Original Research

KLF4 suppresses the proliferation and metastasis of NSCLC cells via inhibition of MSI2 and regulation of the JAK/STAT3 signaling pathway

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ARTICLE INFO	ABSTRACT
	Background: Non-small cell lung cancer (NSCLC) remains an aggresive tumor with poor survival rates. Krüppel-like factor 4 (KLF4) is known to be involved in progression of NSCLC; however, the detailed mechanism by which KLF4 regulates the progression of NSCLC remains unclear. *Methods:* In order to investigate the function of KLF4 in NSCLC, cell proliferation was measured by MTT and colony formation assays. The migration and invasion of NSCLC cells were detected via wound healing and Transwell assays, respectively. Then, the interaction between KLF4 and MSI2 was confirmed using a dual-luciferase reporter assay, and the mechanism by which KLF4 regulates the tumorigenesis of NSCLC was assessed by RT-qPCR and Western blotting. *Results:* The results showed that KLF4 was downregulated, while MSI2 was upregulated in NSCLC. Additionally, KLF4 could inhibit transcription of MSI2, and overexpression of KLF4 or knockdown of MSI2 could inhibit the proliferation, migration and invasion of NSCLC cells. Moreover, KLF4 could inhibit JAK2/STAT3 signalling pathway. *Conclusions:* In conclusion, KLF4 significantly inhibited the proliferation, invasion and migration of NSCLC cells via inactivation of MSI2/JAK2/STAT3 signalling pathway. Thereby, our finding might shed new lights on exploring the new strategies against NSCLC.

Introduction

Lung cancer is responsible for >1.38 million deaths worldwide annually and typically starts when cells of the lung become abnormal and grow uncontrollably [1]. Non-small cell lung cancer (NSCLC) is the most common lung cancer type, accounting for 80-85% of all lung cancers [1]. NSCLC mainly includes large cell carcinoma (8–16%), adenocarcinoma (32–40%) and squamous cell carcinoma (25–30%) [1]. In addition, metastases are a frequent clinical problem for the treatment of NSCLC, and growth of lung cancer cells may lead to the metastasis of tumor. At present, 30–40% of patients with NSCLC are diagnosed with metastatic lung cancer, which is usually found at sites such as the bone, brain, liver and adrenal glands [2]. Substantial efforts have been made to extend the survival times of patients with NSCLC, however, the outcomes remain still not ideal [3]. Therefore, it is `necessary to explore molecular mechanisms underlying the progression of NSCLC for the

treatment of NSCLC.

The Musashi family of RNA-binding proteins are involved in the post-transcriptional regulation of target mRNAs. Musashi-2 (MSI2), as one of the two members of the Musashi family, mainly contributes to the regulation and maintenance of the stem cell phenotype in haematopoietic stem cells [4]. Thus, MSI2 is highly expressed in multiple haematological malignancies. More recently, MSI2 has been linked to the development and progression of various cancers. He et al [5]. studied the function of MSI2 in hepatocellular carcinoma, and reported that MSI2 induced the invasion and progression of hepatocellular carcinoma. Guo et al [6]. found that MSI2 plays a similar role in pancreatic cancer, collectively indicating that MSI2 could be regarded as an oncogene in several types of cancer.

Krüppel-like factor 4 (KLF4) is an epithelial zinc finger transcription factor that is involved in not only the induction of the maintenance of stem cell pluripotency, but also in the development of a wide range of

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solid tumours [7]. It is primarily expressed in the post-mitotic epithelial cells of the skin and gut [8,9]. KLF4 functions as both a tumour enhancer and suppressor. The downregulation of KLF4 may result in cellular hyperproliferation and malignant transformation in colon adenomas and gastric cancer [10,11]. By contrast, the upregulation of KLF4 was reported in oral squamous carcinoma and primary breast ductal carcinoma [12]. Although KLF4 was reported to be an inhibitor in malignant tumors, its role in NSCLC needs to be further reported.

The JAK/STAT pathway is a putative intracellular signalling pathway that regulates the effects of numerous cytokines and growth factors [13]. MSI2 was reported to activate the JAK2/STAT3 pathway and lead to cell migration and invasion in bladder cancer [5]. Another report demonstrated that MSI2 was upregulated by the suppression of KLF4 in pancreatic ductal adenocarcinoma (PDAC). The dysregulated KLF4/MSI2 signalling pathway may, in turn, promote PDAC progression and metastasis. Although the previously published paper described the role of KLF4 in the JAK/STAT pathway in bladder cancer and the association between KLF4 and MSI2 in PDAC, the crosstalk among KLF4, MSI2 and the JAK2/STAT3 signalling pathway was not reported in NSCLC. Therefore, to the best of our knowledge, the present research was the first to demonstrate that KLF4 can inhibit MSI2, which further mediates the JAK2/STAT3 pathway to suppress metastasis of NSCLC cells. Based on these previous studies, the present research aimed to investigate the function of KLF4 in NSCLC. In addition, this study sought to explore the mechanism underlying the function of KLF4 in NSCLC.

Materials and methods

Human tissue specimens

A total of 30 patients with NSCLC and 20 normal lung tissues were included in this research to assess the role of MSI2 and KLF4 in NSCLC, and 60 patients with NSCLC were included to evaluate the association between the expression of MSI2 and clinicopathological features in patients with NSCLC. In addition, the tissues were acquired from the Union Hospital of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China) between July 2013 and December 2015. The NSCLC samples and paracancerous tissues excised from surgery were immediately stored in a liquid nitrogen tank for long-term preservation. The collection of clinical specimens was approved by the Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). All participants were required to provide written informed consent.

The patient inclusion criteria were as follows: i) Individuals who met the diagnostic criteria for NSCLC, and they had not undertaken radiotherapy or chemotherapy 6 months before surgery; ii) tissue specimens were diagnosed by the Department of Pathology, and the distance between the normal tissues adjacent to the cancer and the lesion was >5 cm; iii) patients who were not pregnant or lactating, or patients with other lung diseases as determined via imaging examinations, such as ultrasound and bronchoscopy. The patient exclusion criteria were as follows: Individuals diagnosed with malignant tumours of other systems or individuals who suffered injury of vital organs.

Cell culture

The NSCLC cell lines (H1975, H460, A549, H520 and NCI-H1299) and human pulmonary epithelium 16HBE were purchased from Cell Applications, Inc. DMEM (Gibco; Thermo Fisher Scientific, Inc.) was used to culture NCI-1299, A549, H460 and 16HBE cells, and minimum essential media (Gibco; Thermo Fisher Scientific, Inc.) was used to culture H1975 and 16HBE cells. All the media contained 10% foetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 0.5% penicillin and streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.). All the cell lines were cultured in a humidified atmosphere containing 5% CO_2 at 37C. For experiments with colivelin (STAT3 activator, 0.5

µmol/L) treatment, the cells were seeded in DMEM supplemented with 10% FBS in a 96-well plate (5×10^3 cells/well) and incubated overnight at 37C. After culture for 1 day, cells were washed twice with phosphate buffered saline and cultured in the presence or absence of the indicated reagents in serum-free DMEM for another day, and then cells were subjected to the cell viability assay. All the experiments were repeated four times.

