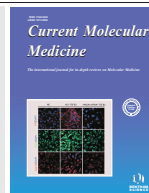


## RESEARCH ARTICLE

BENTHAM  
SCIENCE

## ZNF424 Induces Apoptosis and Inhibits Proliferation in Lung Carcinoma Cells



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**Abstract: Background:** Previously, we showed that the Zinc finger-containing transcription factor ZNF424 inhibits p21 transcription, which has been widely associated with various cancers. However, because the roles of ZNF424 in tumorigenesis have not been characterized, we correlated ZNF424 expression with tumorigenesis in lung cancer.

**Results:** The present immunohistochemical analyses show significantly lower ZNF424 expression levels in 43 of 60 lung cancer tissues compared with adjacent tissues. Moreover, flow cytometry assays indicated that overexpression of ZNF424 induces apoptosis in A549 human lung carcinoma cells, and overexpression of ZNF424 significantly increases numbers of G1 phase cells and decreases numbers of S phase cells, suggesting that ZNF424 inhibits proliferation. Western Blot analyses show that overexpression of ZNF424 decreases protein expression levels of the mitogen-activated protein kinase (MAPK) signaling proteins P-P38 and P-ERK in A549 cells.

**Conclusion:** These are the first data to associate ZNF424 with tumorigenesis and demonstrate an inhibitory role in lung cancer, indicating the potential of ZNF424 expression as a diagnostic marker of lung tumorigenesis.

## ARTICLE HISTORY

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## 1. INTRODUCTION

Zinc-finger (ZNF) proteins containing the Krüppel-associated box (KRAB-containing proteins) have been investigated since 1991 [1], and the KRAB-ZNF family is the largest family of transcription factors (TFs) in mammals [2, 3]. Accordingly, among various functions, KRAB-ZNF genes maintain embryonic stem cells in an undifferentiated state [4] and regulate the cell cycle [5] during brain and neuron development [6, 7].

Lung cancer is the leading cause of cancer-related death worldwide, and non-small-cell lung cancer (NSCLC) comprises about 80% of all lung cancers. Among tumorigenic regulatory networks, KRAB-ZNF genes have been associated with various pivotal biochemical functions in lung cancers. However, the biochemical functions of ZNF424 have not been characterized in lung cancer. Interestingly, our screening with the lymph cancer cell lines HL-60, K-562, Jurkat, Reh and the lung cancer A549 cell line suggested that ZNF424 had negative effects on prohibiting proliferation and promoting apoptosis only in the lung cancer A549 cell line, so we determined ZNF424 expression in A549 cells, and investigated the downstream effects on apoptotic signaling.

Previously, we cloned the novel KRAB-related zinc finger gene ZNF424 and showed that it acts as a transcriptional repressor in the NFAT-p21 pathway in

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HEK-293 cells [8]. Few studies report biological functions of *ZNF424* in tumor cells. Thus, we collected 60 lung cancer specimens from the Hunan Cancer Hospital and revealed associations of *ZNF424* using immunohistochemical analyses. Subsequently, we transfected A549 cells with *ZNF424*-containing plasmid vectors and evaluated the effects of *ZNF424* on tumor proliferation, apoptosis. Our data suggested that *ZNF424* overexpression in A549 cells induced apoptosis, and promoted cell cycle arrest at the G1/S transition. Finally, we showed that *ZNF424* suppressed proliferation and induced apoptosis *via* the mitogen-activated protein kinase (MAPK) signaling pathway in A549 cells.

## 2. MATERIALS AND METHODS

### 2.1. Cells and Reagents

A549 cells (epidermal growth factor receptor wild type cells, WELL BIOLOGICAL SCIENCE) were cultured in RPMI1640 medium containing 10% fetal bovine serum (FBS) and 100-U/ml streptomycin/penicillin (Trans Gen Biotech) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells were subcultured using 0.25% trypsin and were seeded into 10-cm plates for protein and nucleic acid extraction, and into 12-well plates for Dual-Luciferase reporter assays.

