



The novel DNA methylation marker FIBIN suppresses non-small cell lung cancer metastasis by negatively regulating ANXA2

Mingyu Peng ^{a,d,1}, Li Yang ^{a,1}, Jiaxin Liao ^a, Xin Le ^a, Fengsheng Dai ^a, Ran Sun ^b, Fan Wu ^a, Yu Jiang ^a, Rui Tian ^d, Bianfei Shao ^d, Li Zhou ^d, Mingjun Wu ^{c,*,**}, Shuliang Guo ^{a,**}, Tingxiu Xiang ^{a,d,*}

^a Department of Respiratory and Critical Care Medicine, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China

^b Department of Oncology, Jiulongpo People's Hospital, Chongqing 400050, China

^c Institute of Life Science, Chongqing Medical University, Chongqing 400016, China

^d Chongqing Key Laboratory of Translational Research for Cancer Metastasis and Individualized Treatment, Chongqing University Cancer Hospital, Chongqing 400030, China

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ABSTRACT

Objectives: The clinical T1 stage solid lung cancer with metastasis is a serious threat to human life and health. In this study, we performed RNA sequencing on T1 advanced-stage lung cancer and adjacent tissues to identify a novel biomarker and explore its roles in lung cancer.

Methods: Quantitative reversed-transcription PCR, reverse transcription PCR and Western blot, MSP and Meth-target were utilized to evaluate FIBIN expression levels at both the transcriptional and protein levels as well as its methylation status. Differential target protein was evaluated for relative and absolute quantitation by isobaric tags. Co-IP was performed to detect the interactions between target protein. Precise location and expression levels of target proteins were revealed by immunofluorescence staining and component protein extraction using specific kits, respectively.

Results: We reported that FIBIN was frequently silenced due to promoter hypermethylation in lung cancer. Additionally, both *in vitro* and *in vivo* experiments confirmed the significant anti-proliferation and anti-metastasis capabilities of FIBIN. Mechanistically, FIBIN decreased the nuclear accumulation of β -catenin by reducing the binding activity of GSK3 β with ANXA2 while promoting interaction between GSK3 β and β -catenin.

Conclusion: Our findings firstly identify FIBIN is a tumor suppressor, frequently silenced due to promoter hypermethylation. FIBIN may serve as a predictive biomarker for progression or metastasis among early-stage lung cancer patients.

1. Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide [1]. The widespread utilization of CT screening has led to an increasing detection of pulmonary nodules, which often represent early-

stage lung cancer [2,3]. Nevertheless, in clinical practice, there are cases where patients with aggressive characteristics present with local and/or distant metastasis despite being diagnosed with T1 (cT1) stage lung cancer (≤ 3 cm in diameter) [4–6]. These T1 advanced-stage lung cancer pose a significant threat to the life and health of patients and are

Abbreviations: ANXA2, Annexin A2; Aza, 5-Aza-2-deoxycytidine; CHX, Cycloheximide; Co-IP, Co-immunoprecipitation; ECM, Extracellular matrix; FIBIN, Fin Bud Initiation Factor Homolog; GEPPIA, Gene expression profiling interactive analysis; GSK-3 β , Glycogen Synthase Kinase 3 Beta; IHC, Immunohistochemistry; iTRAQ, Isobaric labeling for relative and absolute quantification; LUAD, Lung adenocarcinoma; MSP, Methylation-specific polymerase chain reaction; PLG, Plasminogen; qPCR, Quantitative real-time PCR; RT-PCR, Reverse transcription polymerase chain reaction; TCGA, The cancer genome atlas; tPA, Tissue plasminogen activator; TSG, Tumor suppression gene; WB, Western blotting..

* Corresponding author at: Department of Respiratory and Critical Care Medicine, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China.

** Corresponding authors.

E-mail addresses: wumingjun@cqmu.edu.cn (M. Wu), GUOSL999@sina.com (S. Guo), xiangtx@cqmu.edu.cn (T. Xiang).

¹ Mingyu Peng and Li Yang contributed equally to this work.

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commonly observed as solid nodules in lung adenocarcinoma [2,7]. Therefore, it is imperative to identify novel biomarkers that can predict early metastasis in T1 advanced-stage lung cancer and explore new therapeutic targets for preventing metastasis in current research.

Epigenetics refers to the heritable alteration of gene expression and function without altering the DNA sequence. DNA methylation, an epigenetic modification, frequently induces gene silencing in lung cancer and impacts tumor behavior [8–10]. Changes in epigenetics may play a role in tumor diagnosis and even metastasis progression [11–14]. The objective of this study was to identify and characterize a promising biomarker associated with early prediction and treatment of metastatic T1 lung cancer.

Based on RNA sequencing (RNA-seq) analysis of T1-advanced-stage lung cancer tissues and adjacent tissues, our attention was drawn to FIBIN, which subsequent experiments confirmed as having reduced expression levels and hypermethylated status in lung cancer.