Total RNA extraction and reverse transcription-quantitative PCR (RT-qPCR) analyses

TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA from 60 NSCLC specimens and 20 normal lung tissue specimens. Precellys 24 (Bertin Instruments; CNIM Group) was selected to homogenize the tumour cells, Qubit 2.0 Fluorometer (Invitrogen; Thermo Fisher Scientific, Inc.) was used for RNA quantification and 2100 Bioanalyzer (Agilent Technologies, Inc.) was used for RNA qualification.

RNA was further treated with deoxyribonuclease I (Sigma-Aldrich; Merck KGaA), according to the manufacturer's protocols. Total RNA (1 µg) was incubated with SuperScript™ III Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) to synthesize the first-strand cDNA at 42C for 1 h following oligo priming. After obtaining the cDNA, qPCR was conducted using SYBR® Premix Ex Taq™ (Takara Biotechnology Co., Ltd.) on the ABI 7900HT system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The conditions for qPCR were as follows: Denaturation at 95C for 10 sec, 40 cycles at 95C for 10 sec and 60C for 30 sec. At the end of the amplification procedure, a dissociation stage was added to check non-specific amplification. GAPDH was used as an internal control. KLF4 forward, 5'-CCCTTGGATGCTTGGAGTGA-3' and reverse, 5'-GCGACGGTGCTGAATCTTGT-3'; MSI2 forward, 5'-CGCTCCCAGCAGAAGGTCGTGATTACCAAG-3' and reverse, 5'-GGAGTGACCCCAATCCCTGCCCCAACCA-3'; and GAPDH forward, 5'-TGTGGGCATCAATGGATTTGG-3' and reverse, 5'-ACACCATG-TATTCCGGGTCAAT-3'. The $2^{-\Delta\Delta CT}$ method [14] was used to analyse the data. Each sample was prepared in triplicate.

Cell transfection and plasmid construction

The plasmid pCMV-MSI2 (pMSI2, 10 nM) was obtained from GeneCopoeia, Inc., and Flag-tagged KLF4 overexpression vector (pKLF4, 10 nM) was constructed as previously described [15]. Small interfering RNA (siRNA) against MSI2 (si-MSI2; 10 nM, 5'- CAGACCTCACCAGA-TAGCCTTAGAG-3') was purchased from Thermo Fisher Scientific, Inc. The KLF4 siRNA (si-KLF4-1, 10 nM, AGGAGTTCAACGATCTCCTG-GACCT; si-KLF4-2, 10 nM; 5'- ACGATCTCCTGGACCTGGACTTTAT -3'; si-KLF4-3, 10 nM, CGATCTCCTGGACCTGGACTTTATT; si-KLF4-4, 10 nM, TGGACCTGGACTTTATTCTCTCCAA;) consisted of pools of target-specific siRNAs designed to knock down KLF4 expression (Invitrogen; Thermo Fisher Scientific, Inc.), according to previous studies [16]. Scrambled siRNA (si-NC; 10 nM; Invitrogen; Thermo Fisher Scientific, Inc.) and pCMV vectors (10 nM; Invitrogen; Thermo Fisher Scientific, Inc.) were used as negative controls in this work. si-KLF4-2, si-MSI2, pKLF4 and/or pMSI2 were transfected into A549 and H520 cells (5×10^3) was conducted using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). For transient transfection, the cells were transfected at different doses with plasmids or siRNA for 48 h at 37C before functional assays. A549 and H520 cells treated with the transfection reagent only were included as mock controls. After 48 h of transfection, cells were used for subsequent analysis.

MTT assay

All cell lines (5×10^3) were prepared in 96-well plates and treated with 100 µL sterile MTT dye (0.5 mg/mL; Sigma-Aldrich; Merck KGaA) for 24, 48, 72 and 96 h at 37C for 4 h, followed by the removal of culture
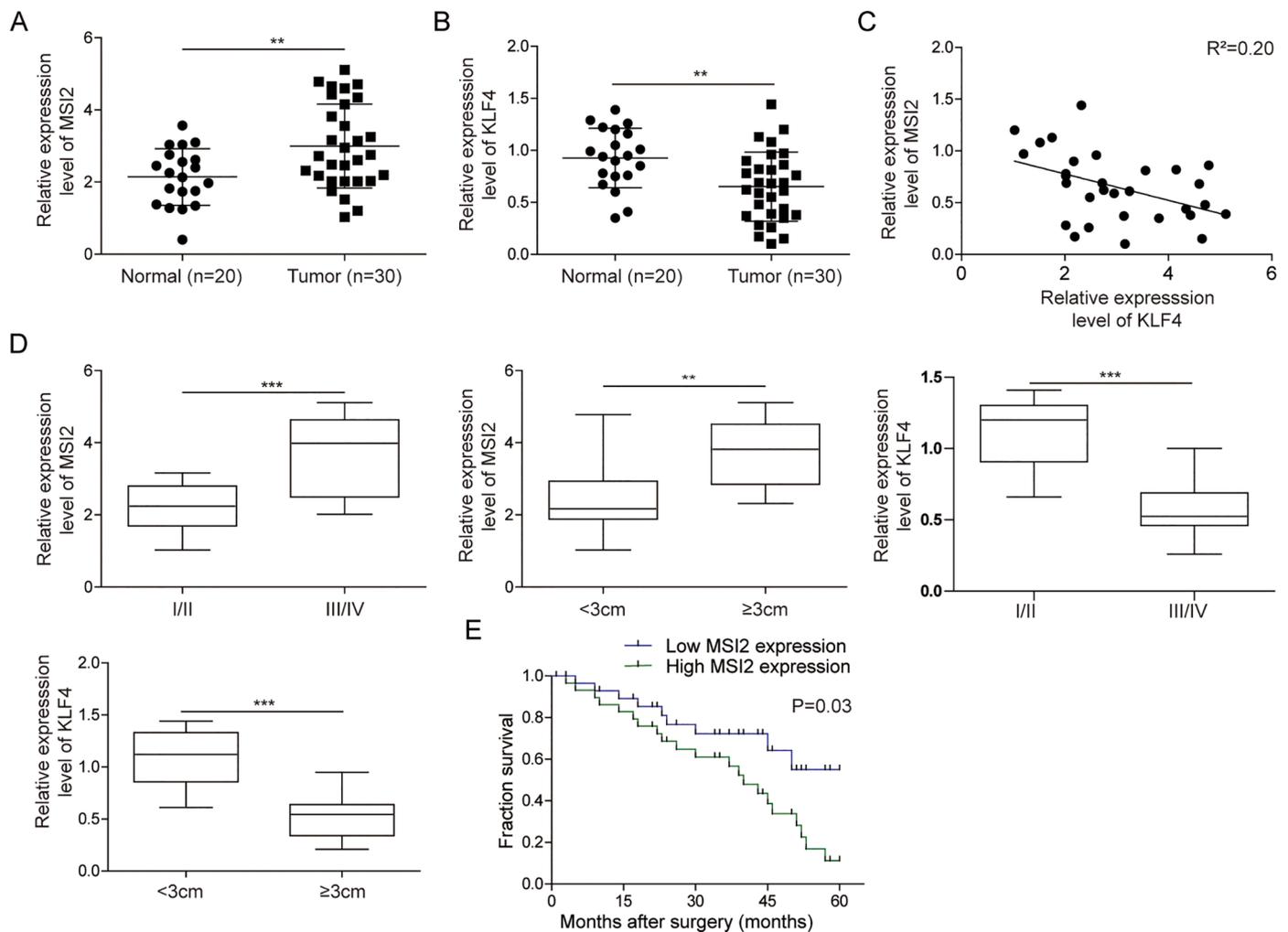
Fig. 1. KLF4 is downregulated and MSI2 is upregulated in NSCLC specimens. (A) MSI2 and (B) KLF4 expression in normal lung tissues (n=20) and NSCLC specimens (n=30), as determined via reverse transcription-quantitative PCR. (C) Correlation between KLF4 and MSI2 expression in NSCLC specimens (n=30), analysed using Pearson's correlation coefficient analysis. (D) Associations between clinicopathological parameters and MSI2/KLF4 expression in NSCLC specimens according to the World Health Organization classification (I/II vs. III/IV) or tumour size ($\geq$3 vs. <3 cm). The results were expressed as the mean $\pm$ SD. **P<0.01, ***P<0.001. (E) Kaplan-Meier survival curves comparing the cumulative overall survival probability in patients with low and high MSI2 expression levels. KLF4, Krüppel-like factor 4; MSI2, Musashi-2; NSCLC, non-small cell lung cancer.

