ZNF251 promotes the progression of lung cancer by activating ERK signaling

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Abstract
Aberrant activation of ERK signaling is a hallmark of lung cancer. Although constitutively activating mutations of EGFR and KRAS contribute to the hyperactivation of ERK1/2, other mechanisms remain elusive. In this study, the zinc finger protein ZNF251 was found to be upregulated in clinical lung cancer samples, and it promoted the growth of lung cancer cells and the growth of primary lung KPC cells from mouse models (Ad-Cre, KrasG12D, and P53f/f). In studying the molecular mechanism, ZNF251 was found to inhibit the expression of dual-specificity phosphatase 6, a negative regulator of ERK activation, by directly binding to its promoter region. Taken together, our data indicate the tumor-promoting effects of ZNF251 in lung cancer and suggest that ZNF251 is a therapeutic target.

KEYWORDS
DUSP6, ERK, lung cancer, ZNF251

1 | INTRODUCTION

Lung cancer is one of the most common malignancies in the world. Non-small-cell lung cancer, which includes adenocarcinoma and squamous cell carcinoma, contributes to the major pathological type of lung cancer. Due to air pollution and smoking, the incidence of lung cancer is increasing. A better understanding of the carcinogenesis of lung cancer would be beneficial for its treatment.

Hyperactivation of ERK promotes the growth and malignant transformation of lung epithelial cells. Aberrant activation of ERK signaling can be caused by a constitutively active mutation of Kras (KrasG12D) and by epidermal growth factor receptor (EGFR). However, other mechanisms also contribute to the dysregulation of this signaling. Multiple reports have shown that dual-specificity phosphatase 6 (Dusp6), an ERK phosphatase, attenuates ERK signaling by the dephosphorylation of ERK. Consistent with these studies, DUSP6 has been found to be downregulated in clinical lung tissues. However, how the expression of DUSP6 is regulated remains unknown.

ZNF251, which belongs to the zinc finger protein family, is one of the somatic genes that is mutated in pulmonary sclerosing pneumocytoma, which presents with diffusely scattered nodules in the right lung. To date, the functions of ZNF251 in mammals remain elusive. In this study, we investigated the expression pattern of ZNF251 in lung cancer and studied its functions and molecular mechanisms in the tumorigenesis of lung cancer.

2 | MATERIALS AND METHODS

2.1 Cell culture and transfection

Lung cancer cell lines (A549, H520, H23, H460, and SPC-A-1) and a normal bronchial epithelial cell line (Beas-2B) were obtained from the cell bank of the Chinese Academy of Science (Shanghai, China). Primary KPC cells were a gift from Dr Ji (Shanghai Institutes of Biological Science, Shanghai, China). All of the cell lines and KPC
cells were maintained in DMEM (11965-084; Gibco) with 10% FBS (10099141; Gibco) as well as penicillin and streptomycin. Cells were transfected with Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer’s instructions. Stable cell lines were selected by treatment with 400 µg/mL G418 (Sangon) for 2 weeks. The resistant cells were pooled, and western blotting was carried out to examine the expression of exogenous ZNF251.

2.2 | Clinical samples

Clinical lung cancer samples were obtained from Shanghai Chest Hospital of Shanghai Jiaotong University. The pathology was determined by 2 pathologists. The samples were collected after obtaining written consent from the patients. These studies were approved by the Ethics Committee of Shanghai Chest Hospital.

2.3 | Quantitative PCR

The RNA was extracted from the clinical tissues and cells using TRIzol (Thermo Fisher Scientific). cDNA was synthesized using a kit (Promega) according to the manufacturer’s instructions. Quantitative PCR was undertaken using a 2× SYBR Green mixture (Takara) according to the manufacturer’s instructions and the Stratagene 3000MP. The sequences for ZNF251 primers were as follows: F, 5′-ttccaggatgtggccgtgta-3′; R, 5′-ccagaagattcaggacccaa-3′.

