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Original Article

# MiRNA-218, a new regulator of HMGB1, suppresses cell migration and invasion in non-small cell lung cancer

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**MicroRNAs (miRNAs) function as negative regulators of gene expression involved in cancer metastasis. The aim of this study is to investigate the potential roles of miR-218 in non-small cell lung cancer and validate its regulation mechanism. Functional studies showed that miR-218 overexpression inhibited cell migration and invasion, but had no effect on cell viability. Enhanced green fluorescent protein reporter assay, real-time polymerase chain reaction and western blot analysis confirmed that miR-218 suppressed the expression of high mobility group box-1 (HMGB1) by directly targeting its 3′-untranslated region. Accordingly, silencing of HMGB1 accorded with the effects of miR-218 on cell migration and invasion, and overexpression of HMGB1 can restore cell migration and invasion which were reduced by miR-218. In conclusion, these findings demonstrate that miR-218 functions as a tumor suppressor in lung cancer. Furthermore, miR-218 may act as a potential therapeutic biomarker for metastatic lung cancer patients.**

*Keywords* microRNA; miR-218; non-small lung cancer; HMGB1

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## Introduction

Lung cancer is the leading cause of cancer-related death worldwide, and it occurs more frequently in men than in women. It has a high mortality and the 5-year survival rate is only about 15%, threatening the human health [1,2]. Once diagnosed, the disease is often at late stage and even metastasizes to other organs. The potential mechanism accounting for the metastasis is less clear, so it is crucial to explore a novel biomarker for early detection and diagnosis.

MicroRNAs (miRNAs) are a family of small non-coding, endogenous single RNA molecules that play important roles in gene expression by binding to the 3′-untranslated region (3′-UTR) of target gene mRNA, leading to mRNA cleavage or translational repression [3]. Studies showed that miRNAs participate in various biological processes, such as cell differentiation, growth, and death and tumor development [4,5]. Until now, about 60% of protein-coding genes have been found to be regulated by miRNAs [6]. About 50% of miRNAs are located in fragile sites or cancer-related gene region [7], suggesting that abnormal expression of miRNAs is related to cancer pathogenesis. For instance, miR-451 inhibits the lung cancer proliferation and colony formation through suppression of *RAB14* (member RAS oncogene family), functioning as a tumor suppressor [8]. MiR-221 promotes the tumorigenesis of hepatocellular carcinoma *in vitro* and *in vivo* [9]. In addition, using meta-analysis for miRNA expression profiling between lung cancer tissues and normal tissues, Guan *et al*. [10] found that many miRNAs are deregulated in lung cancer, including miR-218. In another study, Davidson *et al*. [11] discovered that miR-218 acts as a potential tumor suppressor in lung cancer using bioinformatics mapping approach for identifying novel oncogenic or suppressive miRNAs from genomic-wide array comparative genomic hybridization datasets. However, there is limited report about the roles of miR-218, especially its regulation mechanism in lung cancer cells.

In the present study, we found that miR-218 played an anti-metastatic role by inhibiting cell migration and invasion. Further, we validated that high mobility group box-1 (HMGB1) was a direct target gene for miR-218 in lung cancer cells.

## Materials and Methods

### Cell culture

Human lung cancer cell lines, A549 and H1299, were cultured in RPMI1640 medium (Invitrogen, Carlsbad, USA), supplemented with 10% fetal bovine serum (FBS) and 1% PS (100 U/ml penicillin, 100 μg/ml streptomycin). The cells were maintained in a humidified incubator with 5% $CO_2$ at 37°C.

### Cell transfection

The miR-218 mimics, mimics control, HMGB1 siRNA, siRNA control and pcDNA3/HMGB1, and the control vector were purchased from GenePharma Company (Shanghai, China). Transfection was performed using Lipofectamine$^{TM}$ 2000 (Invitrogen,) according to the manufacturer's protocol. At 48 h after transfection, the cells were harvested for the subsequent experiments.