medium and the addition of 150 μL dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA). The absorbance was measured at 490 nm. All the experiments were conducted in triplicate.

Wound healing assay

A549 and H520 cells were seeded in a 12-well plate (1×10^4 cells/well) and cultured until they reached 70-80% confluence. A 200-μL sterile pipette tip was used to create a scratch (wound) in each confluent monolayer, after which the cells were further incubated in serum-free minimum essential media for 24 h in an atmosphere of 37C, 5% CO_2. The distances between the opposite edges of the wound were used to determine wound closure, and the result was observed by a light microscope. After scratching at an identical location, plates were imaged at 0 and 24 h, and the width (W) of the scratch was measured. The wound closure was calculated as ($W_{0 h}$ - $W_{24 h}$) / $W_{0 h}$ x100%. Fourindependent experiments were conducted.

Colony formation assays

A549 and H520 cells transfected for 24 h were seeded in 60-mm dishes (0.5×10^3 cells/plate) and cultured for 10 days at 37C in 5%

CO_2. Next, the generated colonies (defined as >50 cells) were fixed with 10% formaldehyde for 5 min at room temperature and stained with 1.0% crystal violet at room temperature for 30 s. Three random fields were selected. The result was observed under a light microscope.

Cell invasion assays

For the cell invasion assay, A549 and H520 cells (5×10^3) transfected for 24 h were seeded into the upper Transwell chambers of the 24-well plates in 200 μL serum-free DMEM (Gibco; Thermo Fisher Scientific, Inc.). The upper chambers were precoated with 100 μL 100% Matrigel (BD Biosciences) at 37C for 4 h. The lower chambers were treated with DMEM containing 10% FBS as the chemoattractant. After 48 h of incubation, cells from the upper layer were removed through the membrane, fixed with 95% alcohol for 10 min at room temperature, and stained with 0.1% crystal violet for 5 min at room temperature. Cells in at least five random fields were counted under the IX71 inverted microscope (Olympus Corporation).

ChIP assay

EZ ChIP Chromatin Immunoprecipitation Kit (Millipore, Bedford,

MA, USA) was used to perform ChIP assay according to the manufacture's instruction. In brief, the chromatin was immunoprecipitated by anti-KLF4 (cat. no. ab272860, Abcam). Finally, the enrichment was measured by RT-qPCR.

Western blotting

A549 and H520 cells were harvested and lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology). The BCA assay kit (Bio-Rad Laboratories, Inc.) was used to determine protein concentration. The protein samples (40 μg per lane) were loaded and electrophoresed via SDS-PAGE on a 10% gel, and then transferred to PVDF membranes (EMD Millipore). Membranes were blocked with 5% skimmed milk at room temperature for 1 h, and subsequently co-incubated with the primary antibodies at 4C overnight. Subsequently, the bound primary antibodies were incubated with the corresponding secondary antibodies (1:5000, cat. no. ab6721, Abcam) overnight at 4C, and then detected by enhanced chemiluminescence reagent (Thermo Fisher Scientific, Inc.). The primary antibodies used were purchased from Abcam and were as follows: Anti-KLF4 (1:1000; cat. no. ab215036; Abcam), anti-MSI2 (1:2000; cat. no. ab76148; Abcam), anti-JAK2 (1:1000; cat. no. ab108596; Abcam), anti-phosphorylated (p)-JAK2 (1:1000; cat. no. ab32101; Abcam), anti-STAT3 (1:2000; cat. no. ab68153; Abcam), anti-p-STAT3 (1:1000; cat. no. ab76315; Abcam), anti-Wiskott-Aldrich syndrome protein family member 3 (WASF3; 1:2000; cat. no. ab68031; Abcam) and anti-GAPDH (1:1,000; cat. no. ab9484; Abcam). ImageJ software (version 2.0; National Institutes of Health) was used for semi-quantification of the band densities.

Dual-luciferase reporter assay

A549 and H520 cells were transfected with *MSI2* promoter reporters, si-KLF4-2, or pKLF4. The mutation was generated using a site directed mutagenesis kit (Promega Corporation). The partial sequences of KLF4 and MSI2 were synthesized by Shanghai GenePharma Co., Ltd. These were then cloned into the vectors (pmirGLO; Promega Corporation). Subsequently, pKLF4/si-KLF-4-2 and pMSI2 were co-transfected into NSCLC cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The activity of the *MSI2* promoter was normalised by co-transfection with a β-actin/*Renilla* luciferase reporter containing the full-length *Renilla* luciferase gene. The intensity of luciferase activity was quantified after 24 h of transfection using the Dual-Luciferase Reporter Assay System (Promega Corporation).

Statistical analysis

The results were obtained from four independent experiments and are expressed as the mean ± SD. SPSS 16.0 (SPSS, Inc.) was used to perform statistical analyses. Comparisons between groups were performed using the unpaired Student's t-test to determine the statistical significance. The association between MSI2 expression and clinicopathological characteristics was analysed with a χ^2 test. The overall survival was measured as the time from the date of primary surgery to the date of death or cancer recurrence. Survival curves were plotted and compared using the Kaplan-Meier method and the log-rank test, respectively, and the value of MSI2 expression was obtained by median. ANOVA followed by Tukey's post hoc test for multiple comparisons were used for statistical analysis between multiple groups. The correlation between the expression levels of KLF4 and MSI2 was analysed using the Pearson's correlation coefficient. P<0.05 was considered to indicate a statistically significant difference.

Table 1

Association between the expression of MSI2 and clinicopathological features in patients with non-small cell lung cancer (n=60).

