operation to death or, for living patients, the date of last follow-up. This study was approved by the Research Ethics Committee of our hospital, and written informed consent was obtained from each patient.

Cell lines and miR transfection

Four NSCLC cell lines (A549, H460, 95D and HCC827) and normal lung epithelial cells (NLEC) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin G sodium, and 100 ng/mL streptomycin sulfate. All the cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

For RNA transfection, the cells were seeded into each well of a 24-well plate and incubated overnight, then transfected with mature miR-154 mimics, miR-154 inhibitors (anti-miR-154), or a negative control (miR-NC or anti-miR-NC) (GenePharma, Shanghai, China) at a concentration of 50 nM using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the cells and tissues with TRIzol reagent (Invitrogen). Complementary DNA (cDNA) was reverse-transcribed from total RNA samples using specific miR primers from the TaqMan MicroRNA Assays and reagents from the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). The products were amplified by PCR using a TaqMan Universal PCR Master Mix kit (Applied Biosystems). Small nuclear RNA U6 was used as an internal standard for normalization. All reactions were carried out in triplicate, and the 2^{-DCt} method ($\Delta Ct = Ct_{miR-154} - Ct_{U6}$) was used to quantify the relative amount of miR-154.

Analysis of cell proliferation *in vitro*

The *in vitro* cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Briefly, following transfection, the cells were seeded onto 96-well plates (2 x 10^4 cells per well) and incubated at 37°C. At different time points (24, 48, 72, or 96 h), the culture medium was removed and replaced with fresh medium containing 0.5 mg/mL MTT. Cells were then incubated for another 4 h and resolved using dimethyl sulfoxide (DMSO) (Sigma). The absorbance was measured at 490 nm.

Detection of apoptosis by flow cytometry

Apoptosis was detected by flow cytometric analysis. Brieñy, the cells were washed and resuspended at a concentration of 1 x 10^6 cells/mL. The cells were then stained with Annexin V and propidium iodide, using the Annexin V apoptosis detection kit. After incubation at room temperature in the dark for 15 min, cell apoptosis was investigated on a FACSCalibur cell analyzer (Becton, Dickinson and Company, San Jose, CA, USA).

Transwell invasion assay

The invasion assay was performed using 24-well Transwell chambers (8 mm; Corning). After transfection, tumor cells were resuspended in serum-free RPMI-1640 medium, and 2 x 10^5 cells were seeded into the upper chambers and covered with 1 mg/mL Matrigel. RPMI-1640 (0.5 mL) containing 10% FBS was added to the bottom chambers. Following a 24-h incubation, the non-filtered cells were gently removed with a cotton swab. Filtered cells located on the lower side of the chamber were stained with 0.1% crystal violet (Sigma) and counted under a microscope (Olympus Corp., Tokyo, Japan).

Scratch migration assay

A scratch migration assay was performed to observe the influence of miR-154 on NSCLC cell migration. When the cells transfected with miR-154 mimics, miR-154 inhibitors, or NC had grown to confluence, a scratch in the cell monolayer was made with a cell scratch spatula. After the cells had been incubated under standard conditions for 24 h, pictures of the scratches were taken using a digital camera system coupled with a microscope.

Luciferase reporter assays

A fragment of the *HMGA2* 3'-UTR and a mutated 3'-UTR of *HMGA2* that contained the putative miR-154 binding sites were prepared to construct reporter plasmids containing the 3'-UTR regions of *HMGA2*. DNA fragments were cloned into the downstream of the luciferase gene in the pGL3-REPORT luciferase vector (Invitrogen). For the luciferase assay, cells were seeded onto 24-well plates and cultured for 24 h. The cells were then co-transfected with pGL3-3'-UTR and the control reporter plasmid, miR-154 or NC mimics, and miR-154 inhibitor or NC inhibitor. Two days later, the cells were harvested and lysed in passive lysis buffer, and reporter activity was measured using a dual-luciferase reporter assay system (Promega). Firefly luciferase activity was normalized to *Renilla* luciferase activity for each transfected well.

