



# miR-204 suppresses non-small-cell lung carcinoma (NSCLC) invasion and migration by targeting JAK2

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**ABSTRACT.** Aberrant expression of microRNA is associated with the development and progression of cancers. MicroRNA-204 (miR-204) down-regulation has been previously demonstrated in non-small-cell lung carcinoma (NSCLC); however, the underlying mechanism by which miR-204 suppresses tumorigenesis in NSCLC remains elusive. In this study, miR-204 expression was found to be down-regulated, and that of Janus kinase 2 (JAK2) was found to be up-regulated in four NSCLC cell lines (A549, H1299, H1650, and H358) compared to the normal lung cell line. The overexpression of miR-204 suppressed the invasive and migratory capacities of H1299 cells. A luciferase assay confirmed that the binding of miR-124 to the -untranslated region of JAK2 inhibited the expression of JAK2 proteins in H1299 cells. JAK-2 overexpression effectively reversed miR-204-repressed NSCLC metastasis. Taken together, our findings revealed that miR-204 functions as a tumor suppressor in NSCLC by

targeting JAK2, and that miR-204 may therefore serve as a biomarker for the diagnosis and treatment of NSCLC.

**Key words:** MicroRNA-204; NSCLC; Metastasis; Janus kinase 2

## INTRODUCTION

Lung cancer is one of the most common aggressive malignancies; non-small-cell lung carcinoma (NSCLC) is responsible for almost 80% of the lung cancer-related deaths (Buyukcelik et al., 2004; Torre et al., 2015). Despite the recent (considerable) improvements in chemotherapy and molecular-targeted therapy, lung cancer prognosis remains poor. The invasiveness and metastasis of tumor cells are critical challenges influencing the clinical management of NSCLC. Therefore, the identification of novel molecules that can repress the invasiveness and metastasis of lung cancer cells is the need of the hour.

MicroRNA (miRNA), an abundant class of ~22 nucleotide small-noncoding RNA, are known to post-transcriptionally regulate the gene expression by binding to multiple target mRNA (Bartel, 2004; Calin and Croce, 2006; Tutar et al., 2014). miRNA are important regulators that are significantly involved in the development of human diseases. Recent studies have shown a close correlation between dysregulated miRNA and carcinogenesis and cancer progression (Del Vescovo et al., 2014; Xu et al., 2015). For example, miR-126, miR-200c, and miR-125a-5p expression is associated with the progression and prognosis of non-small cell lung cancer (Kim et al., 2014; Zhu et al., 2014). miR125b, an oncogenic miRNA, is up-regulated in lung cancer, and has been reported to promote cancer progression (Wang et al., 2015), whereas miR-216a, miR-27b, miR-338-3p, miR-30, and miR-99a, which function as tumor-suppressors, are down-regulated in breast cancer cells (Han et al., 2014; Jiang et al., 2014; Wang et al., 2014; Zhong et al., 2014; Chen et al., 2015). Recent studies have shown that some miRNA function as a tumor suppressors in NSCLC (Xia et al., 2014). However, the underlying mechanism remains unclear.

The JAK signaling pathway is a common signaling pathway for many cytokines, and plays an important role in proliferation, differentiation, and apoptosis. JAK2, a member of the Janus kinase (JAK) family, is a crucial intracellular mediator of cytokine and hormone signaling (Aaronson and Horvath, 2002; Levine et al., 2007). Recent studies have provided evidence of a correlation between aberrant JAK2 expression and tumor progression (Constantinescu et al., 2008; Lai and Johnson, 2010; Zhuang et al., 2012) in cancers, such as gastric cancer (Ding et al., 2010), esophageal squamous cell carcinoma (Fang et al., 2015), oral cancer (Kowshik et al., 2014), and breast cancer (Lakshmanan et al., 2012). Although these studies indicate the importance of JAK 2 in cancer progression, its roles in NSCLC remains to be investigated.

In this study, we sought to investigate the underlying mechanism of miR-204 in NSCLC. JAK2 was found to be a direct target of miR-204 in NSCLC, while miR-204 was found to suppress NSCLC metastasis by inhibiting JAK2 expression.