Clinicopathological features	N	MSI2 expression		P-value	X-value
		Low	High		
Age, years				0.781	0.524
≤56	31	15	16		
>56	29	14	15		
Sex				0.777	0.629
Male	35	17	18		
Female	25	13	12		
Tumour size, cm				0.005	5.527
≤3	32	10	22		
>3	28	8	20		
TNM stage				0.026	4.337
I-II	28	10	18		
III-IV	32	12	20		
Metastasis				0.011	5.019
Absent	27	8	19		
Present	33	11	22		

MSI2, Musashi-2; TNM, Tumour Node Metastasis.

Results

KLF4 is downregulated, while MSI2 is upregulated in NSCLC tissues

To evaluate the role of MSI2 and KLF4, RT-qPCR was performed. The data revealed the expression of MSI2 was much higher in NSCLC, whereas KLF4 expression was significantly lower than that in normal lung tissues (Fig. 1A and B). Additionally, MSI2 was weakly negatively correlated with the expression of KLF4 in NSCLC (Figs. 1C and S1A). Next, the associations between clinicopathological parameters and MSI2 expression were analyzed in NSCLC tissues according to the World Health Organization classification (I/II vs. III/IV) or tumour size (≥3 vs. <3 cm) [17]. Total MSI2 expression was positively correlated with the histological classification (P<0.001) and increased tumour size, and KLF4 expression was significantly lower in III/IV stage tumor or tumor that were not smaller than 3 cm (Fig. 1D). The overall survival probability of 60 patients with NSCLC (the time from the date of primary surgery to the date of death or recurrence) was further evaluated using the Kaplan-Meier method, and it was found that the overall survival of patients with high MSI2 expression (n=30) was markedly poorer than those with low MSI2 expression (n=30) (Fig. 1E). Additionally, the clinical data revealed that MSI2 expression in patients was associated with the overall survival rate, tumour size and lymph node metastasis; however, the level of MSI2 was not associated with age or gender (Table 1).