### 2.2. Immunohistochemistry

Samples fixed in 10% neutral formalin were embedded in paraffin and were then sliced into 4-μm thick sections. Immunostaining was performed using the streptavidin-peroxidase method. Briefly, sections were incubated with a monoclonal mouse anti-*ZNF424* antibody (1: 200; Absin121843) at 4°C overnight, followed by biotinylated goat anti-mouse IgG secondary antibody. After washing, sections were incubated with horseradish peroxidase-conjugated streptavidin-biotin (Service bio G1211) and signals were developed using 3, 3'-diaminobenzidine tetrahydrochloride (Sino pharm Chemical Reagent Co., Ltd.).

### 2.3. Flow Cytometry

More than  $1 \times 10^5$  cells were harvested and analyzed using a FACSCalibur flow cytometer (Becton Dickinson, USA). Apoptotic cells were identified using flow cytometry analyses with FITC-Annexin V Staining Detection Kits. Cells in culture flasks were transfected with plasmid containing *ZNF424* for 48 h and were then trypsinized and resuspended in 500 μl of binding buffer containing 10-mM HEPES, (pH 7.4), 140-mM NaCl, 1-mM MgCl<sub>2</sub>, 5-mM KCl, 2.5-mM CaCl<sub>2</sub>, 5 μl of FITC-conjugated Annexin V, and 5 μl of propidium iodide (PI). After a 10-min incubation at room temperature in the dark, cells were harvested and analyzed on a FACSCalibur flow cytometer. Separate cells were transfected with a plasmid vector containing *ZNF424* for 48 h, were washed three times with ice-cold PBS prior to fixing in 70% ice-cold ethanol overnight at 4°. Before testing, cells were stained with 50-μg/ml Plin

the presence of RNase (100 μg/ml). Cells were then harvested within 30 min and cell cycle phases were determined using flow cytometry. PI was excited at 488 nm using an argon ion laser and emissions were quantitated at 630 nm. To eliminate adherent cells and cell debris, 10 000 cells were collected through the FSC/SSC scatterplot using Gate technology.

### 2.4. Protein Extraction and Western Blotting

Total protein extracts were lysed in cold RIPA buffer containing freshly added proteinase and phosphatase inhibitors. Lysates were then centrifuged at 12,000 × g for 15 min, and supernatants were collected. Equal amounts of protein lysates were then separated using SDS-PAGE and were then transferred onto nitrocellulose membranes. Membranes were blocked using 5% non-fat dried milk in TBS with containing 0.1% Tween 20 for 2 h at room temperature, and were then incubated overnight at 4°C with primary antibodies against human cyclinE1, cyclinD1, CDK2, CDK4, P16, P27Kip1, P-ERK/ERK, P-P38/P38, P-JNK/JNK, cleaved-caspase3, which were purchased from UNIV (Shanghai, China). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (Vazyme) at room temperature for 2 h and were finally washed with TBS containing 0.1% Tween 20 and scanned using an Odyssey Infrared Imaging System. Scanned bands were quantified using ImageJ software, and were normalized to β-actin protein expression levels. The phosphorylated proteins p-p38, p-erk, and p-jnk were normalized against their non-phosphorylated counterparts p38, erk, and jnk, respectively. Presented blots are representative of at least three experiments.