Western blotting analysis

Protein lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After blocking, the membranes were incubated with purified rabbit anti-HMGA2 antisera at 4°C overnight. The following day, the membranes were washed with phosphate-buffered saline and then incubated with peroxidase-conjugated goat anti-rabbit IgG. Immunodetection was carried out using enhanced chemiluminescence reagents (Pierce) and exposure on an X-ray film. b-actin was used as an internal reference for relative quantification.

Statistical analysis

All data are reported as means ± SD. Statistical analysis was carried out using the SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). Differences between groups were analyzed using the Student *t*-test or the chi-square test. Relationships between miR-154 expression and HMGA2 protein levels were explored by Pearson correlation analysis. Survival curves were constructed using the Kaplan-Meier method and compared by log-rank tests. To evaluate independent prognostic factors associated with survival, a multivariate Cox proportional hazard regression analysis was used. $P < 0.05$ was considered to be statistically significant.

RESULTS

Decreased expression of miR-154 in NSCLC and its correlation with HMGA2 levels

We performed qRT-PCR analysis to detect miR-154 expression in NSCLC tissues and cell lines. As can be seen in Figure 1A, the results showed that the expression levels of miR-154 were significantly lower in NSCLC specimens (mean ± SD: 13.21 ± 3.46) than in the corresponding adjacent non-cancerous tissues (mean ± SD: 22.65 ± 5.24; $P < 0.001$). miR-154 expression in the four NSCLC cell lines was also clearly downregulated (Figure 1B). Because A549 cells exhibited the lowest miR-154 expression while 95D cells expressed relatively high levels of miR-154 among the four NSCLC cell lines, these two cell lines were selected for miR-154 mimic- or miR-154 inhibitor-transfection in the subsequent studies.

HMGA2 protein levels were detected using western blotting analysis. The results showed that HMGA2 protein levels in the tumor samples were higher than in the adjacent normal tissues ($P < 0.001$; Figure 1C). HMGA2 levels in the NSCLC cells were also higher than in the NLEC cells (Figure 1D). In addition, we observed an obvious inverse correlation ($R = -0.4142$, $P = 0.004$) between HMGA2 levels and miR-154 expression in the NSCLC tumor tissues (Figure 1E).

miR-154 expression and clinicopathologic features in NSCLC

The associations between miR-154 expression and various clinicopathological parameters of NSCLC tissues are summarized in Table 1. Using the median miR-154 expression in all 123 NSCLC patients as a cutoff, the patients were divided into high and low miR-154 expression groups. As shown in Table 1, the miR-154 expression level was lower in samples with lymph node metastasis ($P = 0.004$) and advanced TNM stage ($P < 0.001$). No significant difference was observed between miR-154 expression and patients' age, gender, smoking habits, cell types, T stage, or tumor differentiation.