Overexpression of KLF4 suppresses NSCLC cell migration and invasion

To further confirm the role of KLF4 in NSCLC, its expression was determined in human NSCLC cell lines. The data revealed that the expression of KLF4 was much lower in H1975, H460, A549, H520 and NCI-1299 than in that in normal pulmonary epithelium 16HBE cells (Fig. 2A). The expression of KLF4 was significantly lower in A549 and H520 cells, while the expression of MS2 was significantly highest in A549 and H520 cells among lung cancer cells, compared with 16HBE (Fig. 3A). Thus, A549 and H520 cells were selected of use in subsequent analysis. Firstly, A549 and H520 cell lines were overexpressed KLF4 (pKLF4) by transfection. The result revealed KLF4 was stably overexpressed in NSCLC cells (Fig. 2B), and overexpression of KLF4 could significantly lead to the inhibition of the proliferation rate and number of cell colonies after 24 h of incubation (Fig. 2C and D). Consistently, the migration and invasion of NSCLC cells was significantly decreased by KLF4 overexpression (Fig. 2E and F). Since activation of JAK2/STA3 signalling pathway could promote the tumorigenesis of NSCLC [18], the relationship between KLF4 and JAK2/STAT3 pathway in NSCLC was
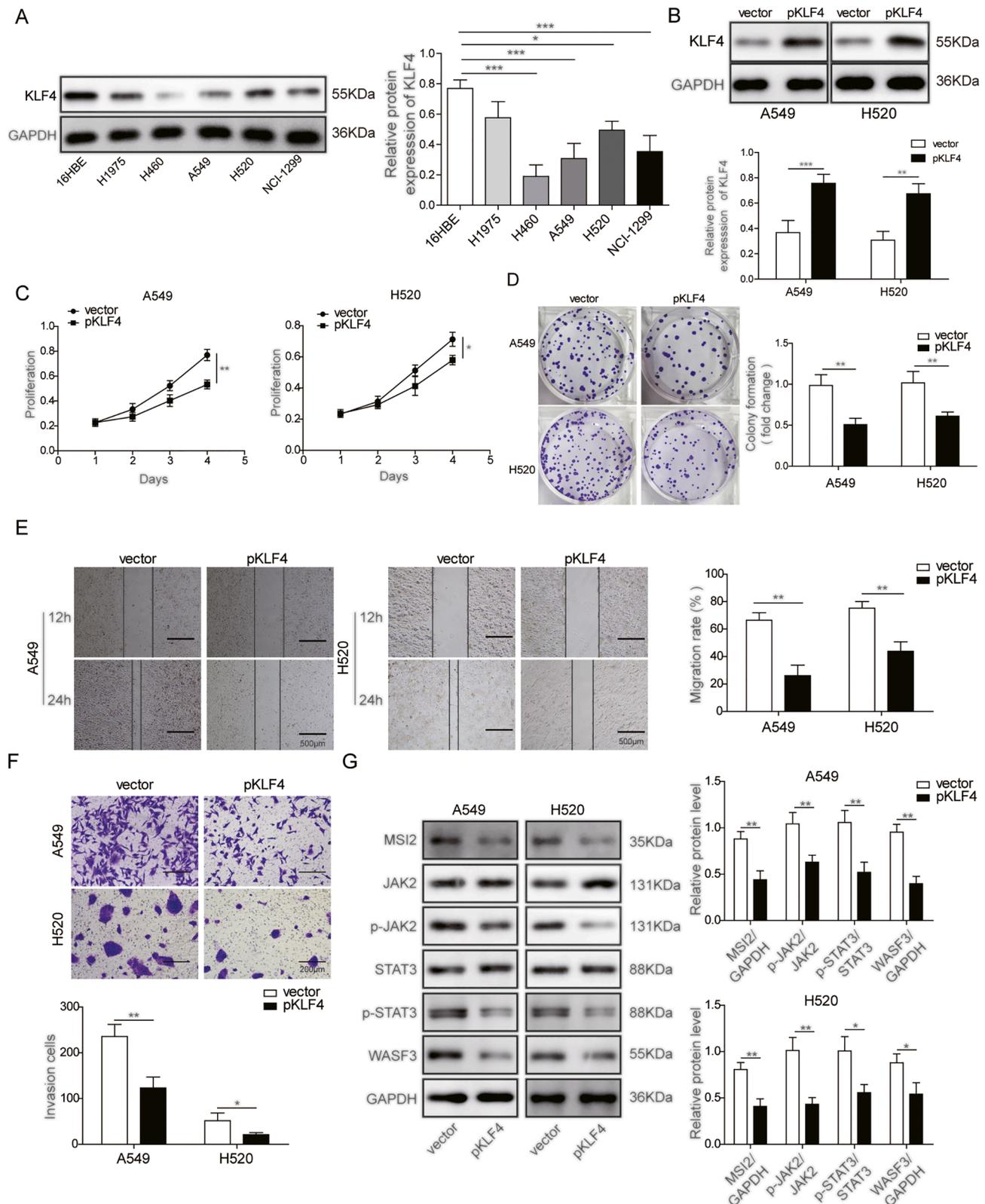
Fig. 2. Overexpression of KLF4 suppresses NSCLC cell migration and invasion via inhibition of the JAK2/STAT3 signalling pathway. (A) Western blotting assay of KLF4 expression in human NSCLC cell lines (H1975, H460, A549, H520 and NCI-1299) and normal pulmonary epithelium cell (16HBE). (B) Western blotting assay of KLF4 expression in A549 and H520 cells transfected with empty vector or KLF4-overexpression plasmids. (C) Cell proliferation, (D) colony formation, (E) migration (scale bar, 500 μm) and (F) invasion assays (scale bar, 200 μm) were performed with KLF4-transfected A549 and H520 cell lines. (G) Western blotting analysis of the expression of JAK2/STA3 signalling pathway markers in KLF4-transfected A549 and H520 cell lines. The results were obtained from four independent experiments and are expressed as the mean ± SD. *$P<0.05$, **$P<0.01$, ***$P<0.001$. KLF4, Krüppel-like factor 4; p-, phosphorylated; WASF3, Wiskott-Aldrich syndrome protein family member 3; NSCLC, non-small cell lung cancer; MSI2, Musashi-2.
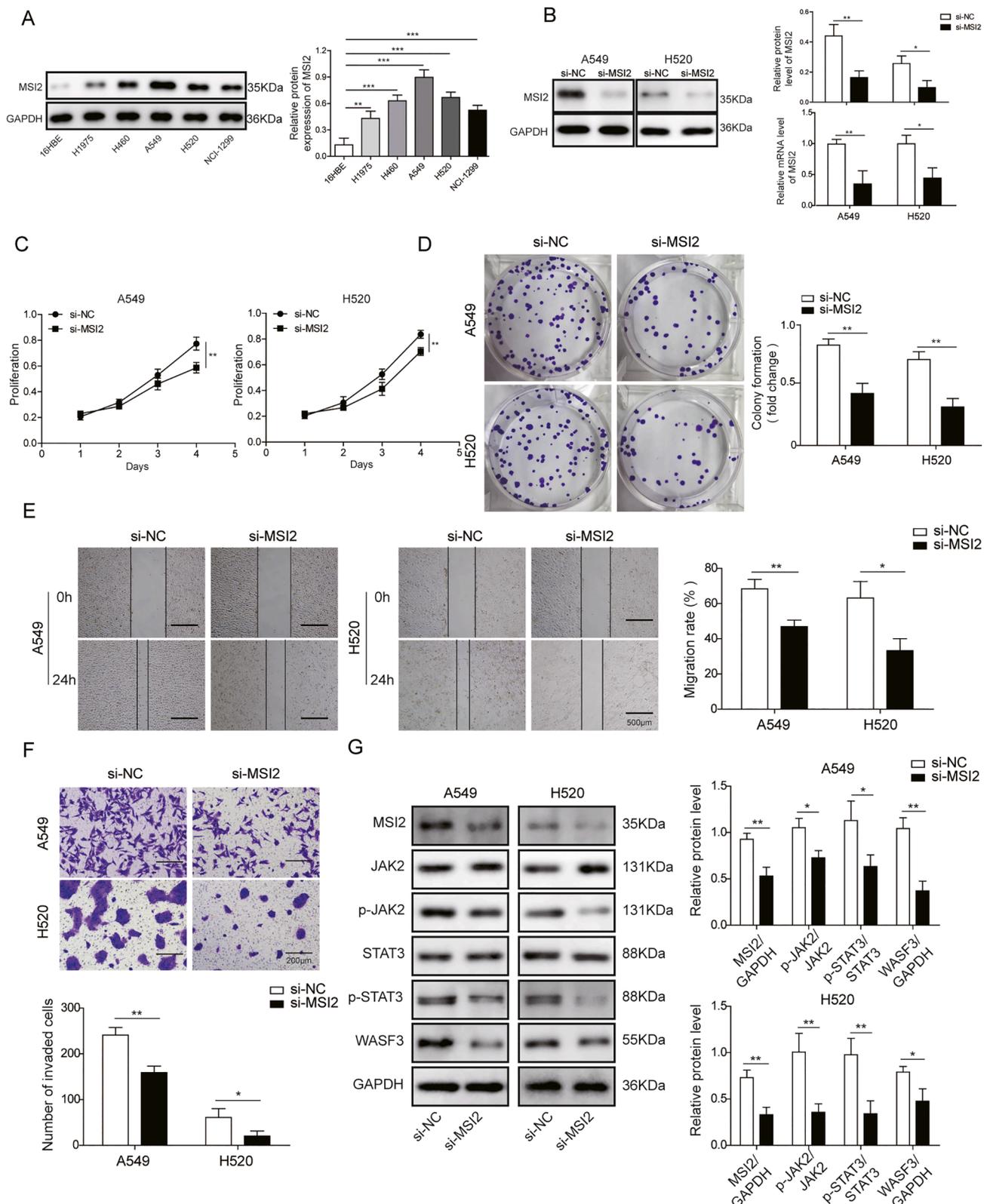
Fig. 3. Knockdown of MSI2 suppressed the migration and invasion of NSCLC cells by activating the JAK2/STAT3 signalling pathway. (A) Western blotting assay of MSI2 expression in human NSCLC cell lines (H1975, H460, A549, H520 and NCI-1299) and normal pulmonary epithelium cell (16HBE). (B) The mRNA and protein expression of MSI2 in A549 and H520 cells transfected with si-NC and si-MSI2, as determined via reverse transcription-quantitative PCR and Western blotting, respectively. (C) Cell proliferation, (D) colony formation, (E) migration (scale bar, 500 μm) and (F) invasion assays (scale bar, 200 μm) were performed using A549 and H520 cells transfected with si-MSI2. (G) Western blotting analysis of the expression of JAK2/STA3 signalling pathway markers in A549 and H520 cells transfected with si-MSI2. The results were obtained from four independent experiments and are expressed as the mean ± SD. *P<0.05, **P<0.01, ***P<0.001. MSI2, Musashi-2; p-, phosphorylated; WASF3, Wiskott-Aldrich syndrome protein family member 3; NSCLC, non-small cell lung cancer; si-, small interfering RNA; NC, negative control.
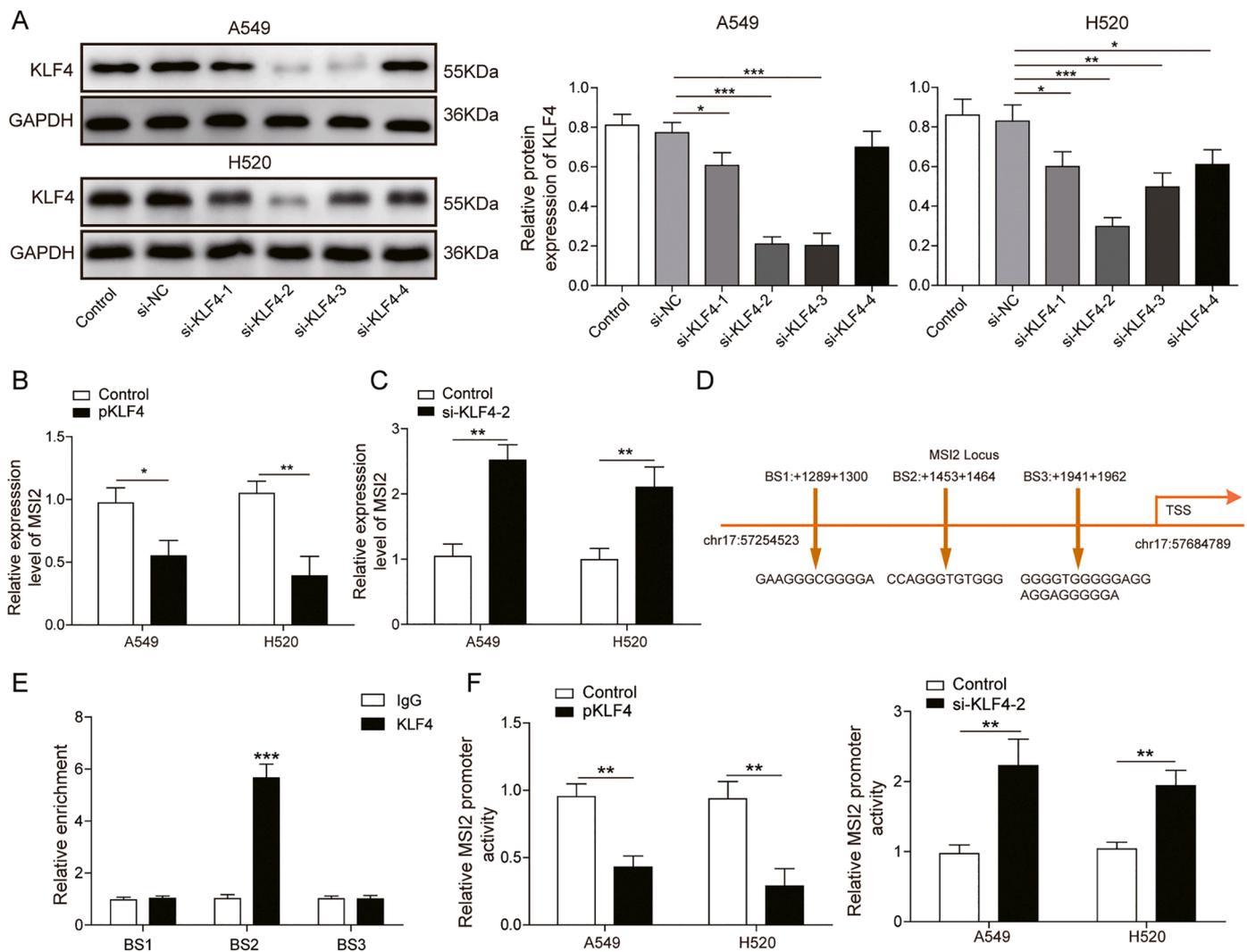
Fig. 4. KLF4 inhibits the transcription of MSI2 expression in non-small cell lung cancer cell lines. (A) Western blotting assay of KLF4 expression in A549 and H520 cells transfected with siRNA targeting KLF4 (si-KLF4-1, si-KLF4-2, si-KLF4-3 and si-KLF4-4). Reverse transcription-quantitative assays of MSI2 expression in A549 and H520 cells transfected with (B) pKLF4 or (C) si-KLF4-2. (D-E) Fold enrichment of ChIP assay in A549 demonstrated the direct binding of KLF4 to MSI2's promoter. (D) A549 and H520 cells were co-transfected with MSI2 and pKLF4, si-KLF4-2 or control vector. Promoter activity in the cells was examined using a dual-luciferase reporter assay. The results were obtained from four independent experiments and are expressed as the mean ± SD. *$P<0.05$, **$P<0.01$, ***$P<0.001$. KLF4, Krüppel-like factor 4; MSI2, Musashi-2; si-, small interfering RNA; NC, negative control; pKLF4, KLF4 overexpression plasmid.