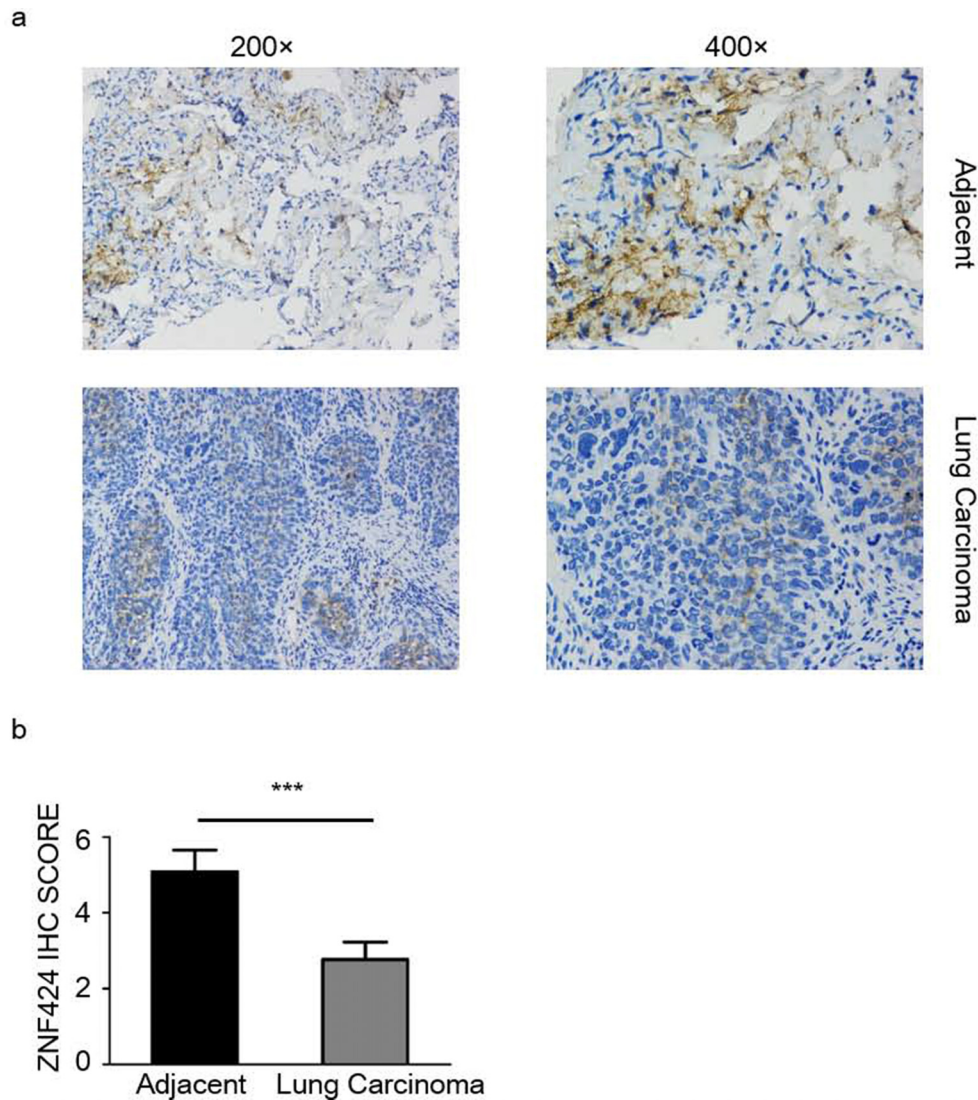
### 2.5. Statistical Analyses

Data are presented as the means ± standard deviations (S.D.) and statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., LaJolla, CA, USA). Immunohistochemical differences and clinical correlations were identified using Chi-square tests. Differences in continuous variables between groups were analyzed using Student's t-test and were considered significant when P < 0.05. All reported P-values are two-tailed.

## 3. RESULTS

### 3.1. *ZNF424* Protein Expression was Downregulated in Lung Carcinoma

To identify clinically relevant correlations between *ZNF424* expression and lung cancer progression, 60 samples of lung carcinoma were collected from Hunan Cancer Hospital and were analyzed using immunohistochemistry (IHC). The ensuing data show significantly lower *ZNF424* protein expression levels in lung cancer tissues than in adjacent tissues (Fig. 1a, 1b), as identified using chi-square tests. Moreover, decreased *ZNF424* protein expression was found in 43 tumor tissue specimens compared with that in para-



**Fig. (1). ZNF424 is upregulated in lung cancers.** (a) Immunohistochemistry (IHC) analyses of 60 lung carcinoma samples from the Hunan Cancer Hospital; (b) Quantitative analysis,  $P < 0.01$ .

carcinoma tissues, suggesting associations between, ZNF424 and lung cancer.