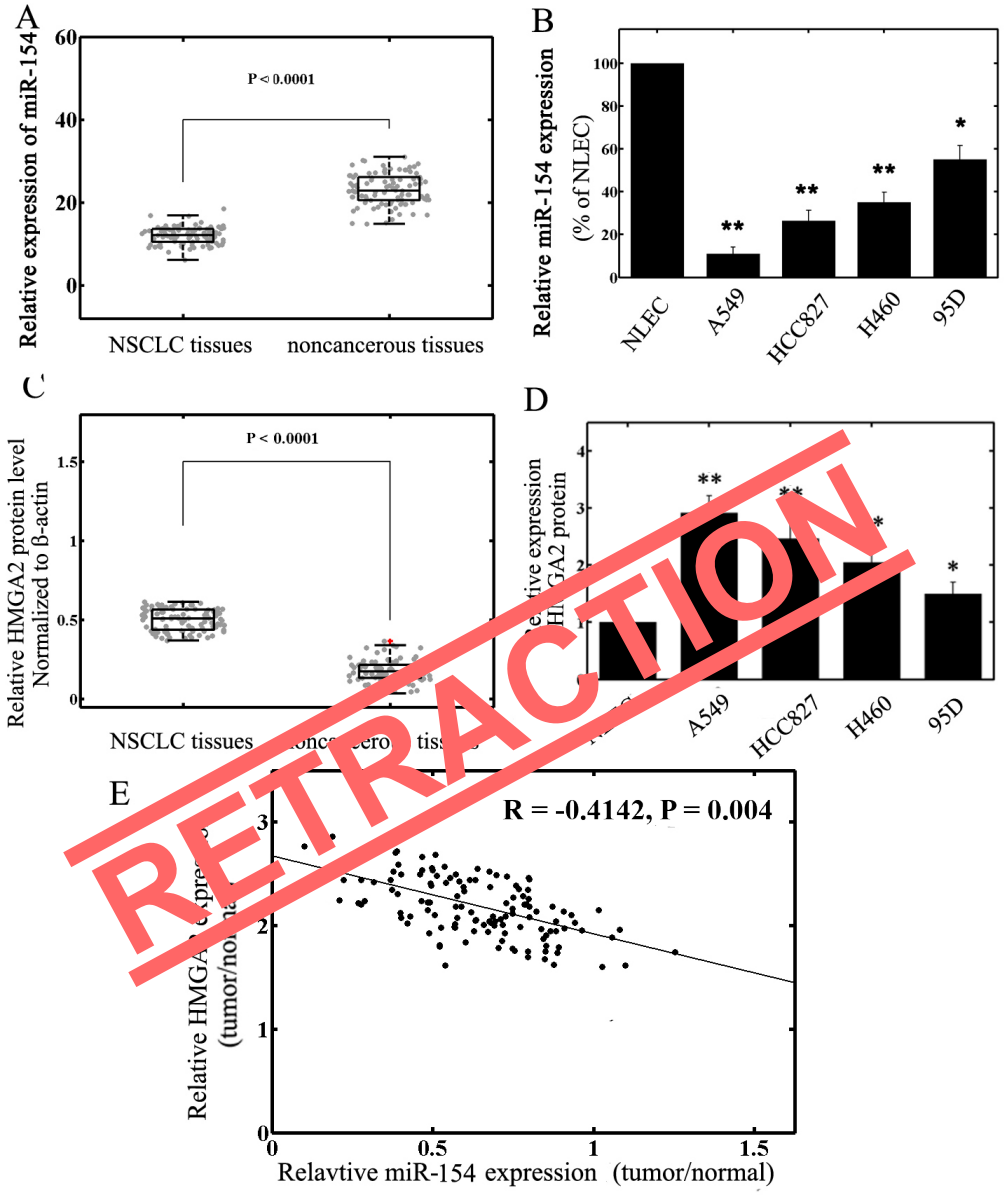

Figure 1. Expression of microRNA-154 (miR-154) and HMGA2 in non-small cell lung cancer (NSCLC) tissues and cell lines. **A.** miR-154 expression was significantly lower in NSCLC tissues than in the corresponding non-cancerous tissues. miR-154 expression levels were calculated by the $2^{-\Delta Ct}$ method and normalized to U6 small nuclear RNA. **B.** miR-154 expression was downregulated in NSCLC cell lines A549, H460, 95D, and HCC827, compared with normal lung epithelial cells (NLEC). *P < 0.05; **P < 0.01. **C.** Relative HMGA2 protein levels in NSCLC and corresponding non-cancerous tissues. HMGA2 protein levels were measured by western blotting analysis and normalized to β-actin. **D.** HMGA2 protein levels in NSCLC cells were higher than in normal lung epithelial cells. *P < 0.05; **P < 0.01. **E.** The inverse correlation of HMGA2 protein levels with miR-154 expression was examined by Pearson correlation analysis.

Table 1. Correlation between microRNA-154 (miR-154) expression and different clinicopathological features in non-small cell lung cancer.