**FIGURE 1** Expression of ZNF251 was elevated in lung cancer. A, mRNA levels of ZNF251 in 50 lung cancer tissues and paired noncancerous tissues were examined using quantitative PCR. B, Statistical analysis of the data in (A). C, Protein levels of ZNF251 in lung cancerous tissues and paired noncancerous tissues were examined using immunohistochemistry (IHC). Magnification is shown. TTF-1 acted as the bio-mark for lung adenocarcinoma. D, Statistical analysis of the data in (C). E, Protein levels of ZNF251 in normal lung epithelial cells, Beas-2B, and in cancer cells was examined using western blot. F, Western blot analysis was used to examine the protein levels of ZNF251 in 3 panels of lung cancer cells. G, H, IHC was carried out to examine the protein levels of ZNF251 in lung cancer tissues with/without Kras mutation (mut), and statistical analysis was undertaken. **P < .01
2.4 Immunohistochemistry

Tissues were embedded in paraffin and cut into 5-µm sections. Dewaxing and rehydration were carried out by placing slides in a decreasing gradient series of xylol and ethanol. Antigen recovery was undertaken by incubating the sections in a pH 6.0 citrate sodium solution at 100°C for 20 minutes. After blocking the endogenous peroxidase activity, the tissues were incubated with the primary Ab at 4°C overnight. Then the secondary Ab was added and incubated with the tissues at room temperature for 1 hour. The signals were developed using DAB (ZSGB-Bio), and the sections were stained with hematoxylin. Tissues were examined under a microscope, and the scoring was carried out as described.16

2.5 Western blot analysis

Protein was extracted from the clinical tissues and cells with RIPA buffer (Cell Signaling Technology). After the concentration of the protein was determined, the protein was separated by SDS-PAGE. The proteins were then transferred to a PVDF membrane (Millipore) and blocked with 5% BSA (Sangon) at room temperature for 1 hour. The membrane was incubated with the primary Ab at 4°C overnight. Then the secondary Ab was added and incubated with the tissues at room temperature for 1 hour, and the signals were developed using an ECL kit (Pierce).

Anti-ZNF251 (ab86535, 1:2000) and anti-DUSP6 (ab76310, 1:3000) Abs were obtained from Abcam. Anti-GAPDH (#5174, 1:1000), anti-myc (#2276, 1:1000), anti-total ERK (#4695, 1:1000), anti-phosphorylated ERK (Thr202/Tyr204) (#4370, 1:1000), and anti-tubulin (#2146, 1:3000) Abs were obtained from Cell Signaling Technology. The HRP-coupled secondary Abs were obtained from Cell Signaling Technology.

2.6 Lung cancer mouse model

The mouse models LKB f/f;KrasG12D and p53 f/f;KrasG12D were housed under standard conditions with 12:12-hour dark : light cycles. To induce lung cancer, we anesthetized 8-week-old mice with 2.5% tribromoethanol, making sure that the mice were fully anesthetized and had no reaction to pain. Ad-Cre (adenovirus) virus (OBIO) was inoculated (1 × 10^9 per mouse) using the intranasal/orthotropic infection protocol as described.17 Eight weeks later, the lungs were harvested, and western blot analysis was carried out.

2.7 Cell viability assay

Cells were plated in a 96-well plate with 200 µL DMEM at a density of 1 × 10^5 cells/well. Then 20 µL MTT (Sangon) per well was added and incubated for 3 hours with the cells at 37°C. After incubation, cells were treated with MTT solvent for 15 minutes at room temperature. Absorbance was measured at optical density of 540 nm.

2.8 Soft agar assay

Soft agar assays were carried out using 10-cm dishes. The bottom layer of soft agar was prepared using DMEM containing 0.75% agar and 10% FBS. The top layer of soft agar was prepared using DMEM containing 1 × 10^4 cells/mL, 10% FBS, and 0.36% agar. The dishes were labelled and put into a 37°C incubator for 3 weeks. Then the colonies were photographed, and the number of colonies (more than 50 µm) of 4 fields for each group was counted.

2.9 Chromatin immunoprecipitation assay

Cells were cross-linked in formaldehyde for 10 minutes at 37°C and resuspended in 300 µL lysis buffer (10 mmol/L EDTA, 50 mmol/L Tris, pH 8.1, 1 mmol/L PMSF, and 1% SDS). The genome was fragmented by sonication. A herring sperm DNA/protein G-Sepharose (Sigma) slurry was used to preclear the supernatant. After mixing with the protein

FIGURE 2  Expression of ZNF251 was elevated in mouse models for lung cancer. A, B, KrasG12D;P53 f/f mice (A) and KrasG12D;LKB f/f mice (B) were treated with 1 × 10^9 Ad-Cre virus or 1 × 10^9 Ad-GFP virus per mouse. Twelve weeks later, the mice were killed, the protein from the lung tissues was extracted, and western blotting was carried out. C, Western blot was undertaken to examine the effects of KrasV12 expression on the protein levels of ZNF251 in 2 lung cancer cell lines
G-Sepharose beads and herring sperm DNA, the supernatants were incubated with a ChIP grade Ab or a control IgG for 4 hours. DNA was eluted from the beads with buffer (1% SDS and 1.1 mol/L NaHCO₃) at 65°C for 4 hours, and quantitative PCR was carried out.