### 3.2. Overexpression of ZNF424 Induces Apoptosis in A549 Cells

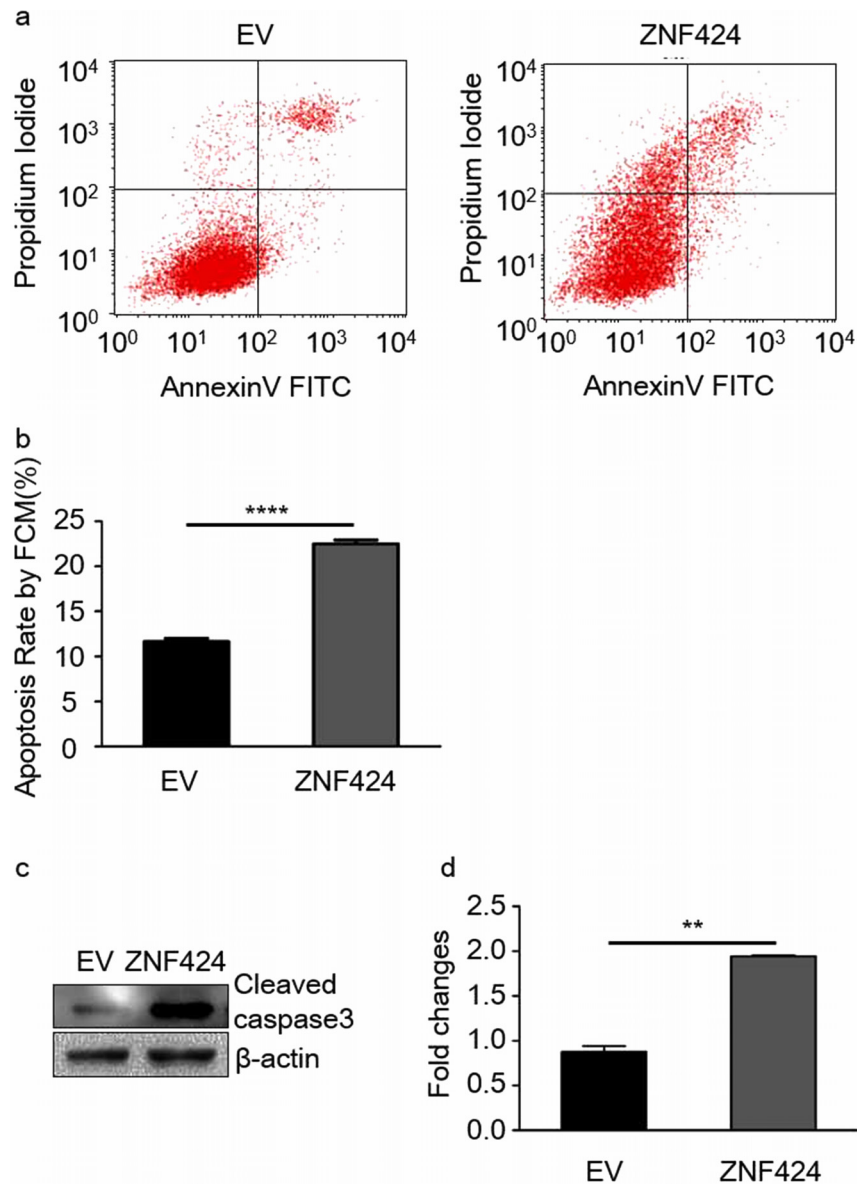
A549 cells were transiently transfected with *ZNF424* and the effects of increased ZNF424 were examined in A549 cells. In flow cytometry analyses, 21.92% of A549 cells overexpressing ZNF424 were apoptotic, whereas only 11.63% of control A549 cells were apoptotic (Fig. 2a). Western blotting analyses showed increased cleaved caspase 3 protein expression in ZNF424 overexpressing A549 cells (Fig. 2b), suggesting that ZNF424 induces apoptosis.