Clinicopathological features	No. of cases	miR-154 expression		P
		Low (N, %)	High (N, %)	
Age (years)				
<60	62	36 (58.1%)	26 (41.9%)	0.106
≥60	61	26 (42.6%)	35 (57.4%)	
Gender				
Male	81	42 (51.9%)	39 (48.1%)	0.709
Female	42	20 (47.6%)	22 (52.4%)	
Smoking status				
Smoking	72	40 (55.6%)	32 (44.4%)	0.203
No smoking	51	22 (43.1%)	29 (56.9%)	
Histological type				
Squamous cell carcinoma	44	25 (56.8%)	19 (43.2%)	0.213
Adenocarcinoma	63	27 (42.9%)	36 (57.1%)	
Others	16	10 (62.5%)	6 (37.5%)	
Histological grade				
G1+G2	65	29 (44.6%)	36 (55.4%)	0.208
G3	58	33 (56.9%)	25 (43.1%)	
T classification				
T_{1+2}	81	38 (46.9%)	43 (53.1%)	0.343
T_3	42	24 (57.1%)	18 (42.9%)	
N classification				
Positive	84	50 (59.5%)	34 (40.5%)	0.004
Negative	39	12 (30.8%)	27 (69.2%)	
TNM stage				
I + II	73	27 (37.0%)	46 (63.0%)	<0.001
III	50	35 (70.0%)	15 (30.0%)	

Downregulation of miR-154 corresponds to poor prognosis in patients with NSCLC

We then determined whether miR-154 expression had prognostic potential for the overall survival of NSCLC patients. Using the Kaplan-Meier method and the log-rank test, we found that the survival rate of patients with high miRNA-154 expression was higher than in patients with low miRNA-154 expression ($P < 0.001$; Figure 2). Moreover, survival benefits were also found in those with negative N classification ($P = 0.035$) and early TNM stage ($P < 0.001$; Table 2).

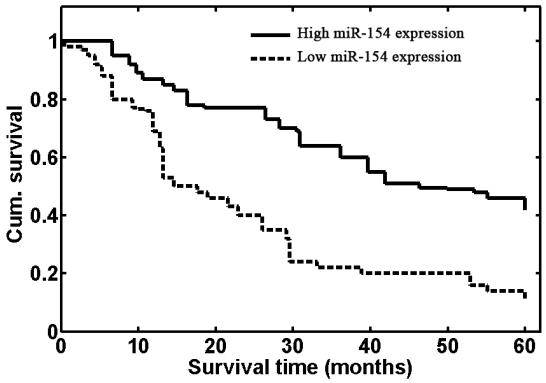

Figure 2. Overall survival curves for two groups defined by low and high expression of microRNA-154 (miR-154) in patients with non-small cell lung cancer (NSCLC). Low miR-154 expression levels were significantly associated with poor outcome ($P < 0.001$, log-rank test).

Table 2. Univariate and multivariate analysis of overall survival in 123 patients with non-small cell lung cancer.

Variables	Univariate log-rank test (P)	Cox multivariable analysis (P)	Relative risk (RR)
Age at diagnosis (years)			
<60 *vs* ≥60	0.56	-	-
Gender			
Male *vs* female	0.44	-	-
Smoking status			
Smoker *vs* never smoked	0.38	-	-
Histological type			
Squamous cell carcinoma *vs* others	0.65		
Histological grade			
(G1+G2) *vs* G3	0.21	-	-
T classification			
T_{1+2} *vs* T_3	0.18	-	-
N classification			
Positive *vs* negative	0.035	0.026	3.474
TNM stage			
I-II *vs* III	<0.001	0.003	6.552
miR-154 expression			
High *vs* low	<0.001	0.0	4.985

Multivariate Cox regression analysis enrolling the significant parameters mentioned above revealed that miR-154 expression (relative risk (RR) 4.985; P = 0.012), lymph node metastasis (RR 3.474; P = 0.026), and TNM stage (RR 6.552; P = 0.003) were independent prognostic markers for survival in NSCLC patients (Table 2).