2.10 | Luciferase assay

The promoter of human DUSP6 was cloned into the pGL3 basic vector. A549 cells were cotransfected with DUSP6 promoter and TK renilla as an internal control. The reporter activity was examined using the kit from Promega.

2.11 | Statistical analysis

The experiments were carried out in triplicate. SPSS 13.0 software was used for statistical analysis, and the data are presented as the mean ± SEM. Statistically significant differences were determined with Student’s t test or ANOVA, and were defined as *P < .05 and **P < .01.
3 | RESULTS

3.1 | ZNF251 mRNA and protein levels increased in clinical lung cancer samples and cell lines

We first examined the expression pattern of ZNF251 in lung cancer samples and cell lines. Quantitative PCR was undertaken to examine the mRNA levels of ZNF251 in 50 lung cancer samples and paired normal tissues. Upregulation of ZNF251 mRNA levels was observed in the cancer tissues (Figure 1A,B). To further confirm these observations, the protein levels of ZNF251 were examined in 20 lung cancer tissues and paired noncancerous tissues. Consistently, higher ZNF251 protein levels were observed in cancerous tissues than in the noncancerous control tissues (Figure 1C,D). In addition, we assessed the protein levels of ZNF251 in a panel of lung cancer cell lines and normal lung epithelial cell lines. Higher ZNF251 protein levels were found in lung cancer cell lines (A549, H520, H23, H460, and SPC-A-1), and lower ZNF251 protein levels were found in normal Beas-2B cells (Figure 1E).

We next examined the correlation between the expression of ZNF251 and the Kras mutation; we examined the expression of ZNF251 in a panel of lung cancer cells. As shown in Figure 1F, the highest expression of ZNF251 was found in SK-LU-1 and H441 cells, which harbored the mutant Kras, and the lowest expression of ZNF251 was found in H1299, H1437, H1993, and H2228 cells, which harbored WT Kras. In addition, it was found that the expression of ZNF251 was upregulated in the cells (PC-9, HCC4006, and HCC827) carrying mutated EGFR to some degree, possibly due to the activation of Kras signaling downstream of EGFR (Figure 1F). Furthermore, we examined the correlation between the protein levels of ZNF251 and Kras mutation in the lung cancer tissues. Among
the 50 lung cancer tissues, 12 lung cancer tissues harbored mutant Kras (Table S1). Higher ZNF251 protein levels were found in the lung cancer tissues with mutant Kras (Figure 1G,H). In summary, these data suggested that ZNF251 was upregulated in lung cancer.

### 3.2 | ZNF251 mRNA and protein levels increased in mouse models of lung cancer

Loss-of-function of P53 and LKB, and gain-of-the-function of the onco-gene Kras are frequently observed in lung cancer. Therefore, we examined the expression of ZNF251 in the lungs of the P53<sup>−/−</sup>;KrasG12D and LKB<sup>−/−</sup>;KrasG12D mouse models. As shown in Figure 2A,B, the level of ZNF251 protein was dramatically upregulated in the lung cancer models. To further confirm the causal relationship between ZNF251 protein levels and Kras status, we overexpressed KrasV12 in H1299 and H1703 cells. As shown in Figure 2C, overexpression of KrasV12 upregulated the protein level of ZNF251.

### 3.3 | ZNF251 promoted growth and tumorigenicity of lung cancer cells

To study the biological functions of ZNF251 during the progression of lung cancer, we first established a primary culture from the lungs of the P53<sup>−/−</sup>;KrasG12D mice and treated the cells with adenovirus expressing Cre (Ad-Cre) to suppress the expression of P53 and activate the expression of KrasG12D (the KPC cell line) (Figure 3A). ZNF251 was overexpressed in H520, A549, and KPC cells (Figure 3A). An MTT assay was carried out to examine the effects of ZNF251 overexpression on the growth of lung cancer cells in liquid culture. As shown in Figure 3B, forced expression of ZNF251 in H520 and A549 cells promoted the growth of cancer cells in a liquid culture. Moreover, we assessed the tumorigenicity of H520, A549, and KPC cells after overexpression of ZNF251 using the soft agar assay. The upregulation of ZNF251 enhanced the anchorage-independent growth of H520, A549, and KPC cells on soft agar (Figure 3C-F).
To further confirm the observations above, the expression of ZNF251 was downregulated in H520, A549, and KPC cells using 2 shRNAs with different sequences (Figure 4A). Knocking down the expression of ZNF251 inhibited the growth of H520 and A549 in liquid culture (Figure 4B) as well as the anchorage-independent growth of H520, KPC, and A549 cells on soft agar (Figure 4C-F).