Exogenously expressed ZNF424 in A549 cells induced G1/S cell cycle arrest. Moreover, assessments of cell cycle distributions in A549 cells transfected with pCMV-ZNF424-tag2B plasmid or pcDNA3.1 (negative control; Fig. 3a) showed an 18.52% increase in numbers of ZNF424-overexpression cells in the G1

phase (Fig. 3b). To define the mechanisms behind these changes in numbers of G1 phase cells, we examined protein expression levels of CyclinD1, CyclinE1, CDK2, CDK4, P16, P27, and Kip1 in ZNF424 overexpressing A549 cells using Western blotting. In accordance with immunohistochemical and flow cytometry data, protein expression levels of CyclinD1, CyclinE1, CDK2, and CDK4 were remarkably decreased, whereas those of P27 Kip1 were augmented in A549 cells. However, expression levels of P16 were not significantly changed (Fig. 3c, 3e), suggesting that ZNF424 overexpression leads to G1/S cell cycle arrest and inhibits proliferation in A549 cells.

### 3.3. ZNF424 Overexpression in A549 Cells was Associated with the p38/ERK MAPK Pathway

Multiple extracellular growth regulatory signals are transduced from cell surfaces to the nucleus via MAPK signaling cascades. Thus, to determine whether ZNF424 expression inhibits A549 cell proliferation via



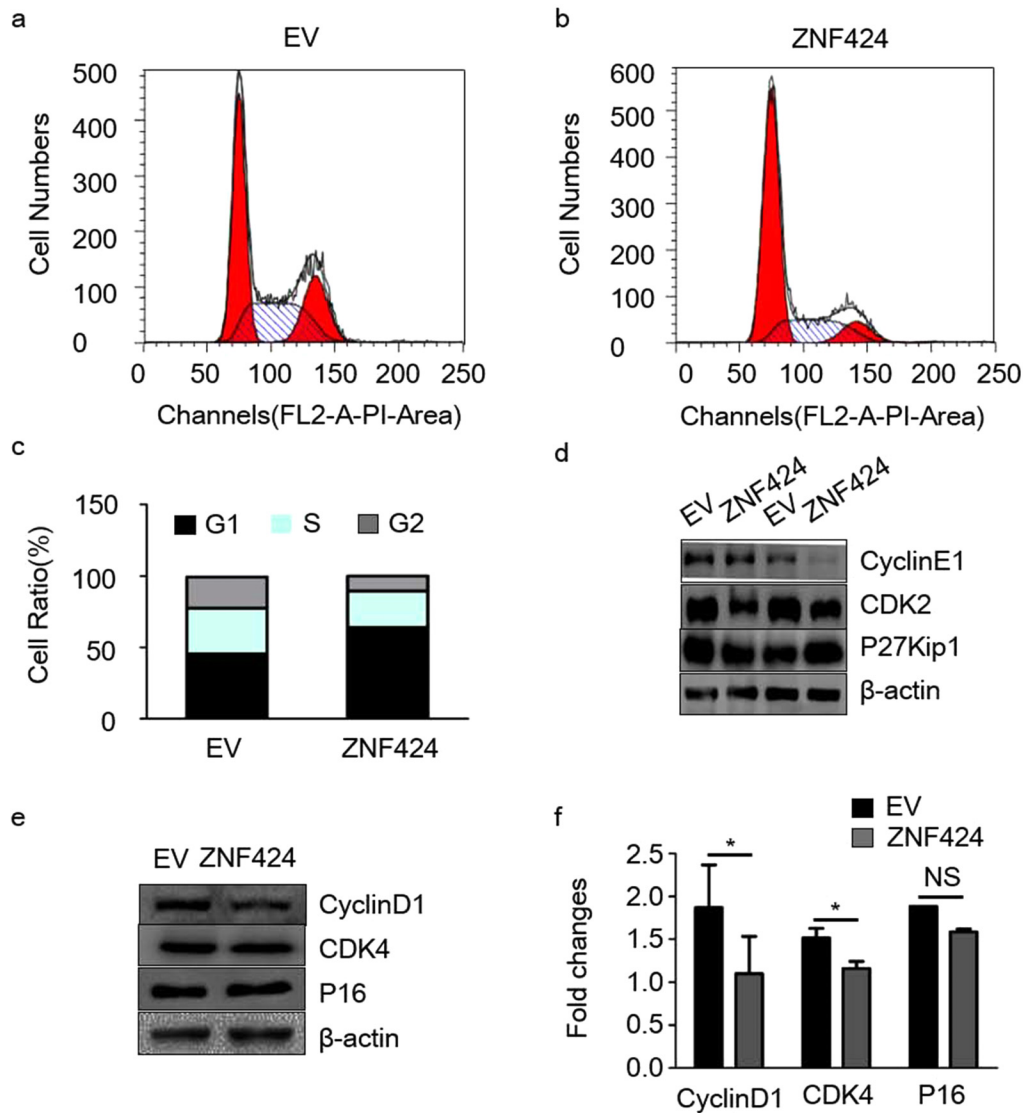
**Fig. (2). Overexpression of ZNF424 induces apoptosis in A549 cells.** (a) Apoptotic A549 cells were counted using flow cytometry after transfection with ZNF424 overexpression plasmid and empty vehicle; (b) Quantitative analysis of flow cytometry data; (c) Western blot analysis of cleaved caspase 3 in ZNF424 overexpressing A549 cells; (d) Protein expression levels were quantified and analyzed using ImageJ and GraphPad software, respectively.