### RNA isolation and real-time polymerase chain reaction

The total RNA (including miRNA) was extracted by Trizol (Invitrogen) according to the manufacturer's instructions. For miRNA reverse transcription (RT), special miR-218 RT primer (5′-GTCGTATCCAGTGCAGGGTCCGAGGTGCA CTGGATACGACACATGGTT-3′) was used. *RNU6B* (U6 small nuclear B non-coding RNA) was used as an internal control. The sequences of U6 RT primer are: 5′-GTC GTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGAT ACGACAAAATATGGAAC-3′). For the RT of total RNA, oligo (dT) was used as a common primer and *β-actin* was used as an internal control. The real-time polymerase chain reaction (PCR) was performed by the SYBR Green PCR master Mix (Applied Biosystems, Foster City, USA) according to the following conditions: 95°C for 5 min followed by 40 cycles of amplification at 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s.

### MTT assay

The transfected cells were plated into 96-well plates at a density of 5000 cells per well. At 48 h after transfection, the cells were incubated with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] for 4 h at 37°C. Then the cells were agitated with MTT solvent on an orbital shaker for 10 min avoiding light. The absorbance at 570 nm ($A_{570\text{nm}}$) was measured with a spectrophometer.

### Transwell migration and invasion assays

The migration and invasion assays were performed using Transwell chamber (Millipore, Billerica, USA). For migration assay, the transfected cells were seeded into the upper chamber with serum-free medium ($2.5 \times 10^4$ cells), and the bottom of the chamber contained the RPMI1640 medium with 10% FBS. While for invasion assay, the chamber was coated with Matrigel, and the following steps were similar to migration assay. When the cells migrated or invaded for 20 h, the cells were fixed and stained with crystal violet. Then the pictures of migratory or invading cells were taken under a microscope and the cell numbers were counted.

### Enhanced green fluorescent protein reporter assay

The *HMGB1* 3′-UTR was amplified and inserted into the downstream of pcDNA3/EGFP vector. While the mutant *HMGB1* 3′-UTR was amplified using pcDNA3/EGFP-HMGB1 3′-UTR as the template. The cells were co-transfected with miR-218 mimics and wild-type or mutant *HMGB1* 3′-UTR, and the construct expressing red fluorescent protein (RFP) was as the spiked-in control. At 48 h after transfection, the cells were lysed with RIPA buffer [(50 mM Tris-HCl, pH 8.8, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)] and the fluorescence intensity was measured by an F-4500 Fluorescence Spectrophotometer (HITACHI, Tokyo, Japan).

### Western blot analysis

The cells were harvested at 48 h after transfection and lysed with RIPA buffer for 30 min at 4°C. The protein (20 μg) was loaded into 15% SDS–polyacrylamide gel electrophoresis for analysis. The first antibody was polyclonal rabbit anti-HMGB1 (1 : 200 dilutions; Merck Millipore) and anti-GAPDH antibody (1 : 1000 dilution; Abcam, San Francisco, USA). The secondary antibody was goat anti-rabbit IgG conjugated with horseradish peroxidase with a dilution of 1 : 1000. The bound antibodies were detected using enhanced chemiluminescence Plus western Blotting Detection system (GE Healthcare, Bethesda, USA). GAPDH was used as an internal control to normalize HMGB1 expression level.

### Statistical analysis

All the data were shown as mean ± standard deviation (SD) and the experiments were repeated three times. The difference was determined by two-tailed Student's *t*-test and $P < 0.05$ was considered statistically significant.

## Results

### MiR-218 overexpression inhibits cell migration and invasion in A549 and H1299 cells, without affecting cell growth

Although previous studies have shown that miR-218 is downregulated in lung cancer [11], the roles of miR-218 have not been elucidated. In this study, we transfected miR-218 mimics or controls into A549 and H1299 cells to investigate the functional significance of miR-218. The expression of miR-218 was analyzed by real-time PCR. The results showed that the cells transfected with miR-218 mimics had a higher miR-218 expression level compared with the cells transfected with controls [**Fig. 1(A)**]. MTT assay showed that miR-218 overexpression had no effect on cell growth in A549 and H1299 cells [**Fig. 1(B)**]. Furthermore, we compared the migration abilities of the transfected cells. As shown in [**Fig. 1(C)**], miR-218 overexpression reduced the number of migratory cells by about 50% in A549 and H1299 cells, compared with the controls. In consistent with migration, the cell invasion ability was also reduced by miR-218 in A549 and H1299 cells [**Fig. 1(D)**]. Since migration and invasion abilities are two essential aspects for cancer cell metastasis, these

