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KIFC1 aggravates non-small-cell lung cancer cell proliferation and metastasis via provoking TGF-β/SMAD signal

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ABSTRACT

Worldwide, non-small-cell lung cancers (NSCLC) are considered one of the deadliest cancers. Very early onset of distant metastasis is an important reason for the lower survival rate of NSCLC patients. Kinesin family member C1 (KIFC1) with high protein levels in various cancers contributes to the initiation and development of many cancers. KIFC1 has also been suggested as a possible marker of NSCLC. Nevertheless, the effects of KIFC1 on NSCLC metastasis have not been researched. To investigate the role of KIFC1 in NSCLC and related mechanisms. Western blot and quantitative real-time PCR were conducted to test the levels of KIFC1 in NSCLC cancerous tissues and NSCLC cancerous cell lines. Colony formation assay, CCK-8, transwell assay and wound healing assay were conducted to detect the functions of KIFC1 on proliferation, migration and invasion of NSCLC cell lines. WesternBlot was conducted to test the role of KIFC1in EMT and TGF-β/SMAD pathway. We discovered that the protein levels of KIFC1 were upregulated in NSCLC cancerous cell lines and cancerous tissues from mankind. KIFC1 was positively related to worse clinical staging and lymph node metastasis of NSCLC patients in clinical data. Overexpressed KIFC1 aggravated expansion, migration and invasion in NSCLC cells, whereas silencing of KIFC1 had the opposite effect in vitro. Mechanistically, the progression of NSCLC was promoted by KIFC1 through induction of EMT and TGF-β/SMAD signal. KIFC1 promoted proliferation and metastasis through accommodating TGF-β/SMAD signal, which is a hint that KIFC1 might offer a prospective therapeutic target for the NSCLC treatment.

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Introduction

As a highly malignant cancer, lung cancer is a larger component of cancer-associated deaths worldwide and exhibits an overall five-year survival rate of less than 11% (1). Of these, non-small-cell lung cancers (NSCLC) accounted for 85% of total lung cancer cases that occurred (2). Typically, NSCLC has been identified and classified into three different categories: large-cell cancer, adenocarcinoma, and squamous cell carcinoma (3). Although many strategies have been developed to treat NSCLC, including surgery, chemotherapy, radiotherapy and immunotherapy like PD-1/PD-L1 antibodies, the death rate from NSCLC remains high, and the major cause is that the tumor has metastasized before diagnosis (4). Therefore, further elucidation of the mechanisms of NSCLC progression and metastasis and as well as identification of new potential targets for intervention are critical for the treatment of this cancer.

As a member of the kinesin-14 family, kinesin family member C1 (KIFC1), also termed HSET, is a nonessential kinesin motor protein and plays a key role in microtubule transport, centrosome clustering and spindle formation during mitosis (5). KIFC1 is reported to exert it function in spermatogenesis (6), oocyte development (7), vesicle and organelle trafficking (8), and double-stranded DNA transport (9). Recently, a study showed that KIFC1 is necessary for stable pole focusing, spindle assembly and normal division of cancer cells with excess centrosomes, suggesting that it has a more pervasive and critical function in the development of cancer cells (10). In fact, KIFC1 has been reported to be highly expressed in multiple cancer types, including hepatocellular cancer (11), breast cancer (12) and ovarian cancer (13), and it facilitates cancer initiation and progression. A newly published study also showed that KIFC1 is overexpressed in NSCLC tissues and knockdown of KIFC1 induces growth inhibition of cancer cells by arresting the cell cycles (14). However, the functions of KIFC1 in the migration and metastasis of NSCLC have not been investigated.

Transforming growth factor beta (TGF-β), as a vital signaling pathway mainly capable of inducing epithelial-mesenchymal transition (EMT) (15, 16), plays a variety of roles in the progression of various cancers (17). In canonical SMAD-dependent signaling, TGF-β binds to a type II receptor located on the outer surface of cell membranes, which recruits a type I receptor to activate its serine/threonine protein kinase. Activated type I receptor induces C-terminal phosphorylation of SMAD2 and SMAD3, subsequently initiating their translocation to the nucleus by forming a transcriptional complex with SMAD4, to modulate the expression of specific molecules (18; 19). Many

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investigations have demonstrated that activation of the TGF-β/SMAD signal is correlated with the expansion, metastasis, and sensitivity to chemotherapy of NSCLC (20-23). However, whether KIFC1 can promote NSCLC metastasis by provoking the TGF-β/SMAD pathway is still not investigated.