the MAPK signaling pathway, we examined expression levels of the related proteins P38, ERK, and JNK. In these analyses, P38 and ERK were suppressed whereas JNK was upregulated in ZNF424 overexpressing A549 cells (Fig. 4a). Hence, overexpression of ZNF424 influences the upstream p38/ERK pathway. Taken together, our data show that ZNF424 inhibits proliferation of A549 cells, in part by inhibiting MAPK signaling.

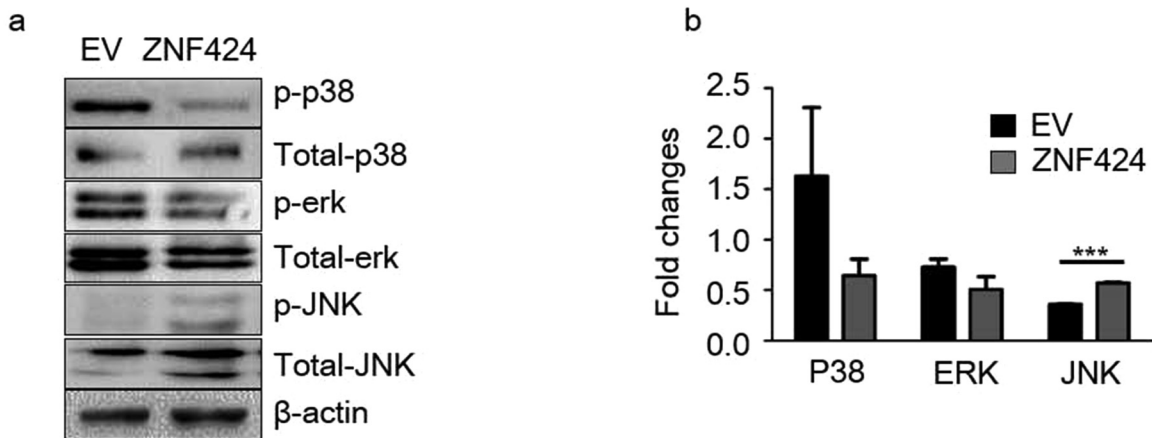
#### 4. DISCUSSION

Zinc-finger-containing transcription factors are the largest known single family of transcriptional regulators in mammalian cells, and play essential roles in differentiation, proliferation, apoptosis, and neoplastic

transformation. ZNF424 is expressed in multiple human tissues and is localized primarily in the nucleus, where it acts as a transcriptional repressor [8]. Previous reports show that zinc finger proteins containing KRAB and C2H2 domains play important roles in lung carcinoma. Moreover, numerous zinc finger family proteins have been associated with lung cancers. For example, knockdown of the zinc finger protein snail increases the sensitivity of A549 cells to cisplatin [9], and inhibition of ZNF746 suppressed invasive activities and blocked the epithelial to mesenchymal transition in H460 non-small cell lung cancer cells [10]. Although Kruppel-Like Factor 6 was frequently downregulated and induced apoptosis in non-small cell lung cancer cells [11], no previous studies report the roles of ZNF424 in lung cancer. In our study, 60 specimens



