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Original Research

Fibrillin 2 gene knockdown inhibits invasion and migration of lung cancer cells

Qiaojun Hong[1,2], Rong Li[3], Yiyan Zhang[1], Kangsheng Gu[1*]

[1] Department of Oncology, First Affiliated Hospital of Anhui Medical University, Hefei, Anhui, China
[2] Department of Oncology, Anhui No.2 Provincial People's Hospital, Anhui, China
[3] Department of Oncology, Affiliated Taikang Xianlin Drum Tower Hospital, Medical School of Nanjing University, Department of Oncology, Taikang Xianlin Drum Tower Hospital Clinical College of Wuhan University, China

[*]**Correspondence to:** 13805692145@163.com

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Abstract: To investigate the effect of Fibrillin 2 (FBN2) expression on the invasion and migration of lung cancer cells, and the underlying mechanism. Protein and mRNA expressions of FBN2 were assayed. The relationship between FBN2 protein expression and clinical characteristics of lung cancer patients was analyzed. Correlation between FBN2 expression level and patient survival time was analyzed. Moreover, the mRNA and protein expression of FBN2 in lung cancer cells and human normal lung epithelial cells were assayed. After constructing low-expressing FBN2 cells, the cell proliferation, clone formation, migration and invasion capabilities were tested. Lung cancer cells proliferation with low FBN2 expression in nude mice was measured with a nude mouse tumorigenic experiment. The mRNA and protein expressions of FBN2 in lung cancer tissues were significantly higher than those in para-cancerous tissues ($p<0.05$). FBN2 protein expression was significantly correlated with TNM stage, lymph node metastasis and histological type ($p<0.05$). Survival time was markedly reduced in patients with high FBN2 expression ($p<0.001$). The expressions of FBN2 mRNA and protein were markedly higher in lung cancer cells than in human normal lung epithelial cells. The proliferation, migration and invasion of lung cancer cells were significantly inhibited by FBN2 knockdown. The FBN2 knockdown significantly inhibited the protein expressions of p-FAK, p-MEK and p-ERK. FBN2 is highly expressed in lung cancer tissues, and as an oncogene, it affects the pathogenesis of lung cancer. The knockdown of the expression of FBN2 significantly inhibits the proliferation, invasion and migration abilities of lung cancer cells.

Key words: Fibrillin 2; FBN2; Fibrillin 2 gene knockdown; Lung cancer cells.

Introduction

Lung cancer is one of the most malignant tumors with the highest cancer-related mortality in the world, and it is a serious threat to the health of Chinese people (1, 2). Clinically, lung cancer is divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). It has been reported that NSCLC, which is more common than SCLC, accounts for about 80% of all cases of lung cancer (3). The disease is characterized by molecular subtypes of gene mutations, and its treatment methods are mainly chemotherapy and surgery. However, there are no specific clinical symptoms at the early onset of lung cancer. Thus, most patients are usually diagnosed with the disease at the advanced stages. This is a serious threat to the long-term survival of patients. Therefore. It is important to identify early markers of lung cancer so as to improve the early diagnosis of the disease.

Multiple genes may be involved in the development of cancer, and different genes go through different stages in tumorigenesis. In addition, different genes may be involved at a particular stage of cancer. The occurrence of lung cancer involves two types of molecular genetic changes, namely the activation of proto-oncogenes and the inactivation of tumor suppressor genes. Studies have shown that changes in gene promoter methylation influence gene expression and changes in gene expression usually precede the pathogenesis of tumors (4-7). Therefore, it is necessary to search for new cancer-related genes in order to understand the mechanism involved in the pathogenesis of lung cancer and provide a scientific basis for its treatment.

Fibrillin-2 (FBN2) gene, which was first found to be expressed in the contact parts of epithelial cells and mesenchymal cells, affects lung development by participating in epithelial and mesenchymal interactions (8). The FBN2 gene has been reported to be down-regulated in pancreatic cancer cells (9). However, it has not been studied in detail in lung cancer. In this study, the protein and mRNA expressions of FBN2 were assayed in 97 lung cancer tissues. Furthermore, FBN2 knockdown was carried out in lung cancer cell lines using lentivirus transfection, and the effect of the knockdown on the proliferation, invasion and migration of tumor cells was determined. Western blotting was used to assay the influence of the FBN2 gene on the expressions of proteins related to the FAK-ERK signaling pathway in lung cancer cell lines. *In vivo* nude mice, tumorigenic experiments were performed to determine tumorigenic ability after FBN2 mutation.