## MATERIAL AND METHODS

### Cell culture

The lung cancer cell lines (A549, H1299, H1650, and H358) and normal lung cells

(BEAS-2B) used in this study were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and penicillin (100 U/mL). The cells were cultured at 37°C in the presence of 5% CO<sub>2</sub>.

### miRNA and siRNA transfection

Negative control (NC), miR-204 mimics (mimics), and miR-204 inhibitors (inhibitors) were purchased from GenePharma (Shanghai, China). The cells were transfected with NC, mimics, and inhibitors using Lipofectamine 2000 (Invitrogen) according to the manufacturer protocols. The transfection efficiency was monitored by quantitative real time polymerase chain reaction (qRT-PCR).

### Real-time PCR

Total RNA was extracted from human tissues and cell lines using the TRIzol reagent (Invitrogen) according to the manufacturer protocols. *miR-204* and *JAK2* mRNA levels were quantified by qRT-PCR (quantitative real-time PCR) using a combination of the miRNA Detection Kit (Ambion) and the SYBR Green qPCR Master Mix (Tiangen), on an ABI 7300 (Applied Biosystems, Foster City, CA, USA) (Shan et al., 2009). The following JAK2 primers were used for PCR detection: 5'-GGGAGGTGGTCGCTGTAAAA- (forward); 5'-ACCAGCACTGTAGCACACTC- (reverse). The relative expression levels of miR-204 and JAK2 were calculated by normalizing to their internal controls (U6 and GAPDH, respectively).

### Western blot

The protein lysates were separated by electrophoresis on a 10% sodium dodecyl sulfate polyacrylamide gel, and subsequently electro-blotted onto polyvinylidene difluoride (PVDF) membranes. The primary antibodies against JAK2 was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA), using a GAPDH antibody (Cell Signaling Technology) as the internal control. Densitometric analysis was performed using the Labworks Image Acquisition and Analysis Software (UVP, Upland, CA, USA).

### Luciferase reporter assays

H1299 cells were seeded onto a 24-well plate (~3 x 10<sup>4</sup> cells/well) 24 h prior to transfection; the cells were then co-transfected with Renilla luciferase and luciferase reporter plasmids, containing miR-204 or the vector control, and wild-type or mutated target gene -untranslated region (UTR) using Lipofectamine 2000 (Invitrogen). The results of the luciferase reporter assay were measured using a Victor 1420 Multilabel Counter (Wallac, Finland) 48 h after transfection, using a Luciferase Assay System (Promega, USA) according to the manufacturer protocols.

### 3-(4,5-dimethylthiazal-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay

The 3-(4,5-dimethylthiazal-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was

used to estimate the cell viability, as described in a previous study (Liu et al., 2013). Briefly, the cells were plated at a density of  $1 \times 10^4$  cells per well on 96-well plates. Following exposure to the specific treatment, the cells were incubated with MTT at a final concentration of 0.5 mg/mL for 4 h at 37°C. The medium was removed, and 150 mM dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The absorbance was read at 570 nm using a multi-well scanning spectrophotometer reader. Cells in the control group were considered to be 100% viable.

### **Invasion assay**

The cells were cultivated up to 80% confluence on 12-well plates. The cellular growth process was then observed after 24 h. All experiments were performed in triplicate. Trans-well invasion chambers were used to evaluate cell invasion. The cells that invaded the membrane were then counted under a light microscope.

### **Wound healing assay**

For the wound healing assay, the cells were seeded on 12-well plates and grown to 90% confluence. Monolayers in the center of the wells were scraped with pipette tips and washed with PBS. Cell movement into the wound area was monitored and photographed at 0 and 24 h under a light microscope, using a camera (DFC350 FX; Leica). The migration distance between the leading edge of the migrating cells and the edge of the wound was compared with that seen in a previous study (Liang et al., 2007).