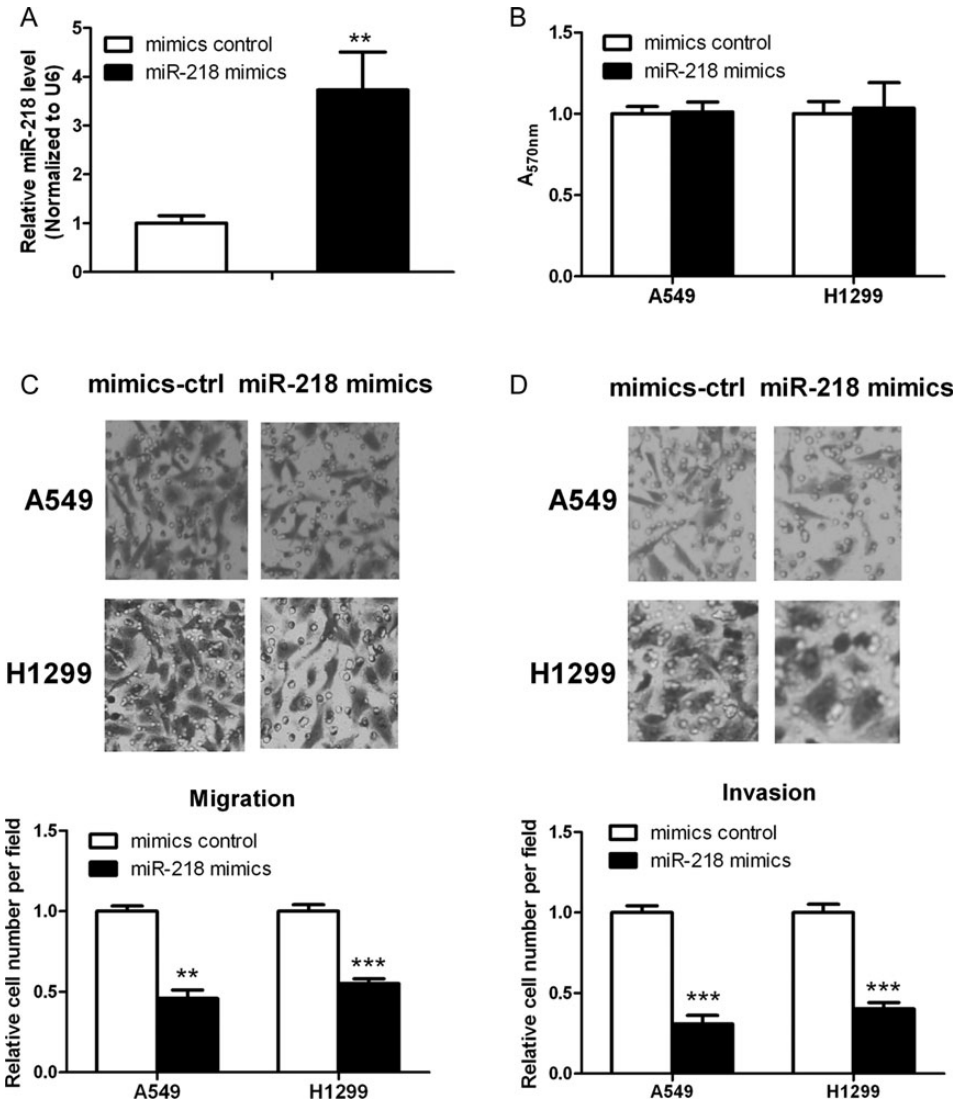


**Figure 1 miR-218 suppresses the cell migration and invasion in A549 and H1299 lung cancer cell lines** (A) The expression of miR-218 was determined by real-time PCR and U6 was used as an internal control. The relative level of controls was assigned as 1. $^{**}P < 0.01$. (B) The cell viability was determined by MTT assay in A549 and H1299 cells with miR-218 mimics or controls. (C,D) Top: representative migratory and invading A549 and H1299 cells with miR-218 mimics or controls, and the cells were stained with crystal violet. Bottom: representative number of migratory and invading cells ($\times 100$). The relative cell number of control in all experiments was assigned as 1. $^{**}P < 0.01$, $^{***}P < 0.001$.

data suggest that miR-218 may play an important role in inhibiting lung cancer metastasis.