Materials and Methods

Clinical samples

A total of 97 lung cancer patients who were admitted

to the Second People's Hospital of Anhui Province from January to September 2019 were selected. They comprised 45 female patients and 57 male patients, aged 53 to 78 years old (mean age = 66.58±7.12 years).

Inclusion criteria: All patients who satisfied the WHO diagnostic criteria for lung cancer in 2015, and did not receive any form of treatment (including targeted drug therapy, chemotherapy and radiotherapy) before enrollment, were included. Lung cancer tissue samples were obtained from the patients using puncture biopsy. Para-cancerous tissues were obtained from some lung cancer patients who needed increased surgical area. Complete clinical data were collected from the patients. The study was approved by the ethics committee of the Second People's Hospital of Anhui Province.

Assay of FBN2 mRNA expression using RT-PCR

Total RNA was extracted from about 50 mg of frozen tissue using Trizol reagent. The ratio of absorbance at 260 nm and absorbance at 280 nm i.e. A260/A280 was measured in a nucleic acid-protein detector, and the RNA content and purity were calculated. The sequences of the primers used were: FBN2: forward = 5'-GGCGAGGACAGCAGGAC-3', reverse = 5'-TGATATTTGCCCACTGGAACA-3'.

Immunohistochemical determination of protein expression of FBN2 in lung cancer tissues and paracancerous tissues

Paraffin tissue sections of 4μm thickness were routinely prepared, dewaxed in xylene, and dehydrated with a gradient concentration of alcohol. After incubation with 0.01 mol/L citrate buffer solution for 20 min, the specimens were incubated in H_2O_2 for 10 min. The primary antibody was added and incubated overnight at 4°C. After washing with PBS buffer, the secondary antibody was added and incubated at 37°C for 30 min, followed by washing with PBS buffer. Then, the sections were subjected to staining which was controlled under the microscope, and re-staining with Hematoxylin. Then, the sections were dehydrated in alcohol and cleared in xylene. Following the sealing, protein expression was observed under the microscope. The staining intensity was scored by three pathologists using the following guide: 0 points (negative), 1 point (+), 1-2 points (++), and 2 points (+++). Scores for percentage positive staining were: 0 point (negative), 1 point (1-50%), 2 points (50-90%), and 3 points (90-100%). Total score/grouping was obtained multiplying the "staining intensity score" with "percentage positive staining score". Scores equal to or less than 6 points were classified in low-antibody expression group, while scores greater than 6 points were placed in the high-antibody expression group.

Cell culture

Lung cancer cell lines (PC-9, H1975, H1640, H441 and A549) and human normal lung epithelial cells (BEAS-2B) were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin at 37% in an incubator with saturated humidity and 5% CO_2.

Cell transfection

Lung cancer cell lines (PC-9 and H1640) were digested with trypsin for 1 min, and the cells were re-suspended in complete medium. After cell count, 0.8×10^6 cells were added to 35-mm culture dishes and cultured overnight in a CO_2 incubator. An interference sequence was designed to construct a lentivirus expression vector (shRNA) based on the FBN2 gene sequence in the Genbank database. Viral packaging and collection were performed on 293T cells. The PC-9 (10 Mol) and H1640 (8 Mol) cells were transfected for 72 hours.

***Determination of cell viability with MTT* assay**

A single-cell suspension was prepared using a culture medium containing 10% fetal bovine serum and inoculated at a density of 10^3 to 10^4 cells per well into a 96-well plate (200μl per well). After 3 to 5 days of cell culture, 20μl MTT solution was added to each well and incubated for 4h. Then, the culture medium/supernatant was discarded and replaced with 150μl DMSO, followed by shaking to mix for 10min. Thereafter, the absorbance of each well was measured at 490nm in an enzyme-linked ImmunoMeter. The cell growth curve was plotted, with time as the abscissa and absorbance as the ordinate.

Clone formation experiment

Lung cancer PC-9 and H1640 cells were inoculated into 6-well plates at a density of 500/well. The cells were cultured in an incubator for 2 weeks, stained with crystal violet and photographed under an Epson PerfectionV200 scanner. Cell number equivalent to 50 cells was taken as 1 clone. The number of clones and percentage clone formation was recorded.