Effects of miR-154 on the biological behavior of NSCLC cells

To selectively overexpress or downregulate miR-154, mature miR-154 mimics or miR-154 inhibitors were transfected into A549 or 95D cells. qRT-PCR analysis confirmed increased miR-154 expression after miR-154 mimic transfection and decreased miR-154 expression following miR-154 inhibitor transfection (Figure 3A). The MTT assay showed that cell proliferation was significantly impaired in the miR-154 mimic-transfected A549 cells, while proliferation of 95D cells was increased in the miR-154 inhibitor-transfected cells compared with the controls (Figure 3B).

Flow cytometry was employed to determine the effect of miR-154 on cell apoptosis. The proportion of apoptotic A549 cells transfected with miR-154 mimics was significantly higher than in the negative control group. Moreover, downregulation of miR-154 reduced 95D cell apoptosis (Figure 3C).

A Transwell invasion assay was performed to investigate whether miR-154 had a direct influence on NSCLC cell invasion. As shown in Figure 4A, upregulation of miR-154 impeded the invasion of A549 cells. Conversely, transfection of 95D cells with miR-154 inhibitors promoted cell invasion capability. The scratch migration assay also confirmed the inhibitory effect of miR-154 on NSCLC cell migration (Figure 4B).

HMGA2 is the target gene of miR-154

Analysis of the 3'-UTR sequence of *HMGA2* using TargetScan revealed two putative binding sites for miR-154: one is located between nucleotides 163 and 169, and the other is

located between nucleotides 296 and 302 (Figure 5A). To further validate the predicted target, we fused the predicted *HMGA2* 3'-UTR target site, including binding sites 1 or 2 for miR-154, to the downstream of the firefly luciferase gene. The reporter plasmids were co-transfected into A549 cells along with miR-154 mimics or NC.