### HMGB1 is a direct target for miR-218 in A549 and H1299 cells

Three bioinformatics tools, Targetscan, miRanda, and miRwalk were used to predict the target genes for miR-218, which mediated the anti-metastatic effects of miR-218 in A549 and H1299 cells. Consensually, considering the potential roles of target genes and the binding sites with miR-218, HMGB1 had the potential as a candidate target. First, we determined HMGB1 expression in lung cancer tissues by real-time PCR and found that HMGB1 was up-regulated in lung cancer tissues, compared with the paired non-tumor tissues [**Fig. 2(A)**]. To determine HMGB1 whether the expression was controlled by miR-218, we performed real-time PCR and western blot in miR-218 mimics or controls transfected A549 and H1299 cells. As shown in [**Fig. 2(B,C)**], miR-218 overexpression suppressed HMGB1 expression by about 50% on both mRNA and protein levels compared with the controls, respectively.

To confirm the direct binding sites of HMGB1 with miR-218, we constructed Enhanced green fluorescent protein (EGFP) reporter gene with *HMGB1* 3′-UTR and the mutant *HMGB1* 3′-UTR. The sequences were shown in **Fig. 2(D)**. Then we performed EGFP reporter assay to detect the effects of miR-218 on green fluorescent protein (GFP) intensity controlled by *HMGB1* 3′-UTR or mutant 3′-UTR. We found that miR-218 reduced the HMGB1 intensity by about 40% [**Fig. 2(E)**], while miR-218 had no effect on the mutant *HMGB1* 3′-UTR