### 3.4 ZNF251 activated ERK signaling in lung cancer cells

To understand the molecular mechanisms behind the tumorigenicity observations, we assessed the effects of ZNF251 on the activation of several pathways. Interestingly, ZNF251 was found to activate
ERK signaling, which was indicated by the phosphorylation of ERK Thr202 and Tyr204 (Figure 5A). Consistent with that observation, knocking down ZNF251 expression inhibited ERK phosphorylation (Figure 5A). Moreover, the Gene Set Enrichment Analysis showed that ZNF251 expression positively correlated with Kras-ERK signaling (Figure 5B). For the biological function, treatment with LY321496, an inhibitor of ERK, effectively blocked the colony formation induced by ZNF251 overexpression (Figure 5C,D).

3.5 | ZNF251 inhibited expression of DUSP6

Dual-specificity phosphatase 6 is a broadly expressed dual-specificity phosphatase that binds to and dephosphorylates ERK, which decreases ERK activity. Given that ZNF251 is mainly localized in the nucleus, we hypothesized that ZNF251 enhances ERK signaling by negative regulation of DUSP6. As shown in Figure 6A, knocking down the expression of ZNF251 upregulated the mRNA levels of DUSP6, and overexpressing ZNF251 downregulated DUSP6, as shown in Figure 6B. Consistent with these data, as shown in Figure 6C,D, ZNF251 negatively regulated the protein levels of DUSP6. We next examined whether ZNF251 activated ERK signaling by downregulating DUSP6. As shown in Figure 6E, overexpression of DUSP6 impaired the phosphorylation of ERK that was induced by ZNF251. Further data from ChIP assays indicated that ZNF251 was bound to the DUSP6 promoter (Figure 6F). To better understand the regulation of DUSP6 by ZNF251, we screened the binding site for ZNF251 in the DUSP6 promoter region (Table S2). As shown in Figure 6G, the deletion of −300 bp to −200 bp of the DUSP6 promoter region abolished the inhibition of DUSP6 promoter activity by ZNF251. Further mutation analysis indicated that the sequence CACCC is the binding site for ZNF251 because mutation of CACCC to CTAAT abolished the inhibition of DUSP6 promoter activity by ZNF251 (Figure 6H). Taken together, these observations suggest that ZNF251 negatively regulates the expression of DUSP6 and activates ERK signaling.

4 | DISCUSSION

Activation of ERK signaling is very prevalent in lung cancer, which suggests that ERK is a promising therapeutic target. Extracellular signal-regulated kinase signaling has been reported to be activated in approximately 90% of lung cancers, which is more frequent than the observation of EGFR and Kras mutations; this disparity indicates that there are additional molecular mechanisms that activate the ERK pathway. In this study, we have shown that the zinc finger protein ZNF251 activated ERK signaling by directly binding to and depressing the expression of DUSP6. ZNF251 promoted the phosphorylation of ERK and promoted the anchorage-independent growth of lung cancer cells, and ZNF251 was upregulated in lung cancer samples and lung cancer mouse models. These studies reported the oncogenic roles of ZNF251 in lung cancer. One of the interesting findings of this study is the upregulation of ZNF251 in clinical lung cancer samples, suggesting the application of ZNF251 as a biomarker for diagnosing lung cancer. Moreover, elevation of ZNF251 protein levels was observed in both LKB1f/f;KrasG12D and P53f/f;KrasG12D mouse models, indicating that ZNF251 might be a target of Kras signaling. To our knowledge, the present study is the first to reveal the roles of ZNF251 in tumorigenesis.

Another important finding of this study is the regulation of DUSP6 expression by ZNF251. ZNF251 directly binds the promoter region of DUSP6 and inhibits its expression. Moreover, several studies have shown that higher DUSP6 was correlated with lower ERK expression in squamous cell carcinomas. Based on these observations, screening to identify small molecular chemicals that inhibit the DNA binding activity of ZNF251 would benefit the treatment of lung cancer.

In summary, the present study has revealed the oncogenic roles of ZNF251 in lung cancer by activating ERK signaling and suggested that ZNF251 is a promising therapeutic target for lung cancer treatment.

CONFLICT OF INTEREST

There is no conflict of interest.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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