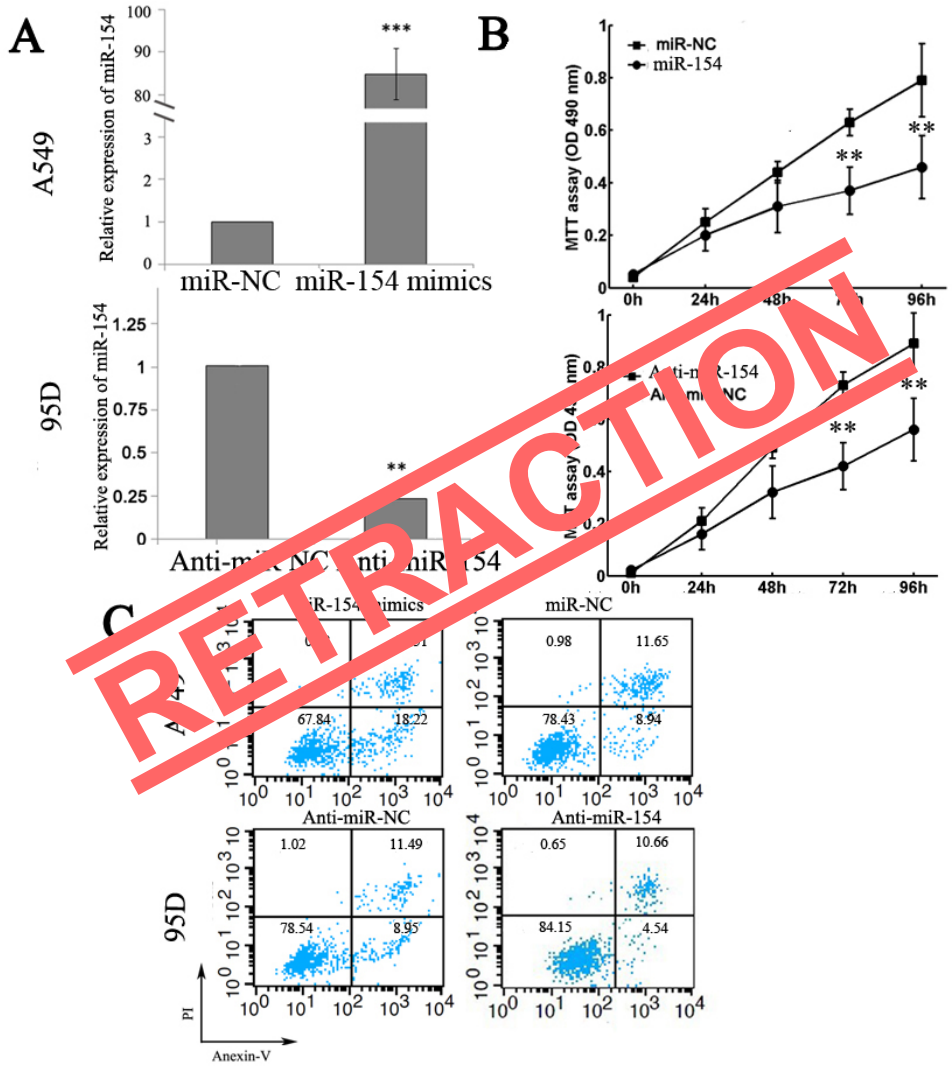

Figure 3. Effects of microRNA-154 (miR-154) mimic or inhibitor transfection on non-small cell lung cancer (NSCLC) cell proliferation and apoptosis. **A.** Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis confirmed increased miR-154 expression in A549 cells transfected with miR-154 mimics, and decreased miR-154 expression in 95D cells transfected with miR-154 inhibitors. U6 RNA was used as an internal control. ***P < 0.001. **B.** MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] showed that miR-154 reduced cell proliferation *in vitro*. Data are reported as means ± SD of the experiments performed in triplicate. **P < 0.01. **C.** Cell apoptosis was detected by flow cytometric analysis after transfection with miR-154 mimics, miR-154 inhibitors (anti-miR-154), or negative control (miR-NC).