Transwell assay

A Transwell chamber was placed on a 2-well culture plate. The serum-free medium was added to the upper chamber, and the set-up was allowed to stand at room temperature for 30min. Matrigel was pre-coated with Matrigel matrix gel (volume ratio of Matrigel: serum-free medium = 1:8), and 50μl Matrigel matrix gel was spread in a small chamber and placed overnight at 37°C. Lung cancer cells in logarithmic growth phase were inoculated in the upper chamber at a density of 1×10^5 cells per ml, and the culture medium containing 10% fetal bovine serum was added to the lower chamber. After incubation at 37°C for 24h, the remaining cells on the upper surface of the membrane were removed and fixed in anhydrous methanol for 30min. The cell membrane was stained with 1% crystal violet, and the number of migrated and invaded cells was counted under the microscope.

Western-blotting assay for protein expressions of FBN2 and Integrin-ERK signaling pathway-related factors

Lung cancer cells in the logarithmic growth phase were inoculated at a density of 2×10^6 per well in a 6-well plate, and cultured for 24h. Then, the RIPA lysis solution was added, and lysing was done on ice for 30min. The mixture was centrifuged at 14000g for 30min at a low temperature to obtain the total protein. The protein was quantified with the BCA method, and 20μg total pro-

tein was taken for SDS-PAGE gel electrophoresis. After electrophoresis, the membrane was washed thrice with 1× TBST and then sealed with skim milk at room temperature for 1 h. The diluted primary antibody (1:1000) was added and incubated overnight at 4°C. Then, the membrane was washed 3 times using TBST buffer and incubated with diluted horseradish peroxide-labeled goat anti-mouse and goat anti-rabbit secondary antibodies (1:1500 dilution). After 50min incubation at room temperature, the membrane was washed 3 times with TBST buffer. Chemiluminescence reagent was added for color development for 10min. Then, the film was pressed and developed.

Statistical analysis

The experimental data were processed with SPSS21.0 and Graph prism 6.0 software. Measurement data are expressed as mean ± standard deviation (SD). Student's *t*-test was used for comparison between two groups, while analysis of variance was used for comparison among multiple groups. Count data are expressed as n, and the chi-square test was used for comparison amongst groups. The Kaplan-Meier method was used to establish survival curves, and differences in survival amongst groups were compared using the Log-rank test. Differences were assumed as statistically significant at $\alpha = 0.05$, and $p<0.05$.

Results

High-frequency mutation of the FBN2 gene in lung cancer tissues

The expression of FBN2 mRNA in lung cancer tissues was significantly increased, when compared with that in para-cancerous tissues ($p<0.05$; Figure 1A). Immunohistochemistry was used to analyze the protein expressions of FBN2 in 97 cases of lung cancer tissues and the results showed that FBN2 protein was highly expressed in lung cancer tissues. Positive staining was mainly located at the edge of the cancer cells. Dark brown staining was seen, with no staining in the nucleus, while the expression of FBN2 protein was relatively low in para-cancerous normal tissues (Figure 1B). These results indicate that the FBN2 gene was highly expressed in lung cancer tissues, suggesting that it may be involved in the pathogenesis of lung cancer.

The pathological scores for expression of FBN2, as recorded by three pathologists, are shown in Table 1. There was a significant difference in FBN2 protein expression between the two groups ($p<0.01$). The number of cases with high FBN2 expression in lung cancer tissues was markedly higher than that in para-cancerous tissues.

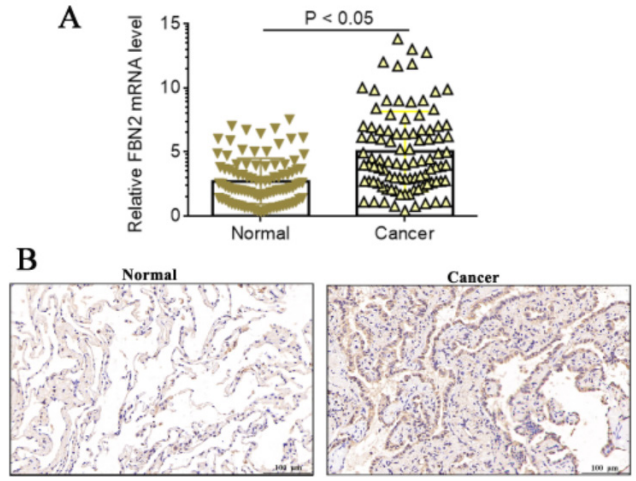

Figure 1. Expressions of FBN2 in lung cancer tissues and para-cancerous normal tissues. A: Transcription level of FBN2 mRNA in lung cancer tissues and para-cancerous normal tissues, as determined using fluorescence quantitative PCR. B: The expression of FBN2 protein in lung cancer tissues and para-cancerous normal tissues, as measured using the IHC method.