**Fig. (3).** Ectopic expression of ZNF424 induces G1/S arrest in A549 cells by regulating cyclin proteins and cyclin-dependent kinase. (a, b) Cell cycle stages were determined using flow cytometry; (c) Quantitative analysis; (d-f) Western blotting analyses of cell cycle-related proteins are presented with quantitative densitometric analyses.



**Fig. (4).** ZNF424 overexpression inhibits proliferation through the P38/ERK MAPK pathway. (a) Western blots of MAPK protein; (b) Quantitative densitometric analysis.

were collected from lung cancer patients for immunohistochemical analyses, which showed that ZNF424 expression was significantly lower in tumor tissues than in adjacent tissues, indicating a negative correlation between ZNF424 expression and lung cancer. Hence, ZNF424 may provide a diagnostic marker for lung tumorigenesis, as indicated in ROC analyses (data not shown).

The present negative correlation with lung cancer suggests a negative regulatory function of ZNF424. Accordingly, data from ZNF424-overexpressing A549 cells show increased numbers of cells in the G1 phase and decreased numbers of S-phase cells, and CyclinD1, CyclinE1, CDK2, CDK4 protein levels were concomitantly decreased. In addition, ZNF424-induced apoptosis was observed in FACS analyses, with significant increases in cleaved caspase-3 protein levels. Collectively, these data suggest that ZNF424 functions as an inhibitor of lung cancer by causing G1/S arrest and inducing apoptosis. To our knowledge, this is the first study to show that ZNF424 induces apoptosis in a human cancer cell line. However, previous studies have identified zinc finger transcription factors that inhibit tumor cell proliferation by inducing apoptosis. In particular, the von kruppel-like factor 6 was frequently downregulated in non-small cell lung cancer cells, and induced apoptosis when overexpressed [11].

ZNF family members have been shown to promote or inhibit cell growth by activating or inactivating MAPK and various other signaling pathways. The actions of Kruppel-like factor 4 on non-small cell lung cancer cell growth and aggressiveness were related to transforming growth factor- $\beta$ 1-mediated ERK/JNK/NF- $\kappa$ B signaling [12], and the novel human KRAB/C2H2 zinc finger protein ZNF418 suppressed MAPK signaling [13].

In our studies, ZNF424 promoted JNK phosphorylation but decreased phosphorylation levels of P38 and ERK. Previous studies demonstrate Ras/Raf/MEK/ERK signaling from cell surface receptors to transcription factors, and Raf reportedly acts *via* MEK- and ERK-dependent and independent mechanisms to induce the phosphorylation of apoptosis-related proteins [14]. Furthermore, Ras and its downstream effectors altered the expression of multiple cell cycle regulators, including p16<sup>Ink4a</sup>, p15<sup>Ink4b</sup>, and p21<sup>Cip1</sup>, and caused premature cell cycle arrest at the G1 phase. Because this process was shown to be dependent on the Raf/MEK/ERK pathway [15-17], the effects of ZNF424 on proliferation and apoptosis are likely mediated by P38/ERK/JNK and MAPK, although further studies are required to confirm the roles of these cascades.

## CONCLUSION

In conclusion, ZNF424 induces apoptosis and inhibits proliferation in A549 cells, in part by acting through the MAPK signaling pathway. These data warrant consideration of ZNF424 as a target for small

molecule inhibitors of the MAPK signal pathway, and for the treatment of lung cancer.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

## HUMAN AND ANIMAL RIGHTS

No humans/animals were used for studies that are the basis of this research.

## CONSENT FOR PUBLICATION

Not applicable.

## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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