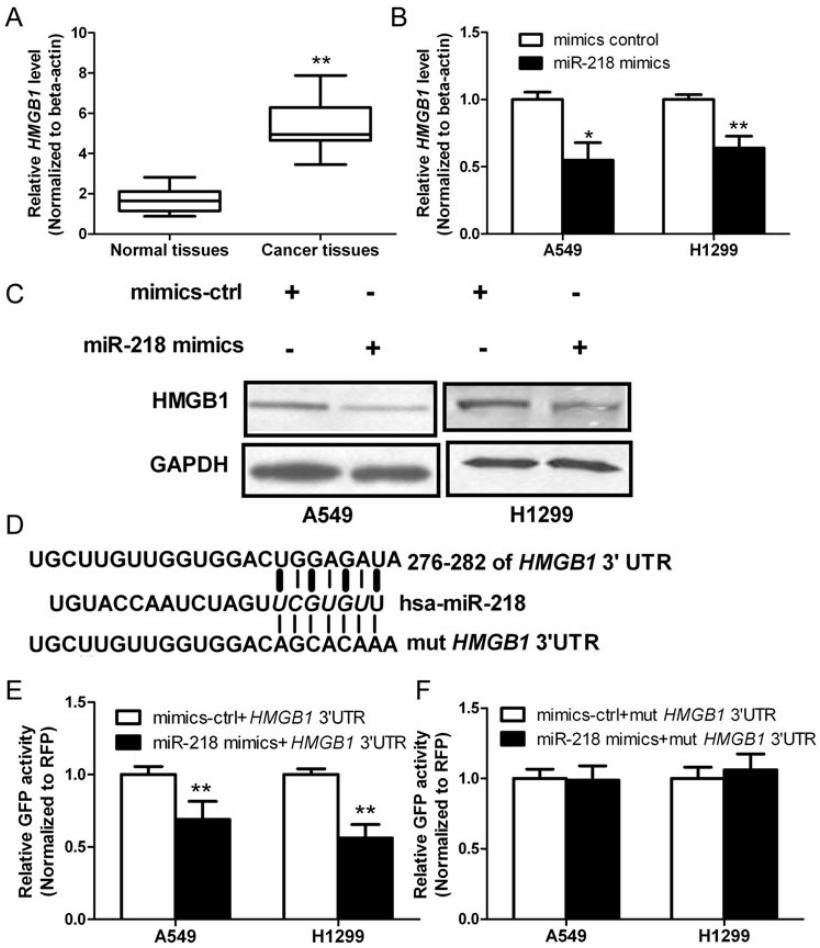


**Figure 2 miR-218 inhibits HMGB1 expression by directly targeting its 3′-UTR** (A) The expression of HMGB1 was evaluated by real-time PCR in non-small lung cancer tissues and adjacent non-tumor tissues. β-Actin was used as an internal control. (B,C) The expression of HMGB1 was evaluated by real-time PCR (B) and western blot (C) in A549 and H1299 cells transfected with miR-218 mimics or controls. β-Actin and GAPDH were used as an internal control, respectively. (D) miR-218 binding sequences with the wild-type or mutant 3′-UTR of *HMGB1*. The 'italic' sequences represented the conserved seed sequences of miR-218 among species. The 'bold' line represented the mutated nucleotides. (E,F) The cells were co-transfected with miR-218 mimics and wild-type or mutant *HMGB1* 3′-UTR, and the GFP intensity was measured by the EGFP reporter assay. The relative level of the controls in all experiments was assigned as 1. $*P < 0.05$, $**P < 0.01$.

[**Fig. 2(F)**]. Overall, these results indicate that miR-218 suppresses HMGB1 expression by binding directly to its 3′-UTR.

### HMGB1 silencing inhibits cell migration and invasion in A549 and H1299 cells

To identify if HMGB1 can mediate the roles of miR-218 in A549 and H1299 cells, we performed cell migration and invasion assays first. **Figure 3(A,B)** showed that HMGB1 was significantly silenced by *HMGB1* siRNA in A549 and H1299 cells. Furthermore, the migration and invasion assays suggested that silencing of HMGB1 suppressed the cell migration and invasion abilities in A549 cells, and similar results was obtained in H1299 cells [**Fig. 3(C,D)**].

### HMGB1 overexpression rescues the cell migration and invasion in A549 and H1299 cells inhibited by miR-218

To clarify that miR-218 plays an important role in the inhibition of cell migration and invasion through the regulation of HMGB1, rather than other genes, we performed rescue experiment by co-transfecting the cells with miR-218 mimics and pcDNA3/HMGB1. **Figure 4(A)** showed that HMGB1 was overexpressed in pcDNA3/HMGB1 transfected cells. In addition, we found that HMGB1 restored the cell migration and invasion abilities reduced by miR-218 mimics [**Fig. 4(B)**]. Taken together, our results suggest that HMGB1 may play an important role in lung cancer metastasis reduced by miR-218 mimics.

## Discussion

Understanding the molecular mechanism of cancer development is important for effective therapy. Aberrant miRNAs expression occurs frequently in human cancers. Therefore, it is crucial to explore the function of deregulated miRNAs in cancers. Previous studies have discovered that miR-218 is down-regulated in gastric cancer [12,13], cervical squamous