Correlation between FBN2 protein expression and clinical characteristics of lung cancer patients

The relationship between the expression of FBN2 protein and the clinicopathological features of lung cancer patients was analyzed. The results showed that there was no significant correlation between the expression level of FBN2 gene and patient's age, gender, tumor size, N stage, degree of differentiation or smoking history ($p>0.05$). However, FBN2 expression was significantly correlated with the patient's TNM stage, lymph node metastasis and histological type ($p<0.05$). This suggests that the expression level of the FBN2 gene may influence the growth and proliferation of tumor cells.

Correlation between FBN2 expression and survival time of lung cancer patients

Results of analysis of the correlation between the expression level of FBN2 and the prognosis and survival time of lung cancer patients showed that, compared with patients in the low-FBN2 expression group, the survival time of patients in high-FBN2 expression was significantly reduced ($p<0.001$). This indicates that high expression of FBN2 was associated with poor prognosis of patients, and could be used as an indicator of the prognosis of patients.

High expression of FBN2 in lung cancer cell lines

The mRNA and protein expressions of FBN2 in lung cancer cell lines (PC-9, H1975, H1640, H441 and

Table 1. Expression of FBN2 in lung cancer tissues and para-cancerous normal tissues.

Tissue	n	FBN2 expression		χ^2	P
		High (%)	Low (%)		
Lung carcinoma tissue	97	67	30	36.47	<0.01
Lung normal tissues	97	25	72		

$P<0.01$ indicates that the differences were statistically highly significant.

Table 2. Correlation of FBN2 protein and clinical characteristics of lung cancer tissues and lung cancer patients.

	Variables	n	FBN2 expression		χ^2	*p*
			low	high		
Age (years)	≤60	43	12	31	0.33	0.57
	>60	54	18	36		
Tumor size	≤ 3cm	40	9	31	2.26	0.13
	> 3cm	57	21	36		
Gender	Female	45	15	30	0.23	0.63
	male	52	15	37		
TNM stage	I/II	59	24	35	6.70	0.00
	III/IV	38	6	32		
N stage	N0	51	18	33	0.96	0.34
	N1/N2/N3	46	12	34		
Differentiation	Well	12	3	9	1.03	0.60
	Moderate	35	13	22		
	Poor	50	14	36		
Smoking history	Smokers	43	13	30	0.02	0.89
	Non-smokers	54	17	37		
Lymph node metastasis	Positive	67	15	52	7.40	0.00
	Negative	30	15	15		
Histological tumor type	Squamous tumor type	73	18	55	5.43	0.02
	Adenocarci-noma	24	12	12		

A549), and in human normal lung epithelial cell BEAS-2B were determined with RT-PCR and Western blotting assays. The results showed that the expressions of FBN2 mRNA and protein in lung cancer cells (PC-9, H1975, H1640, H441 and A549) were markedly higher than the corresponding expressions in BEAS-2B human normal lung epithelial cells, with PC-9 and H1640 cells having the highest expression levels (Figure 3). Thus, in subsequent studies, PC-9 and H1640 cell lines with high expressions of FBN2 were used.

Effect of transfection on mRNA and protein expressions of FBN2 in lung cancer cell lines

Compared with the blank control group, transfection of interference shRNA targeting FBN2 sequence into H1640 and PC-9 cells significantly reduced the mRNA and protein expressions of endogenous FBN2,

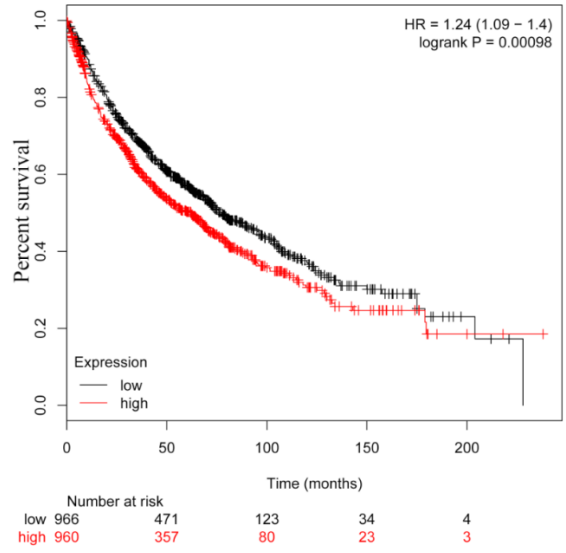

Figure 2. Correlation between FBN2 expression and patient survival time in the TCGA database.