Therefore, our research aims to elucidate the effect of KIFC1 in NSCLC metastasis and reveal its potential molecular mechanisms involved in the TGF-β/SMAD pathway by affecting EMT.

Materials and Methods

Tissue samples from patients

Tumors and paired normal tumor-adjacent tissues were surgically resected and gathered from NSCLC patients who had never previously received chemotherapy, radiotherapy or targeted therapy in Wujin Hospital Affiliated with Jiangsu University. Detailed clinicopathological characteristics of patients are derived from medical records in the hospital's computerized registry databases. The study protocol has been approved by ethics committees of the clinical research, and all patients agreed and signed informed consent forms.

Cells and culture conditions

The cell lines, including BEAS-2B, H1730, SK-MES, PC-9, A549, H1299, and SPC-A1 were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA), and incubated with 5% CO2 at 37°C in a humidified atmosphere using the respective medium. BEAS-2B, SK-MES, PC9, and A549 cells were kept in DMEM medium (HyClone, cat. SH30022.01B, South Logan, UT, USA) supplemented with 10% FBS and 1% penicillin-streptomycin. H1730, H1299, and PC-9 cells were cultured in RPMI-1640 medium (HyClone, cat. SH30809.01B, South Logan, UT, USA) with 1% penicillin-streptomycin and 10% FBS.

Antibodies

See below for all antibodies used: anti-KIFC1 (ThermoFisher, cat. 20790-1-AP, Waltham, MA, USA), anti-E-cadherin (Beyotime, cat. AF6759, Shanghai, China), anti-N-cadherin (Beyotime, cat. AG1554, Shanghai, China), anti-Vimentin (Beyotime, cat. AF1975, Shanghai, China), anti-TGFβ (Beyotime, cat. AF0297, Shanghai, China), anti-SMAD2/3 (Beyotime, cat. AF8001, Shanghai, China), anti-phospho-Smad 2/3 (Beyotime, cat. AF5920, Shanghai, China), anti-SMAD4 (Beyotime, cat. AF1291, Shanghai, China), anti-β actin (Beyotime, cat. AF5003, Shanghai, China), Secondary antibody (Beyotime, cat. A0208, Shanghai, China).

Cell transfection

The plasmids of pcDNA3.1-KIFC1 (OE-KIFC) and pcDNA3.1 empty vector were obtained from GenePharma in China and transfected into PC9 cells to up-regulate KIFC1 expression. KIFC1-targeted shRNA sequences (sh-KIFC1-1, sh-KIFC1-2) were processed by GenePharma in China and used to transfect A549 cells to down-regulate KIFC1 expression. Pointless shRNA can be designed as a negative control (sh-NC). Sequences of the specific short interfering RNAs are as follows: sh-NC, TTCTCCGAACGTGTCACGT; sh-KIFC1#1, TGAGAAGAAACGGACAAGA; sh-KIFC1#2, GGTGGAATTGCAGGAAGAA. The cells were cultured in plates with 96-well until they reached 70% confluence, then using Lipofectamine 3000 (ThermoFisher, cat. L3000001, Waltham, MA, USA) for transfection. Following 48 h of transfection, quantitative real-time PCR or western blot was used to confirm the transfection efficiency.

Quantitative real-time PCR

Cell total RNAs were purified by TRIzol reagents (Invitrogen, cat. 15596018, Carlsbad, CA, USA) and then treated with first chain cDNA transcription kit (Thermo Scientific, cat. #K1682, Waltham, MA, USA) to reverse transcribed into cDNA. The expression of the indicated genes was tested by real-time quantitative PCR via using SYBR Green (TaKaRa, cat. #RR820A, Tokyo, Japan) on ABI7500 real-time PCR System (Bio-Rad, Hercules, CA, USA). The primers used were described below: KIFC1 forward, 5'- GGTGCAACGACCAAAATTACC -3', KIFC1 reverse, 5'-GGGTCCTGTCTTCTTGGAAAC -3'; β-actin forward, 5'-CATGTACGTTGCTATCCAGGC-3', β-actin reverse, 5'- CTCCTTAATGTCACGCACGAT -3'. The relative expression level of target gene was calculated by a comparative Ct ($^{\Delta\Delta}$Ct) method.