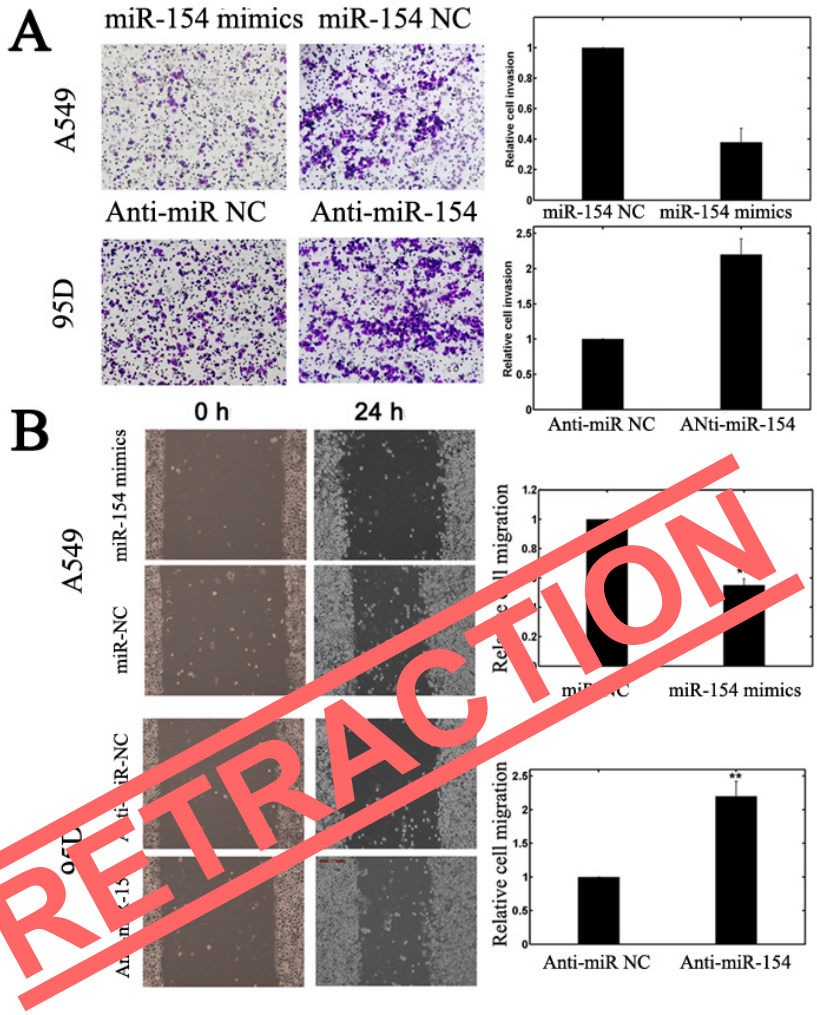

Figure 4. Effects of microRNA-154 (miR-154) mimic or inhibitor transfection on non-small cell lung cancer (NSCLC) cell invasion and migration. **A.** The Transwell invasion assay showed that upregulation of miR-154 impeded the invasion of A549 cells, while transfection of 95D cells with miR-154 inhibitors promoted cell invasion. **B.** The scratch migration assay confirmed the inhibitory effect of miR-154 on NSCLC cell migration. **P < 0.01. For abbreviations, see Figure 3.

The miR-154 mimics significantly suppressed the luciferase activity of the reporter plasmid containing two binding sites compared with the NC of the wild-type reporter, but not the activity of the mutant. In contrast, inhibition of endogenous miR-154 by the miR-154 inhibitor significantly increased the luciferase reporter gene activity compared with co-transfection with the NC inhibitor of the wild-type reporter (Figure 5B and C). Taken together, our results suggest that *HMGA2* is a direct target gene of miR-154.

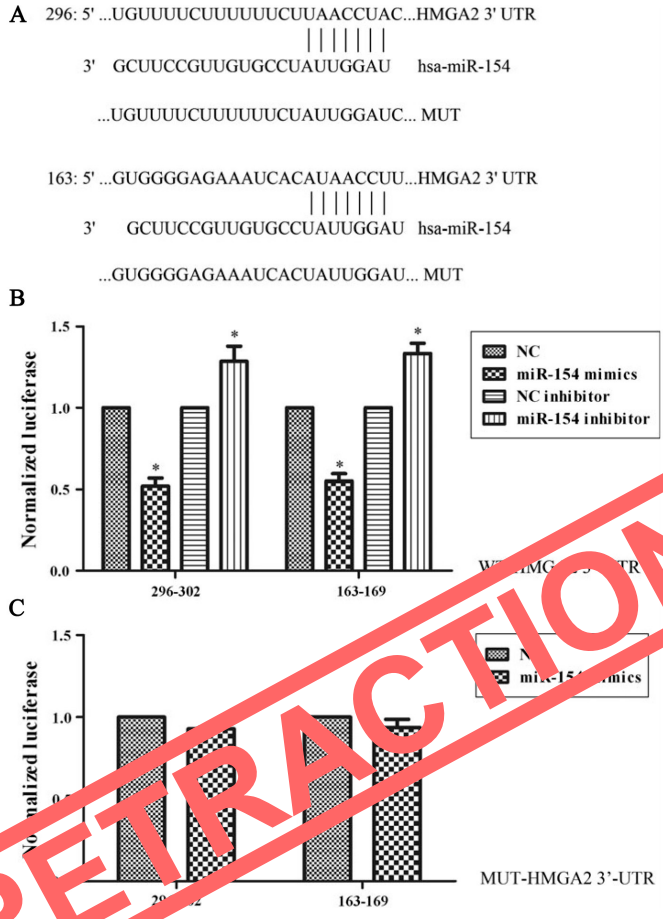

Figure 5. mic[illegible]154 [illegible]154) suppresses HMGA2 expression by binding to the *HMGA2* 3'-untranslated region (UTR). [illegible] Schemat[illegible] description of the *HMGA2* 3'-UTR with two putative binding sites for miR-154. **B.** and **C.** Dual-lucifera[illegible]ssay results for A549 cells suggesting that *HMGA2* is a target gene of miR-154. *P < 0.05.