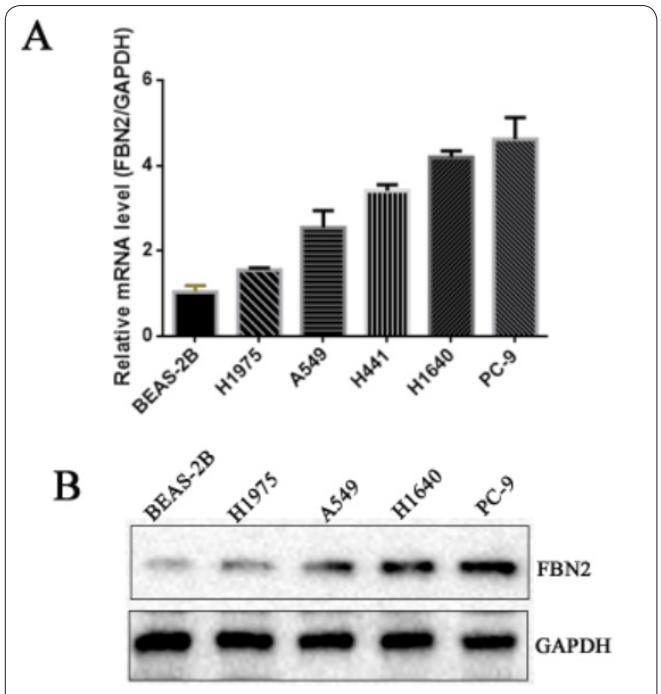

Figure 3. Expressions of FBN2 in lung epithelial cells and lung cancer cell lines. A: Transcription levels of FBN2 mRNA in lung epithelial cells and lung cancer cell lines, as determined using RT-PCR. B: Protein expression of FBN2 in lung epithelial cells and lung cancer cell lines, as assayed using western blotting.

with interference efficiencies of 54 and 53%, respectively (p<0.01; Figure 4). This indicated that the FBN2 knockdown vector was successfully constructed and that it provided a reliable *in vitro* model for subsequent studies on the function of this gene in cells.

FBN2 knockdown significantly inhibited the proliferation of lung cancer H1640 and PC-9 cells

Compared with the blank control group, the proliferation of cells in the transfection group was inhibited, and cell growth was significantly suppressed after 72

hours of culture ($p<0.05$). With the extension of culture time, the inhibitory effect became more and more obvious ($p<0.01$; Figure 5), suggesting that FBN2 inhibited the growth of lung cancer cells.

FBN2 knockdown significantly inhibited clone formation ability of lung cancer H1640 and PC-9 cells

Clone formation assay was used to determine the long-term effect of FBN2 on cell growth and proliferation. The results showed that the clone formation ability of the transfection group was significantly reduced when compared with the blank control group (Figure 6).

FBN2 knockdown significantly inhibited invasion and migration of lung cancer H1640 and PC-9 cells

Compared with the blank control group, knockdown of the FBN2 gene significantly inhibited the migration ability of PC-9 and H1640 cells ($p<0.01$). The migrations of PC-9 and H1640 cells were inhibited by 52.3 and 60.5 %, respectively (Figure 7). In addition, FBN2 significantly inhibited the invasion of PC-9 and H1640 cells by 55.6 and 71.3 %, respectively ($p<0.01$; Figure 8).