Western Blot

The cultured cells were first lysed with RIPA buffers (Beyotime, cat. P0013B, Shanghai, China). The total concentrations of protein were measured by a BCA assay kit (CWBIO, cat. CW0014S). The same amounts of protein were isolated by 10% SDS-PAGE and then transferred to PVDF membranes (Millipore, cat. ISEQ00010). The membranes were blocked with milk and incubated at 4°Covernight with the indicated primary antibodies, followed by incubation with enzyme-labeled secondary antibodies at room temperature. Signal strips were shown by ECL kits (Applygen Technologies, cat. P1030). Beta-actin served as a load control.

Cell counting kit (CCK)-8 assay

The proliferation of cells was determined using the commercial CCK-8 (Beyotime, China, cat. C0037, Shanghai, China). Cells were inoculated into a 96-well plate at the density of 2×10^4 cells/well and incubated in DMEM with 10% FBS for the corresponding time (24 h, 48 h, 72 h, 96 h). Then, each well was added to the culture plate of CCK8 solution and incubated at 37°C for 4 h. Absorbance (OD450) at 450 nm was determined with the microplate readers.

Colony-formation assay

Cell proliferation capacity was assessed by colony-formative assay. A549 and PC9 cells were inoculated at 1×10^5/well in a 96-well plate and maintained for 14 days to form colonies. To make the colony visible, the cells were fixed with methanol for 15 min and then stained in Giemsa for 30 min. Numbers of colonies containing > 50 cells were photographed and counted.

Transwell migration and invasion assay

The migration and invasion capacity of cells was evaluated by transwell assays. To perform cell migration assays, treated cells diluted with DMEM lacking serum were inoculated to the above lumen of a transwell insert (pore

size, 8 μm; Corning, cat. CLS3464, Corning, NY, USA), while DMEM with 20% FBS was filled into the inferior lumen acted as a chemotactic agent. After the cells were cultured at at 37°C for 24 h, the cells migrated below the filter were fixed and then stained in crystal violet. Samples were counted and photographed under light microscopes. To perform cell invasion assays, a transwell insert pre-coated with matrigel (Millipore, cat. E1270, Billerica, MA, USA) in the upper lumen was used for experiments, other procedures are the same as the cell migration assays.

Wound-healing assay

Cellular motion was evaluated by wound-healing assays. Transfected overexpression and knockdown cells were inoculated into 6 wells plate and cultured to reach 80% confluences. Then, 200 μL pipette tips were used to create wound via cutting apart the cellular layer. PBS flushing removed the floating cells. The size of the wound was photographed and measured after 24 h of wound formation. The cell migration area was calculated for statistical analysis.

Statistical analysis

Three separate experiments were repeated to produce data, which was expressed as mean plus standard deviation (SD). Statistical comparison between groups was conducted by GraphPad Prism 8 software (La Jolla, CA, USA). Student test with two-tails was used for statistical difference analysis, and the values were $P<0.05$, $P<0.01$, $P<0.001$, represented by *, ** and ***, respectively.

Results

KIFC1 is upregulated in NSCLC cancerous cells and cancerous tissues

To characterize the effects of KIFC1 on NSCLC progression, we first detected the KIFC1 protein levels in NSCLC cancerous tissues and NSCLC cancerous cell lines, including H1730, SK-MES, PC9, SPC-A1, A549 and H1299. First, the upregulation of KIFC1 in paired NSCLC cancerous tissues (n=20) was verified by detecting the mRNA levels of KIFC1 via Real-time RT-PCR (Figure 1A). Furthermore, we found that both mRNA and protein expressions of KIFC1 were upregulated in cell lines of NSCLC (H1730, SK-MES, PC9, SPC-A1, H1299 and A549) compared with BEAS-2B, a mankind normal bronchiel epithelial cell line (Figure 1B and 1C). Compared with other NSCLC cell lines, A549 cells that expressed the highest KIFC1 expression levels were conducted in the loss-of-functions assay, and PC9 cells with the lowest KIFC1 expression levels were conducted for the gains-of-functions assay. These data suggested that the high expression levels of KIFC1 in NSCLC cancerous tissues and cancerous cell lines may herald its potential role in NSCLC development.