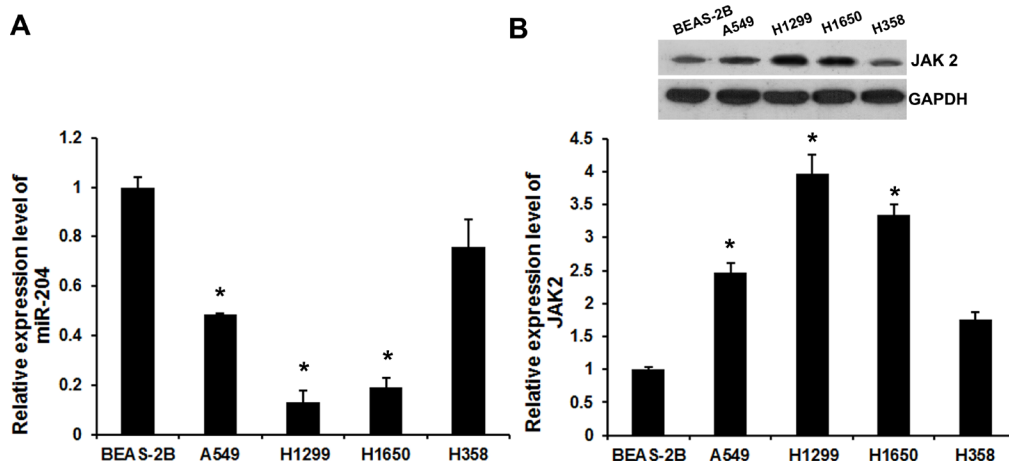
### **Statistical analysis**

All data are reported as means  $\pm$  standard deviation (SD), and analyzed using the Student *t*-test. P values  $< 0.05$  compared to the respective controls were considered to be statistically significant.

## **RESULTS**

### **miR-204 expression is reduced and negatively correlates with JAK2 expression in NSCLC cells**

qRT-PCR was employed to detect miR-204 levels in NSCLC cell lines. The results of the real-time PCR analysis showed that the miR-204 expression was markedly down-regulated in four of the cell lines (A549, H1299, H1650, and H358), compared to the expression levels in normal lung cells (BEAS-2B), which was consistent with the results of a previous study (Sun et al., 2014) (Figure 1A). The JAK2 expression levels were then analyzed in NSCLC cell lines. We observed a significantly higher EphA2 expression in NSCLC cell lines (A549, H1299, H1650, and H358), compared to the corresponding expression levels in normal lung cells (BEAS-2B) (Figure 1B). Taken together, these results indicate that miR-204 may be a tumor inhibitor, and that JAK2 may be an oncogenic regulator modulating the progression of NSCLC.



**Figure 1.** Expression of miR-204 and JAK2 in NSCLC cell lines. **A.** qRT-PCR analysis revealed the expression level of miR-204 in four NSCLC cell lines (A549, H1299, H1650, and H358) and normal lung cells (BEAS-2B). **B.** Western blot analysis revealed the relative expression level of JAK2 in four NSCLC cell lines and normal lung cells. All experiments were performed in triplicate. Each bar represents the mean of three independent experiments. \* $P < 0.01$  versus the BEAS-2B (normal lung) cell line.

### miR-204 directly targets JAK2 in NSCLC

The miRNA target prediction websites [www.microRNA.org](http://www.microRNA.org) and TargetScan were used to identify a conserved miR-204-binding site in the -UTR of *JAK2* mRNA, in order to elucidate whether JAK2 is a potential downstream target gene of miR-204 in NSCLC. This prediction was confirmed and the possibility of JAK2 being a direct target of miR-204 was verified by a dual-luciferase reporter system, where miR-204 and luciferase reporter plasmids containing the -UTR of JAK2, or mutated JAK2 (bearing deletions of the putative miR-204a target sites), were co-transfected. As shown in Figure 2A, the co-transfection of miR-204 mimics suppressed the luciferase activity of the reporter (containing the wild-type JAK2 -UTR sequence). However, miR-204 mimics did not affect the luciferase activity when the target cells were transfected with mutated JAK2. These results suggested that JAK2 may be a direct functional target of miR-204 in NSCLC.