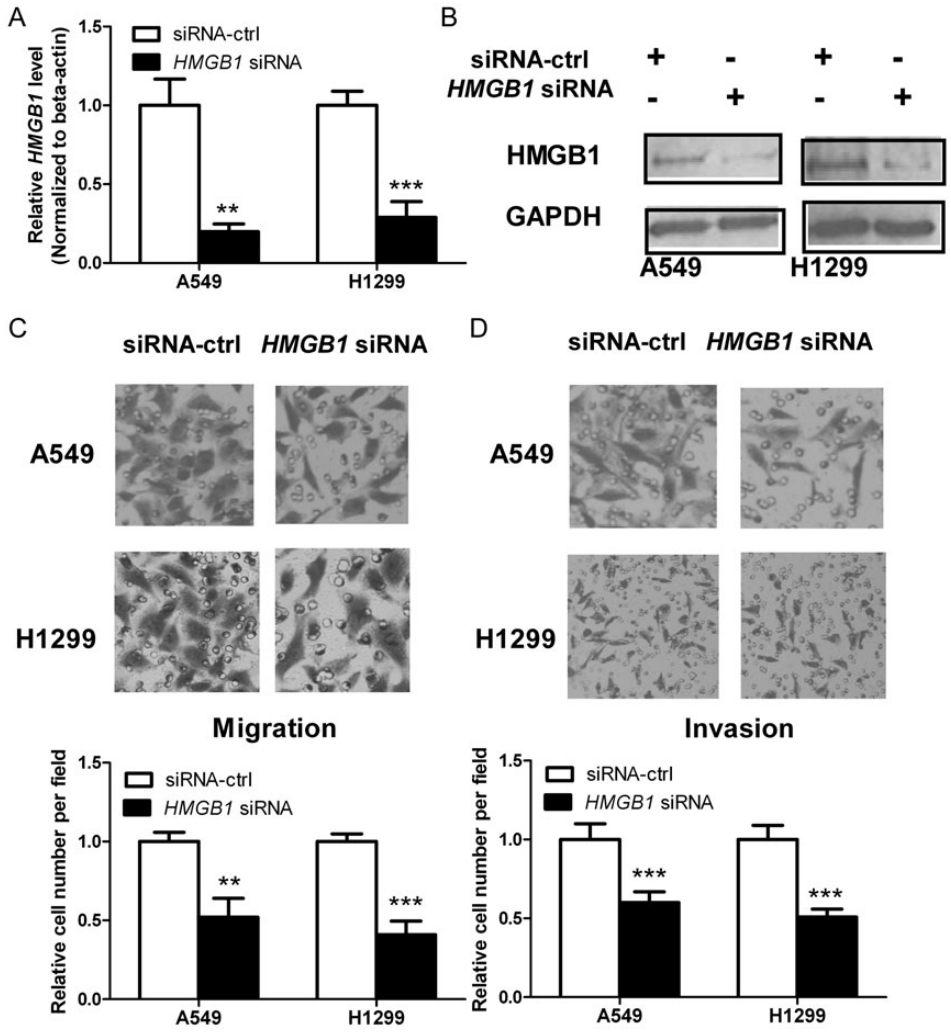


**Figure 3 The silencing of HMGB1 inhibits the cell migration and invasion in A549 and H1299 lung cancer cell lines** (A,B) HMGB1 was silenced by treatment with *HMGB1* siRNA in A549 and H1299 cells. The expression of HMGB1 was evaluated by real-time PCR (A) and western blot (B). β-Actin and GAPDH were used as an internal control, respectively. (C,D) Top: representative migratory and invading A549 and H1299 cells with *HMGB1* siRNA or controls, and the cells were stained with crystal violet. Bottom: representative number of migratory and invading cells (×100). The relative cell number of control in all experiments was assigned as 1. $^{**}P<0.01$, $^{***}P<0.001$.

cell carcinoma [14], medulloblastoma [15], and glioblastoma [16]. MiR-218 is down-regulated in breast cancer and regulates the tumor suppressor genes *RASSF1A* [Ras association (RalGDS/AF-6) domain family member 1] and *Claudin-6* by targeting *HoxB3* (homeobox B3) [17]. In this study, we found that miR-218 inhibited the cell migration and invasion, functioning as a tumor suppressor in A549 and H1299 cells.

As for the silencing of miRNAs in cancers, a large body of evidence shows that methylation may be one of the causes accounting for it. MiR-124 and miR-203 are hypermethylated in hepatocellular carcinoma, leading to its silenced expression [18]. The CpG islands of miR-34a, miR-34b, and miR-129-2 have high methylation ratio, inducing their low expression in esophageal squamous cell carcinoma [19]. The mature miR-218 has two miR-218 precursors, miR-218-1 and miR-218-2, which are located in the intronic region of *SLIT2* (slit homolog 2) and *SLIT3* host genes, respectively [11]. The promoters of *SLIT2* and *SLIT3* are hypermethylated, leading to the silenced expression of both genes [20,21]. Therefore, the down-regulation of miR-218 in lung cancer may be due to the hypermethylation of the promoter of its host genes.