Then, we analyzed the correlation between KIFC1 and general information or clinicopathological characteristics of NSCLC patients. The data suggested that the expression of KIFC1 was remarkably correlated with TNM staging (III-IV) (p=0.006) and lymph node metastasis (P=0.006), but not with age, sex or smoking history (Table 1). Taken together, these clinical data implied that KIFC1 might be

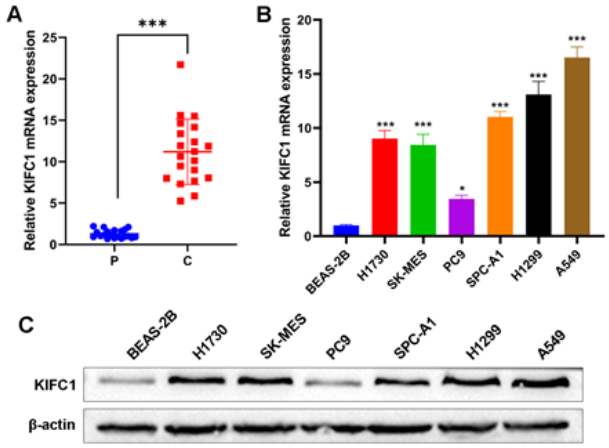

Figure 1. The protein levels of KIFC1 increase in NSCLC tissues and cells. (A) The mRNAs of KIFC1 were analyzed by real-time PCR in 20 paired NSCLC and paracancerous tissues. (B) Real-time PCR analyzes KIFC1 mRNA levels in NSCLC cells and BEAS-2B. (C) The protein levels of KIFC1 were analyzed by western blot in NSCLC cells and BEAS-2B. The data represent the mean±SD of 3 independent expressions. Paired student's *t*-test with two tails, *$P<0.05$, and ***$P<0.001$. C: cancer, P: paracancerous tissue.

Table 1. Association between KIFC1 expression and clinical characteristics.

Characteristics	n	KIFC1 expression		*P* value
		Low	High	
Age				
< 60	14	8	6	0.392
≥60	6	2	4	
Sex				
Male	15	7	8	0.606
Female	5	3	2	
Smoking history				
Yes	16	7	9	0.264
No	4	3	1	
TNM Stage				
I-II	12	9	3	0.006
III-IV	8	1	7	
Lymph metastasis				
Yes	8	1	7	0.006
No	12	9	3	

involved in the process of metastasis of NSCLC.

KIFC1 promotes cell proliferation of NSCLC in vitro

To identify the effects of KIFC1 on NSCLC, loss-of-functions and gains-of-functions assays were conducted by silencing KIFC1 in A549 cells and KIFC1 overexpressed in PC9 cells, respectively. Quantitative RT-PCR and western blot assays were conducted for transfection efficiency confirmation (Figure 2A and 2B). Sh-KIFC#1, which exhibited a higher knockdown efficiency compared to sh-KIFC#2, was selected for the following experiments. Next, growth curves (CCK8 assay) and colony formation assays were conducted to test cell growth capacity. KIFC1 depletion suppressed the cells growth and resulted in a decrease in the numbers of A549 cell colonies (Figures 2C and 2E), whereas KIFC1 overexpression enhanced the cell growth and colony formation of PC9 cells (Figures 2D and 2F). The above data suggest that KIFC1 is an oncogenic gene that aggravates the in vitro proliferation of NSCLC cells.

KIFC1 aggravates in vitro invasion and migration of NSCLC cells

Following, we investigated the effects of KIFC1 on the invasion and migration of NSCLC cells by in vitro transwell assay and wound-healing assay. The results of migration and invasion analysis by transwell displayed that KIFC1 silencing suppressed the migrations and invasions