In addition, the regulatory effect of miR-204 on JAK2 was confirmed by detecting the expression of JAK2 to the changes in miR-204 expression in the H1299 cell line by qRT-PCR and western blot analysis. The assays revealed a negative regulatory effect of miR-204 on JAK2 (Figures 2B and 2C). The up-regulation of miR-204 was found to decrease the expression of JAK2, while miR-204 downregulation increased the level of JAK2 expression.

### miR-204 inhibited the invasion and migration of NSCLC cells by targeting JAK2

The effect of JAK2 on the miR-204-mediated antitumor properties in H1299 cells was verified by performing rescue experiments. pcDNA3.1-JAK2 was transiently transfected to restore the JAK2 expression in H1299 cells, with the pcDNA3.1 vector being used as the negative control. We observed a significant increase in the expression of the JAK2 protein



the underlying mechanism of NSCLC tumorigenesis remains unclear. Therefore, a better understanding of these mechanisms will facilitate the development of novel therapeutic targets and strategies for the treatment of NSCLC. In this study, we focused on miR-204, which was down-regulated in several tumor types, including NSCLC. Our results revealed that JAK2 was a direct functional target of miR-204 in NSCLC cells. NSCLC cell lines also displayed decreased miR-204 and increased JAK2 protein expression compared to the normal lung cell line. Furthermore, ectopic overexpression of miR-204 blocked the NSCLC cell invasion and migration *in vitro*, which was reversed by the overexpression of JAK2.

Aberrant expression of miRNA is associated with cancer progression, including proliferation, apoptosis, migration, and invasion. De-regulation of miRNA, such as miR-21, miR-22, miR-448, miR-654-3p, miR-205, miR-216a, miR-143, and miR-181a in NSCLC is a key factor underlying tumorigenesis (Wang et al., 2014; Xu et al., 2015). Human miR-204 is down-regulated in several tumor types, including NSCLC (Li et al., 2014; Shi et al., 2014; Xia et al., 2014). This suggested the potential role of miR-204 as a tumor suppressor. These findings prompted us to investigate the regulation of miR-204 in NSCLC cells. Recent studies have shown an association between decreased miR-204 expression and poor prognosis in patients with breast cancer (Li et al., 2014). miR-204 was (statistically) significantly down-regulated in gastric cancer, and miR-204 promotes GC cell apoptosis by repressing Bcl-2 expression (Sacconi et al., 2012). However, the role of miR-204 in cancers, especially in NSCLC, remains to be clarified. In this study, the oncogene JAK2 was confirmed to be directly targeted by miR-204 in NSCLC cells. NSCLC cells also displayed decreased miR-204 and increased JAK2 protein expression, which suggested that JAK2 was regulated by miR-204 in human NSCLC.

JAK proteins are activated by cytokine and growth factor receptor signaling to subsequently activate downstream proteins, including the signal transducers and activators of transcription. The de-regulation of the JAK expression is associated with several aspects of tumorigenesis, including increased proliferation and reduced apoptosis (Qian et al., 2011). JAK2 protein is overexpressed in several immune diseases and a variety of cancers, including NSCLC (Looyenga et al., 2012). In this study, the overexpression of JAK2 in NSCLC was suppressed by the miR-204 mimics. Overexpressed miR-204 significantly inhibited NSCLC invasion and migration, which was reversed by the overexpression of JAK2. Given that JAK2 may play an important role in cancer carcinogenesis, our discovery that miR-204 inhibits JAK activity and is commonly down-regulated in NSCLC may provide a novel therapeutic avenue, as miRNA could potentially be used to modulate JAK2.

In conclusion, we have identified that miR-204 modulates NSCLC cell invasion and migration by targeting JAK2. Overexpression of miR-204 may have potential therapeutic applications in NSCLC. The application of miRNA to the treatment of NSCLC should be investigated in future studies.

## Conflicts of interest

The authors declare no conflict of interest.

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