investigated. The data demonstrated the levels of MSI, p-JAK, p-STAT3 and WASF3 in NSCLC cells were significantly inhibited by KLF4 upregulation (Fig. 2G). Altogether, overexpression of KLF4 could suppress the invasion and migration of NSCLC cells via mediation of JAK2/STAT3 signalling pathway.

Knockdown of MSI2 suppresses the migration and invasion of NSCLC cells

Since KLF4 was confirmed to be negatively correlated with the expression of MSI2 (Fig. 1C), the expression of MSI2 in NSCLC cell lines (H1975, H460, A549, H520, NCI-1299) and 16HBE cells was determined. As expected, the level of MSI2 in NSCLC cells was significantly higher, compared with that in normal 16HBE cell lines (Fig. 3A). Next, the efficiency of cell transfection was tested. The result suggested the mRNA and protein expression of MSI2 in NSCLC cells was significantly decreased by MSI2 knockdown (Fig. 3B), while the level of KLF4 was not affected by MSI2 silencing (Fig. S1B). Furthermore, knockdown of MSI2 could obviously decrease the proliferation and colony number of NSCLC cells after 24 h of incubation (Fig. 3C and D). In consistence with these data, the migratory and invasive abilities of A549 and H520 cells were

suppressed in the presence of MSI2 siRNA (Fig. 3E and F). Furthermore, MSI2 knockdown decreased the expression of p-JAK2, p-STAT3 and WASF3 in NSCLC cells (Fig. 3G). To sum up, knockdown of MSI2 could suppress the invasion and migration of NSCLC cells via inactivation of JAK2/STAT3 signalling pathway.