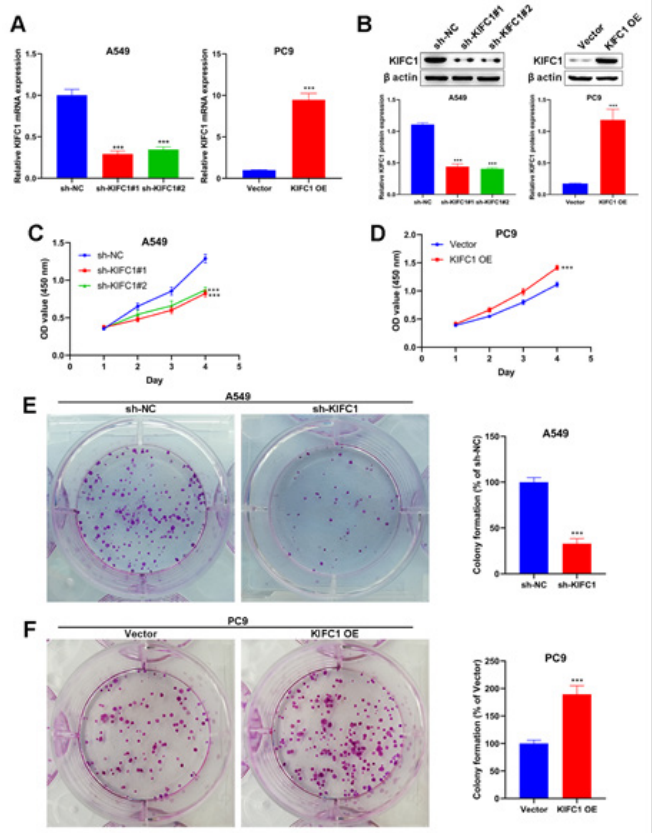

Figure 2. NSCLC cell proliferation was aggravated by KIFC1 in vitro. (A-B) Quantitative real-time PCR (A) and western blot (B) were conducted to assess the transfection efficiency of KIFC1. (C-D) CCK8 assays were used to assess the NSCLC cell proliferation. (E-F) Colony-formation assays were performed to detect the capacity of NSCLC cell proliferation. Data were displayed as mean±SD. Paired student's *t*-test, ***P<0.001.

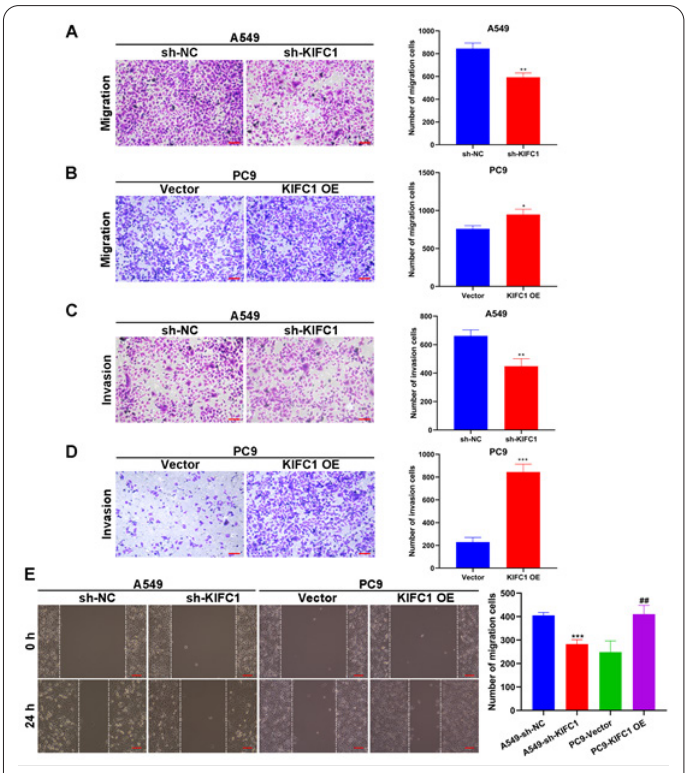

Figure 3. The capacity of NSCLC cell invasions and migrations in vitro was aggravated by KIFC1. (A-B) The migrations of NSCLC cells were evaluated by transwell assays. (C-D) The invasions of NSCLC cells were assessed by transwell assay. (E) Wound-healing assay analyzes the motility of NSCLC cells with KIFC1 overexpression or KIFC1 knockdown. Micrographs were acquired at 0 and 24h, and the offset distances (right panel) were measured by Image J software. All data were present as mean±SD. Paired student's *t* test, $^{\#\#}P<0.01$ from PC9-vector group; $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$.

of A549 cells (Figure 3A and 3C), while overexpressed KIFC1 aggravated the migrations and invasions of PC9 cells (Figure 3B and 3D). Wound healing assays also revealed much narrower blank areas in KIFC1 overexpressing PC9 cells compared to controls, indicating increased migration abilities of PC9 cells overexpressing KIFC1. In contrast, the blank area of A549 cells silenced with KIFC1 was much wider compared to the control, suggesting that the knockdown of KIFC1 reduces the migratory capacity of A549 cells (Figure 3E). Together, the above data suggest that KIFC1 aggravates the migrations and invasions of NSCLC cells in vitro.