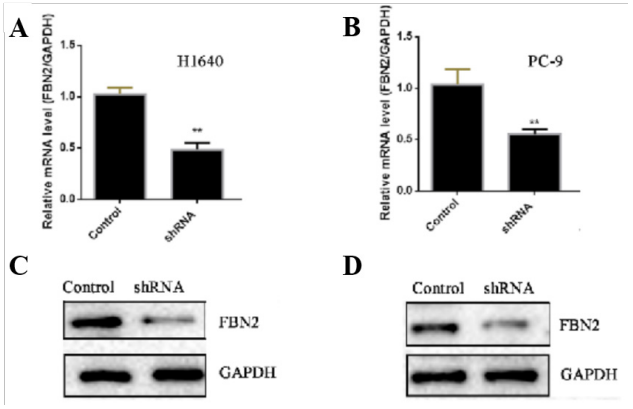

Figure 4. (A) mRNA expression levels of FBN2 in H1640 cells in the blank control group (control) and the transfection group (shRNA). (B) mRNA expression levels of FBN2 in PC-9 cells in the blank control group (control) and the transfection group (shRNA). (C) FBN2 protein expression levels in H1640 cells in the blank control group (control) and the transfection group (shRNA). (D) FBN2 protein expression levels in PC-9 cells in the blank control group (control) and the transfection group (shRNA). $^{**}P < 0.01$.

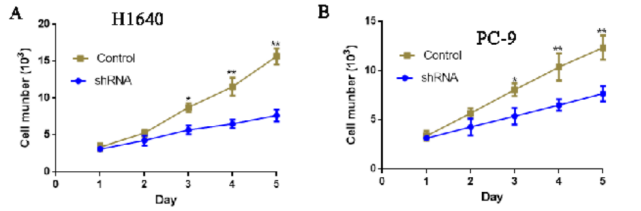

Figure 5. Growth of H1640 and PC-9 cells, as measured using trypan blue staining. $^{*}P<0.05$, $^{**}p<0.01$.

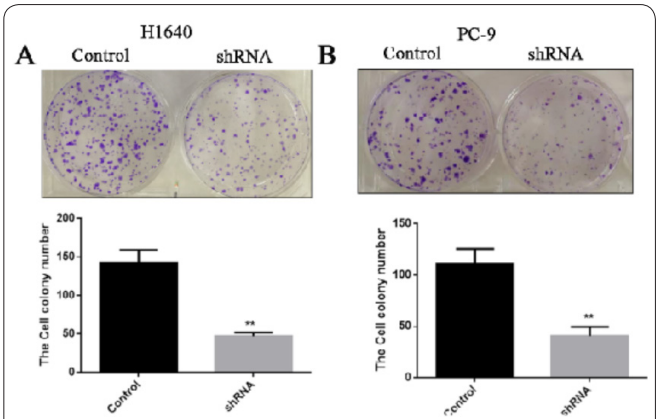

Figure 6. Clone formation ability of the blank control group (control) and transfection group (shRNA) in H1640 and PC-9 cells. $^{**}P<0.01$.

FBN2 knockdown significantly inhibited EMT-related molecule expression of lung cancer PC-9 cells

In PC-9 cells, knockdown of the FBN2 gene with shRNA significantly inhibited the mRNA and protein expression levels of N-cadherin and vimentin, while the mRNA and protein levels of E-cadherin were significantly up-regulated. The FBN2 knockdown had no significant effect on other transcription factors i.e. *Slug, Snail* and *Twist* (Figure 9).

FBN2 knockdown inhibited the FAK/ERK signaling pathway

Western blot results showed that knockdown of FBN2 significantly inhibited the protein expressions of p-FAK, p-MEK and p-ERK, but had no significant effect on total protein expressions of FAK, MEK and ERK in the cells (Figure 10).

Discussion

The FBN2 gene is located on chromosome 5, and the fibrillin it encodes is the main component of microfibril which is involved in the formation of lung tissue (10). It has been widely reported that mutation in the FBN2 gene is associated with a variety of hereditary connec-

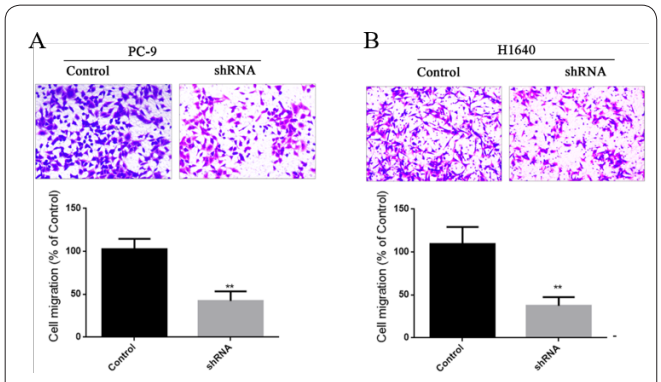

Figure 7. Effect of FBN2 knockdown on the migration ability of H1640 and PC-9 cells, as determined using Transwell migration assay. $^{**}P<0.01$.