KLF4 inhibits the transcription of MSI2 in NSCLC cells

Next, a series of experiments were performed to explore the effect of KLF4 on MSI2 expression. Thus, A549 and H520 cells were successfully transfected with si-KLF4. Since NSCLC cells were more sensitive to si-KLF4-2 compared with si-KLF4-1, si-KLF4-2 was used for further experiments (Fig. 4A). As shown in Fig. 4B and C, KLF4 upregulation significantly reduced the level of MSI2 in NSCLC cells, while si-KLF4-2 significantly increased MSI2 expression. Furthermore, KLF4 exerted an inhibitory effect on the promoter region of MSI2 in NSCLC cells (Fig. 4D). According to the prediction of bioinformatics tool (AnimalTFDB3 (hust.edu.cn)), KLF4 could bind with MSI2 promoter (Fig. 4D), and the result of ChIP confirmed this phenomenon, as shown in Fig. 4E, the binding sites named as BS2(+1453-+1464) of MSI2 were
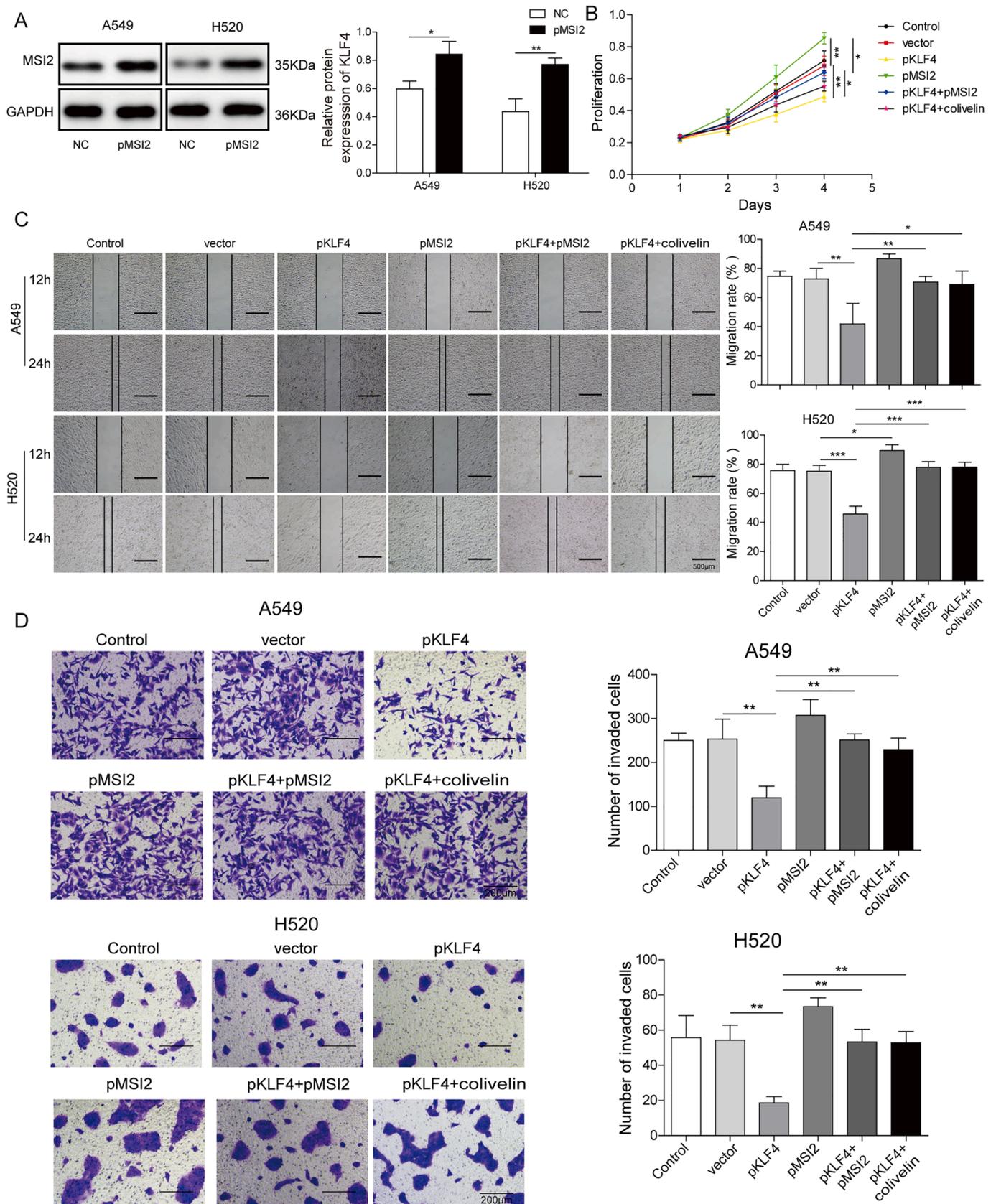
Fig. 5. KLF4 inhibits MSI2 to regulate non-small cell lung cancer via the JAK2/STAT3 signalling pathway. (A) MSI2 expression in A549 and H520 cells transfected with pMSI2 was measured via western blotting. (B) Cell proliferation, (C) migration (scale bar, 500 µm) and (D) invasion (scale bar, 200 µm) assays using A549 and H520 cells treated with pKLF4, pMSI2, pKLF4 + pMSI2, and pKLF4 + colivelin. The results were obtained from four independent experiments and are expressed as the mean ± SD. *$P<0.05$, **$P<0.01$, ***$P<0.001$. KLF4, Krüppel-like factor 4; MSI, Musashi-2; pKLF4, KLF4 overexpression plasmid; pMSI2, MSI2 overexpression plasmid; NC, negative control.
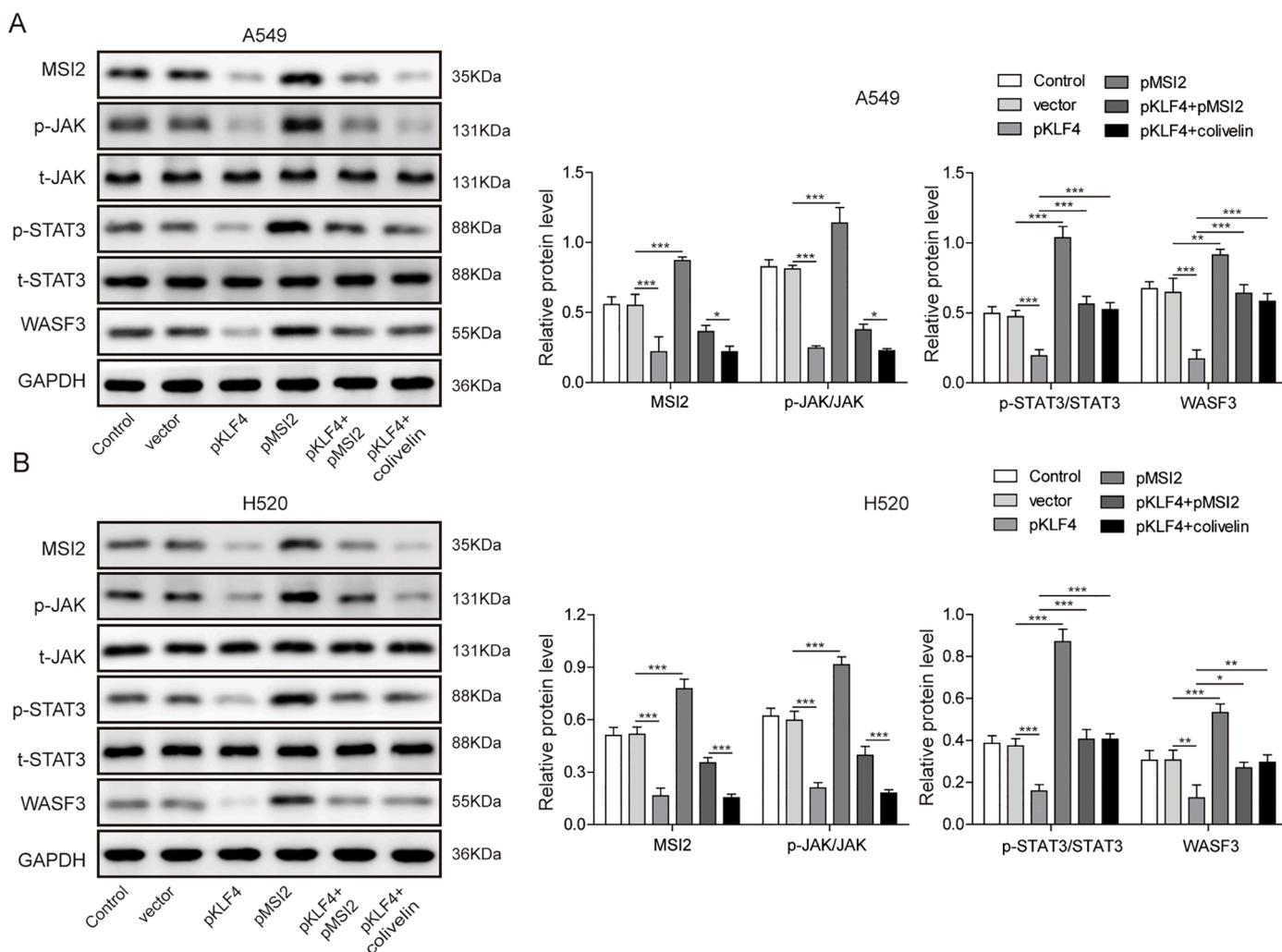
Fig. 6. KLF4 inhibits MSI2 to regulate non-small cell lung cancer via the JAK2/STAT3 signalling pathway. (A) Western blotting analysis of the expression of JAK2/STA3 signalling pathway markers in the transfected A549 cell line treated with pKLF4, pMSI2, pKLF4 + pMSI2, and pKLF4 + colivelin. (B) Western blotting analysis of the expression of JAK2/STA3 signalling pathway markers in the transfected H520 cell line treated with pKLF4, pMSI2, pKLF4 + pMSI2, and pKLF4 + colivelin. The results were obtained from four independent experiments and are expressed as the mean ± SD. *$P<0.05$, **$P<0.01$, ***$P<0.001$. KLF4, Krüppel-like factor 4; MSI2, Musashi-2; pKLF4, KLF4 overexpression plasmid; pMSI2, MSI2 overexpression plasmid; p-, phosphorylated; WASF3, Wiskott-Aldrich syndrome protein family member 3.