KIFC1 activates EMT signals in NSCLC cancerous cells

Extensive evidence suggests that epithelial-mesenchymal transition (EMT) in NSCLC is related with cancer progression and worse prognosis (24, 25). Consistently, the protein levels of epithelial cell marker (E-cadherin) were obviously elevated, while the protein levels of mesenchyme markers (Vimentin and N-cadherin) were significantly down-regulated in KIFC1 depleted A549 cell lines (Figure 4A). In contrast, E-cadherin expression was substantially downregulated, while Vimentin and N-cadherin levels were upregulated in KIFC1overexpressing PC9 cells (Figure 4B). These findings indicated that KIFC1 can induce EMT in NSCLC cells, which was associated with its promotion of proliferation, metastasis and invasion in NSCLC.

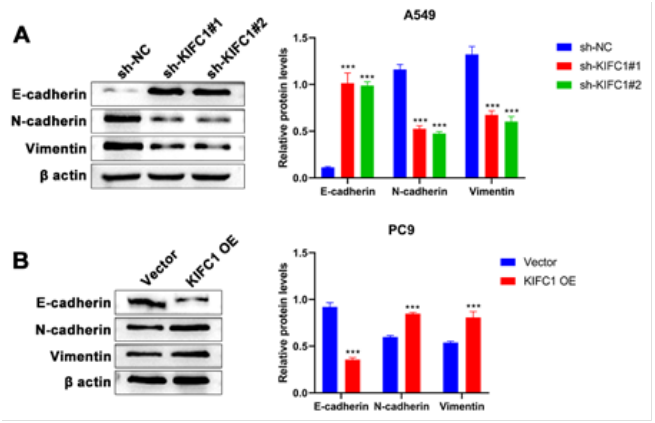

Figure 4. KIFC1 is an inducer of EMT in NSCLC cells. (A) Western blots were conducted to test the protein levels of N-cadherin, E-cadherin and Vimentin in A549 cells with KIFC1 knockdown. (B) Western blots were conducted to measure the protein levels of N-cadherin, E-cadherin and Vimentin in PC9 cells with KIFC1 overexpression. Data were shown as mean±SD. Paired student's *t*-test, ***P<0.001. ns: no significant difference.

KIFC1 exerts its oncogenic effects through activation of the TGF-β/SMAD signal

TGF-β/SMAD signals play crucial roles in NSCLC carcinogenesis and metastasis (23, 26). Moreover, TGF-β triggers EMT mainly through the SMAD signaling pathway (27, 28). Hence, we further explored the roles of KIFC1 in the modulation of TGF-β/SMAD signal. As shown in Figure 5A, depletion of KIFC1 decreased the protein expressions of TGF-β, phosphorylated Smad 2/3, total Smad 2/3 and total Smad 4 in A549 cells. Conversely, overexpressed KIFC1 provoked TGF-β/SMAD signaling by increasing the protein expressions of TGF-β, phosphorylated Smad 2/3, total Smad 2/3 and total Smad 4 in PC9 cells (Figure 5B).In general, these data indicated that the TGF-β/SMAD signal might be involved in KIFC1-mediated proliferation, invasion, migration and EMT of NSCLC cells.

Discussion

NSCLC accounts for the largest proportion of total lung cancer cases (85%) and shows a high rate of distant metastasis with a five-year survival rate of no more than 5% (29). Despite the development of treatments for NSCLC in recent years, its high incidence of tumor metastasis is the foremost reason for recurrence and mortality in NSCLC patients (30, 31). Therefore, the goal of achieving comprehensive control of cancer metastasis by elucidating the genetic aberrations and the underlying signaling pathways that drive NSCLC metastasis is urgently needed. In this project, we disclosed that the protein levels of KIFC1 were elevated in NSCLC tissues and cells, which was remarkably correlated to tumor stage and the occurrence of metastasis in NSCLC patients. Upregulated KIFC1 not only promotes cancer cell proliferation but also has been identified for the first time to promote invasion, migration and EMT in NSCLC cells. Moreover, KIFC1 might modulate the development of NSCLC by provoking the TGF-β/SMAD pathway.