MiRNA plays tumor suppressive or oncogenic roles through binding to the 3′-UTR of the target genes, so exploring the targets of miR-218 in lung cancer is crucial for understanding its regulation mechanism. In this study, we used bioinformatics for target genes prediction. Considering the overlap of the genes searched by TargetScan, miRanda, and miRwalk, we acquired a few of candidates. In addition, we considered the potential roles of the candidates and the expression levels in lung cancer reported in previous studies. Finally, we chose HMGB1 as the potential target for further validation. We found that HMGB1 was up-regulated in lung

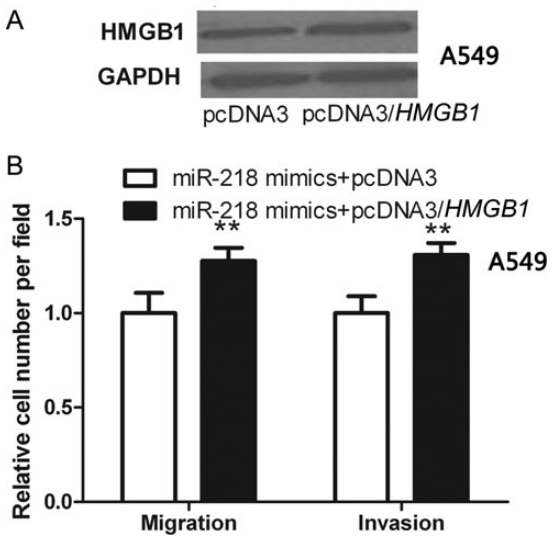


**Figure 4 The overexpression of HMGB1 restores the cell migration and invasion reduced by miR-218** (A) The cells were transfected with pcDNA3/*HMGB1* and controls. The HMGB1 level was evaluated by western blot. GAPDH was used as an internal control. (B) The cells were co-transfected with miR-218 and HMGB1, and then the migration and invasion assays were detected. $^{**}P<0.01$.

cancer, and miR-218 overexpression decreased the HMGB1 expression at both mRNA and protein levels, suggesting that miR-218 negatively regulated HMGB1. Then we performed EGFP reporter assay, which was a direct method for target validation. Consistently, miR-218 inhibited the *HMGB1* 3′-UTR activity, while had no impact on the mutant 3′-UTR. The functional studies indicated that HMGB1 silencing suppressed the cell migration and invasion, which was similar to the function of miR-218 overexpression. Importantly, HMGB1 overexpression could restore the cell migration and invasion which were inhibited by miR-218. All these results validated that HMGB1 was a direct target for miR-218, and miR-218 negatively regulated its expression.

HMGB1 is located on the human chromosome 13q12 [22], and belongs to the high mobility group protein superfamily. HMGB1 has been reported to participate in various biological processes, including inflammation [23], DNA and tissue repair [24,25], and cell mobility [26]. Interaction between HMGB1 and its receptor RAGE (receptor for advanced glycation end-products) is involved in the malignant behaviors in several cancers, such as breast cancer [27,28], colon cancer [29], melanoma [30], and lung cancer [31]. Reports also demonstrated that HMGB1 is associated with cancer cell migration and invasion. HMGB1 promotes the cell invasion via regulation of *MMP-9* (matrix metallopeptidase 9) in lung cancer [32,33]. HMGB1 can activate caspase-1 and contribute to hepatocellular carcinoma invasion and metastasis [34]. Moreover, knockdown of HMGB1 has clinical significance that inhibits human liver cancer growth and metastasis [35]. Our results were consistent with the above reports and suggested that silencing of HMGB1 inhibited lung cancer migration and invasion. With respect to the mechanism of HMGB1 regulation on cell behaviors, we will focus on the genes and signaling pathway regulated by HMGB1, like MMP-9, caspase-3, and HMGB1-RAGE axis in the future study.

In conclusion, our study suggests that miR-218 regulates lung cancer cell migration and invasion via suppression of HMGB1, functioning as a tumor suppressor. Overexpression of miR-218 may act as a novel therapeutic biomarker for metastatic lung cancer patients. Although the application of miRNA biomarker in clinical disease may encounter big barrier, our results provide a basis for the molecular strategy of diseases.

In the future, the miR-218 expression levels among lung cancer patients with different stages of metastasis, and the association of 5-year survival rate of patients with different miR-218 levels will be studied. In addition, the systematic delivery of miRNA into patients via nanoparticles as delivery tools needs intensive design and trials.

## Funding

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