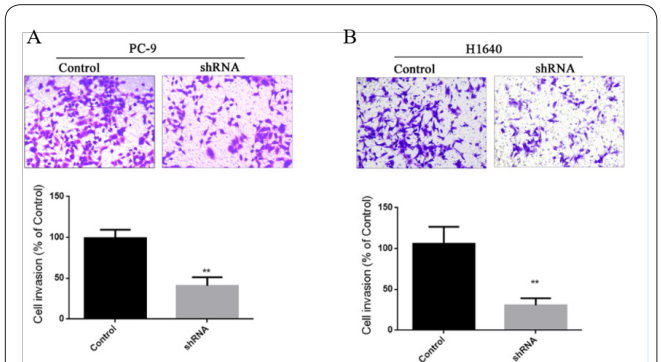

Figure 8. Effect of transfection on the cell invasion ability of H1640 and PC-9 cells, as determined using Transwell migration assay. $^{**}P<0.01$.

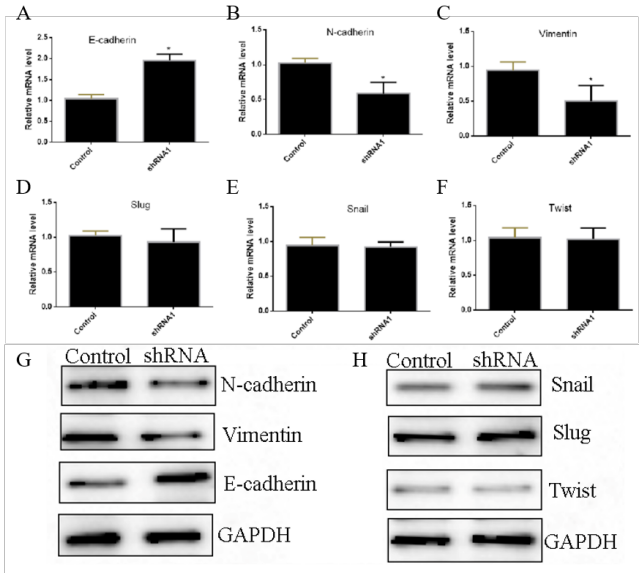

Figure 9. Effect of FBN2 gene knockdown in PC-9 cells on the mRNA and protein expression levels of EMT-related genes. **P*<0.05.

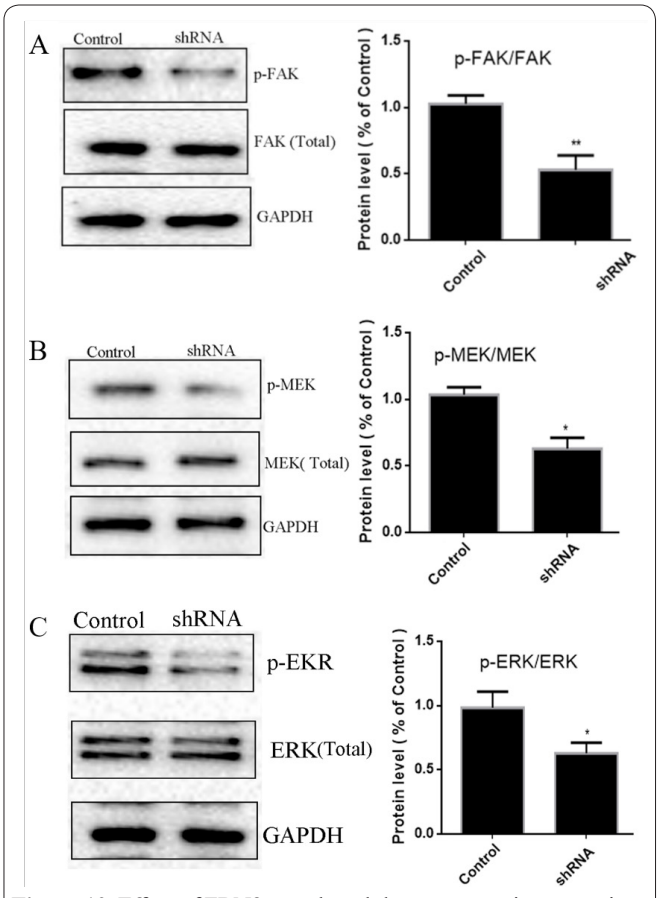

Figure 10. Effect of FBN2 gene knockdown on protein expressions of p-FAK, p-MEK and p-ERK in PC-9 cells. **P*<0.05, ***p*<0.01.

tive tissue diseases. However, there are no relevant studies on the role of FBN2 mutation in the pathogenesis of lung cancer. In this study, FBN2 expression in lung cancer tissues was determined, and the related molecular mechanisms affecting the development of lung cancer were preliminarily studied to determine whether the gene could be a potential therapeutic target for lung cancer.