The expression levels of KIFC1 were elevated in endometrial cancer (EC) and accelerated cancer expansion and metastasis through regulating the PI3K/AKT pathway (32). Of hepatocellular cancers, KIFC1 is also highly expressed and acts as an oncogene that promotes the pathogenesis of HCC and reduces the sensitivity of HCC to paclitaxel (33). KIFC1 has also been shown to be a potential marker for predicting worse prognosis, overall survival and metastasis in ovarian cancer (13). High expression of KIFC1 was associated with genomic instability, PD-L1 expression, alteration of TP53, cisplatin resistance and poor prognosis in bladder cancer (34). Therefore, KIFC1 might have potential as a marker for tumor diagnostic and therapeutic targets. In our study, we collected cancerous and paraneoplastic tissues from NSCLC patients, as well as NSCLC cell lines for analysis. We disclosed that KIFC1 levels were remarkably increased in both NSCLC cancerous tissues and cells. Further analysis of clinical data showed that NSCLC patients bearing high KIFC1 levels have worse clinical stage and earlier lymph node metastasis. The above data indicated that KIFC1 may be a prospective biomarker for clinical diagnosis and a promising therapeutic target for NSCLC.

Previous studies have reported that interfering with KIFC1 expression can significantly suppress cell growth in breast cancer cells (35). KIFC silencing was shown to reduce cell proliferation, migration and expression of CENPE, a tumor promoter, in ovarian cancer (36). Consistent with these studies, our results proved that KIFC1 can aggravate cell expansion, invasion and migration in NSCLC cells, suggesting that KIFC1 might influence NSCLC progression. Epithelial-mesenchymal transition (EMT) is an inducer that drives metastasis during the progression of many cancer types, such as NSCLC (37). In particular, many studies have demonstrated that KIFC1 can induce EMT and promote metastasis in cancer cells (11; 36). Expectantly, our results also displayed that KIFC1 could induce EMT in NSCLC cells. TGF-β/SMAD signaling is identified as a vital inducer of EMT. Whether KIFC1 can provoke the TGF-β/SMAD signal to aggravate metastasis has not been clarified. Our analysis also demonstrated that KIFC1 can promote the NSCLC progression of NSCLC by provoking TGF-β/SMAD signaling.

In summary, we disclosed that the expression levels of

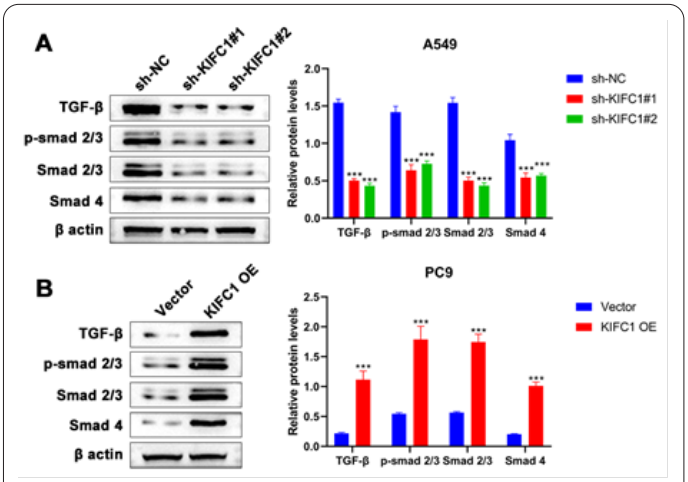

Figure 5. KIFC1 exerts a tumorigenic effect in NSCLC cells via the TGF-β/SMAD signal. (A) Western blots were conducted to measure the protein level of TGF-β, p-Smad 2/3, Smad2/3, and Smad4 in A549 cells with KIFC1 depletion. (B) Western blots were conducted to test the protein expression of TGF-β, p-Smad 2/3, Smad2/3, and Smad4 in PC9 cells with KIFC1 overexpression. Data were shown as mean±SD. Paired Student's *t*-test, ***P<0.001.

KIFC1 are upregulated in mankind NSCLC tissues, and were significantly positively correlated with clinical staging and lymph gland metastasis. KIFC1 played a key role in regulating the expansion, invasion, and migration of NSCLC cells, and the intrinsic mechanisms were at least in part through modulating TGF-β/SMAD signals. Nevertheless, in addition to Smad2/3 and Smad4, the role of other smad molecules in promoting NSCLC progression by KIFC1 deserves further exploration and research. In a word, our findings highlight KIFC1 as a new and prospective target of treatment in NSCLC therapeutic schedules.

Conflict of Interests

The authors declared no conflict of interest.

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