found to be responsible for KLF4 interaction in A549. The relative promoter activity of MSI2 was downregulated in pKLF4 group and upregulated in si-KLF4-2 in both A549 and H520 cells (Fig. 4F). In summary, these results indicated KLF4 could inhibit the transcription of MSI2 in NSCLC cells.

KLF4 suppressed the invasion and migration of NSCLC cells via inhibition of JAK2/STAT3 signalling pathway

To demonstrate whether KLF4 can downregulate the level of MSI2 via JAK2/STAT3 signalling pathway, A549 and H520 cell lines were transfected with pMSI2 (MSI2-overexpression vectors) (Fig. 5A), and then A549 and H520 cells were co-transfected with pKLF4 and pMSI2. Besides, cells were treated with colivelin (a STAT3 activator). The data demonstrated that the overexpression of KLF4 significantly decreased the proliferation, invasion and migration of NSCLC cells, while MSI2 overexpression or colivelin partially reversed this phenomenon (Fig. 5B–D). Overexpression of MSI2 significantly increased the colony number, migration and invasion of NSCLC cells (Fig. 5B–D). Moreover, the levels of p-JAK2, p-STAT3 and WASF3 in NSCLC cells were significantly decreased by KLF4 overexpression; MSI2 upregulation could increase the levels of MSI2, p-JAK2, p-STAT3 and WASF3 in NSCLC cells.

Besides, overexpression of MSI2 or colivelin treatment markedly abolished these phenomena (Fig. 6A). Collectively, these results demonstrated that KLF4 suppressed the invasion and migration of NSCLC cells via inhibiting JAK2/STAT3 signalling pathway.

Discussion

Due to the lack of early detection tools and late stage recognition of the symptoms, NSCLC has a much lower five-year survival rate (17.8%) than other common types of cancers [19]. Metastasis is the most frequent clinical problem in the treatment of NSCLC, for which clinical practice includes molecular-targeted agents, palliative radiotherapy and immune checkpoint inhibitor treatment. In the present research, the potential role of KLF4 in NSCLC was characterised and it was determined that KLF4 could mediate MSI2 via the JAK2/STAT3 signalling pathway, and thus affect NSCLC progression. Yang et al. [20] reported that MSI2 mediates the JAK2/STAT3 signalling pathway in bladder cancer cells, Thus, the findings of the present research were consistent with this previous research.

Both KLF4 and MSI2 expression are closely associated with the clinical progression of patients with malignancies. Previously, a report suggested that the KLF4/MSI2 signalling pathway critically regulated

the growth and metastasis of pancreatic cancer in a mouse model [6]. However, the work could not elucidate the underlying mechanism regarding how this pathway regulates the functions of the downstream targets. In agreement with this previous research, the present study demonstrated that KLF4 expression was downregulated in human NSCLC specimens and cell lines, whereas MSI2 was upregulated. Statistical analysis revealed that their expression levels were negatively correlated. This association was further demonstrated by the subsequent findings that overexpression of KLF4 or knockdown of MSI2 expression had the same inhibitory effects on the proliferative, invasive and migratory abilities of NSCLC cells. Consequently, it was suggested that both KLF4 and MSI2 might be involved in the occurrence and development of NSCLC.

The JAK2/STAT3 signalling pathway is a commonly known pathway that plays an important role in the epithelial-mesenchymal transition (EMT) of cancer cells [18]. MSI2 has been implicated in this signalling pathway in bladder cancer [20]. The present data further supported the important role of MSI2 in NSCLC and provided more evidence that KLF4 has an upstream regulatory role in this process. KLF4 directly targeted the promoter region of MSI2, thus suppressing its transcriptional activity. Overexpression of KLF4 reduced the activity of the MSI2 promoter, whereas KLF4 knockdown enhanced the activity of the MSI2 promoter. Furthermore, the alterations in the expression levels of KLF4 or MSI2 were both closely related to the phosphorylation of JAK2 and its downstream effector STAT3. In addition, the expression of WASF3, which is activated by the JAK2/STAT3 signalling pathway [21], was also demonstrated in the present research. A549 cells transfected with pKLF4 showed decreased proliferation, invasion and migration, along with reduced expression of the JAK2/STAT3 marker proteins. However, following the overexpression of MSI2 or co-culture with a STAT3 activator, these changes partially recovered.

However, there are some limitations of the present study. Additional underlying mechanisms by which KLF4 mediates the tumorigenesis of NSCLC need to be further explored, and more rescue experiments are needed to further verify the association between KLF4 and JAK2/STAT3 signalling. Meanwhile, the mechanism by which KLF4 regulates JAK2/STAT3 pathway in NSCLC remains unexplored. Thus, further studies are needed in the future.

Conclusions

To the best of our knowledge, the present research is the first to illustrate the regulatory role of KLF4 in the suppression of MSI2 via the JAK2/STAT3 signalling pathway in NSCLC. The precise molecular characterization of the abnormal signalling cascades involved in NSCLC must be evaluated as the combination of molecular targeted drugs and multi-gene therapy has gradually emerged as a research trend and would likely become a novel strategy for targeted NSCLC treatment. The present research provided clues for the further exploration of the gene crosstalk among KLF4, MSI2 and JAK2/STAT3 in NSCLC, and warrants further investigation to assist in finding novel lung cancer therapeutic options.

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Ethical approval

The collection of clinical specimens was approved by the Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). All participants were required to provide written informed consent.

Consent for publication

The informed consent was obtained from study participants.

Availability of data and material

All data generated or analyzed during this work are included in this article. The datasets used and/or analyzed during the current research are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Di-Di Luo: Conceptualization, Visualization, Data curation, Writing – original draft, Formal analysis, Supervision. **Feng Zhao:** Project administration, Conceptualization, Visualization, Investigation, Funding acquisition, Methodology, Data curation, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2022.101396.

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