The mRNA and protein expressions of FBN2 were detected in 97 cases of lung cancer tissues, indicating that the FBN2 gene expression is abnormally high in lung cancer tissues. Further analysis of the expression of FBN2 gene in the TCGA database showed that the high expression of FBN2 had a negative effect on the survival time of lung cancer patients, indicating that the abnormal expression of this gene is correlated with the occurrence of lung cancer.

Gene mutation is an important driving factor in tumorigenesis. It can lead to the activation of some oncogenes or the inactivation of tumor suppressor genes, thereby disrupting the normal regulatory mechanism of cells (11). The lung cancer cell lines PC-9, H1975, H1640, H441 and A549, and human normal lung epithelial cells (BEAS-2B) were used in this study. First, the expressions of FBN2 in lung cancer cells and normal lung cells were compared. It was found that FBN2 was highly expressed in lung cancer cells, with PC-9 and H1640 having the highest expression levels. Therefore, these cells were selected for use in subsequent studies on the effect of FBN2 gene knockdown on lung cancer cell proliferation, migration and invasion. The results showed that FBN2 knockdown inhibited the growth and clone formation ability, and the invasion and migration ability of lung cancer cells. In other words, FBN2 may be an important oncogene associated with the pathogenesis of lung cancer, and FBN2 knockdown can inhibit its cancer-promoting effect.

Epithelial-mesenchymal transformation (EMT) refers to the biological process in which epithelial cells are transformed into cells with mesenchymal phenotypes through specific procedures (12). To elucidate the molecular mechanism involved in the EMT process in malignant tumor cells, to understand its pathological significance in the occurrence and metastasis of malignant tumors, it is important to focus on key proteins of EMT and therapeutic methods targeting these key proteins (13). In this study, FBN2 knockdown with shRNA significantly downregulated the expression levels of N-cadherin and vimentin and significantly upregulated the transcription of E-cadherin. The results of fluorescence quantitative PCR and western blotting indicated that FBN2 gene knockdown inhibited the invasion and migration of lung cancer cells by upregulating the expression of E-cadherin protein while downregulating the expressions of N-cadherin and vimentin. These results suggest that FBN2 may affect the expression of these molecules through the downstream signaling pathway.

The high correlation between FAK and tumor invasion and metastasis has been confirmed in some studies. Therefore, FAK is used as a clue in the determination of the relationship between suppression of signal transduction pathway and lung cancer invasion and metastasis, and to elucidate the molecular mechanism of lung cancer invasion and metastasis (14). Studies have reported that FAK regulated the activity of downstream molecule ERK (MAPK/ERK kinase), and thus participated in the regulation of various cellular biological functions (15, 16). The present study showed that FBN2 gene knockdown significantly inhibited the phosphorylation levels of FAK protein, MEK protein and ERK protein, suggesting that FBN2 may activate the MEK/ERK signaling pathway by enhancing the phosphorylation levels of FAK, thereby promoting lung carcinogenesis.

In conclusion, this study has established that the expression of FBN2 is upregulated in lung cancer tis-

sues, and it affects the survival of lung cancer patients. Through *in vitro* and *in vivo* assays, this study has shown that FBN2 gene knockdown inhibits the proliferation, invasion and migration of lung cancer cells; reduces the phosphorylation level of FAK, and blocks the MEK/ERK signaling pathway, thereby inducing apoptosis and inhibiting the survival of lung cancer cells. Therefore, the FBN2 gene can be used as a potential target in the development of drugs for the treatment of lung cancer.

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Conflicts of interest
There are no conflicts of interest in this study.

Author's contribution
All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Kangsheng Gu; Qiaojun Hong, Rong Li, Yiyan Zhang, Kangsheng Gu collected and analysed the data; Qiaojun Hong wrote the text and all authors have read and approved the text before publication.

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