DISCUSSION

Dysregulation of miRs is involved in tumorigenesis and progression in various types of tumor; however, the potential roles of miRs in NSCLC require clarification. In the present study, we revealed decreased miR-154 expression in NSCLC and its correlation with aggressive clinicopathological features and poor survival. Overexpression of miR-154 could significantly inhibit cell proliferation, invasion, and migration, and promote cell apoptosis *in vitro*. Moreover, *HMGA2* was identified as a direct target of miR-154. To the best of our knowledge, this is the first study to investigate the clinical significance and biological function of miR-154 in NSCLC.

miR-154 has the human chromosomal locus 14q32, which is frequently lost in human cancers (Dai et al., 2005; Pécuchet et al., 2013; Manodoro et al., 2014), and miR-154 downregulation has been reported to play an important role in cancer progression. For

instance, Xin et al. (2014) showed that miR-154 expression was reduced in colorectal cancer tissues and cell lines. Ectopic expression of miR-154 markedly suppressed cell proliferation and colony formation, migration, and invasion in colorectal cancer cells. Wang et al. (2011a) found that restoration of intracellular miR-154 suppressed tumor cell malignancy and the G1/S transition in hepatocellular cancer cells. Zhu et al. (2013) reported low miR-154 expression levels in primary prostate cancer compared with nonmalignant samples. Forced expression of miR-154 significantly reduced the migratory and invasive capabilities of prostate cancer cells. Taken together, these results reveal that miR-154 might act as a tumor suppressor in a variety of tumors, and loss of miR-154 might play a critical role in cancer formation and progression.

It is now clear that miRs exert their oncogenic or tumor suppressor functions by regulating the expression of target genes (Liu et al., 2014). With regard to miR-154, several targets have been confirmed in previous research including the cyclin D2 gene (*CCND2*) and the Toll-like receptor 2 gene (*TLR2*) (Xin et al., 2014; Zhu et al., 2014). The *HMGA2* gene has the chromosomal locus 12q14 and encodes a 109-amino acid protein. As a tumor-promoting gene, *HMGA2* is upregulated in different tumor types, and has been identified as a target gene of a number of miRs. The important role of HMGA2 has been strongly underlined in numerous papers, owing to its role in inducing epithelial-mesenchymal transition and facilitating the proliferation and metastasis of cancer cells. Kumar et al. (2014) corroborated the contribution of HMGA2 in NSCLC progression and metastasis. Using a luciferase reporter assay, our study demonstrated that *HMGA2* was a direct target of miR-154 in NSCLC. However, there is no 'one-to-one' connection between miRs and target mRNAs. A typical miR can have more than 100 targets (Brennecke et al., 2005). Conversely, several miRs can converge on a single transcript target (Krek et al., 2005). *HMGA2* is not the only miR-154 target that is dysregulated in NSCLC. Other functional targets of miR-154, such as *CCND2* and *TLR2* (Salskov et al., 2011; Ke et al., 2014), also modulate NSCLC pathogenesis. Therefore, the potential regulatory function afforded by miR-154 is enormous and the exact mechanisms by which miR-154 influences NSCLC progression need further clarification.

In conclusion, our results revealed that miRNA-154 was downregulated in NSCLC and correlated with aggressive clinicopathological features. The overexpression of miR-154 resulted in a tumor suppressive effects in functional analysis. *HMGA2* was identified as a direct target gene of miR-154. These findings suggest that miR-154 may act as a tumor suppressor in NSCLC and is a potential candidate for miR-based therapy against NSCLC.

Conflicts of interest

The authors declare no conflict of interest.

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