FIBIN, located at 17p13.1, was initially discovered in zebrafish and was crucial for the initiation of pectoral fin bud development. Despite being an understudied gene, limited reports have highlighted its biological and cellular effects, including involvement in pectoral fin bud initiation, tumors, myogenic differentiation, and toxicity [15–19]. Recent studies have revealed a reduced copy number and downregulated expression of FIBIN in Wilms tumor, suggesting its potential role as a tumor suppressor[16]. However, the potential mechanisms by which FIBIN mediates T1-advanced-stage lung cancer progression remains largely unknown.

Metastatic T1 lung cancer exhibits aggressiveness and poses challenges for early detection. Therefore, there is an urgent need for advancements in both early prediction and treatment strategies. In this study, we have reported that the silencing of FIBIN is attributed to promoter CpG hypermethylation. Notably, patients with high levels of FIBIN have a more favorable clinical prognosis in lung cancer. Mechanistically, FIBIN exerted its anti-metastatic effects by attenuating the binding activity between GSK3 β and ANXA2 while promoting the interaction between GSK3 β and β -catenin, consequently leading to reduced nuclear accumulation of β -catenin. These findings suggest that downregulated expression and elevated methylation levels of FIBIN may serve as predictive biomarkers for disease progression or metastasis in early-stage lung cancer patients.

2. Materials and methods

2.1. Cell culture and tissues

Cell lines (H1299, H2122, A549, H460, H446, H358, BEAS-2B, 293T) were gained from collaborators or the Cell Bank of the Chinese Academy of Sciences. All cells were cultured in DMEM (Gibco BRL, NY, USA) or RPMI 1640 (Gibco BRL, NY, USA) medium and regularly detected for mycoplasma contamination. Tissues were obtained from the First Affiliated Hospital of Chongqing Medical University and Chongqing University of Cancer Hospital, Chongqing, China. All tissue samples underwent histologically confirmation. Written informed consent was obtained from all patients and the study received ethics approval.

2.2. RNA isolation, quantitative PCR and semiquantitative PCR

Total RNA was isolated from tissues and cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Quantitative and Semiquantitative PCR were performed using Maxima SYBR R _ Green/ROX qPCR Master Mix (Promega) and Go-Taq (Promega, Madison, WI, USA), respectively [20]. The gene expression level was calculated by the $2^{-\Delta\Delta Ct}$ method. All primers sequences are provided in Table S1.

2.3. Bisulfite treatment and methylation-specific PCR

Genomic DNA was extracted from tissues and cells. Bisulfite conversion of DNA samples was performed as previously mentioned [21]. Methylation-specific PCR (MSP) was conducted using AmpliTaq Gold360 DNA polymerase (Applied Biosystems, US) as reported[22].

2.4. Transfection

Cells were cultured to 75% -80% confluence and further transfected with plasmids(FIBIN, ANXA2 or control vector, GeneCopoeia, MD, USA) using Lipofectamine 3000 (Invitrogen). The transfection rate of lung cancer cells was 70–80%. The cells were collected after 48 h.

2.5. 5-Aza-2'-deoxycytidine treatment

Cells were cultured in a medium supplemented with 10 μ mol/L 5-Aza (Sigma-Aldrich, St. Louis, MO, USA) for 72 h.

2.6. Cell proliferation assays

The cell viability was evaluated using Cell Counting Kit-8 (CCK-8; Dojindo Inc., Japan) assays at 24, 48 and 72 h after the cells were grown in 96-well plates. The absorbance of the cells was measured at 450 nm.

2.7. Colony formation assay

Cells were cultured in 6-well plates at a concentration gradient of 200, 400 and 800 cells per well. After approximately 2 weeks, cells were successively treated with 4% paraformaldehyde and crystal violet. Subsequently, the surviving colonies (≥ 50 cells/colony) were counted.

2.8. Cell migration and invasion assays

Migration and invasion assays were performed as previously described[23]. A serum concentration gradient was established in culture medium on both sides of the membrane to facilitate cells migration from the upper chamber across the membrane. Cells that crossed the membrane were retained. Matrigel was added in the upper chamber for invasion assays.

2.9. Flow cytometry

For cell cycle assays, cells were harvested, washed with PBS, fixed with ice-cold 70% ethanol for 24 h at 4 °C and further stained with PI. Cells were washed and double-stained with APC and DAPI following the manufacturer's protocol for apoptosis.

2.10. Immunohistochemistry(IHC) assays

IHC was conducted according to the manufacturer's instructions. The primary antibodies included FIBIN (#122621, Abcam, MA, USA) and Ki67 (#15580, Abcam, MA, USA).

2.11. Immunofluorescence staining

Cells were initially seeded on glass coverslips in advance and then fixed in 4% paraformaldehyde. Cell membranes were permeabilized with 0.1% Triton X-100 and incubated with immunofluorescence blocking solution for 60 min at room temperature to avoid non-specific antibody conjugation. Subsequently, the coverslips were incubated with the primary antibody and the secondary antibody in turn. Finally, DAPI was used to counterstain DNA at room temperature. The primary antibodies included FIBIN (#122621, Abcam, MA, USA), ANXA2 (Cat No. 60051-1-Ig, Proteintech, China) and β -catenin (sc-7963, Santa Cruz, CA, USA).

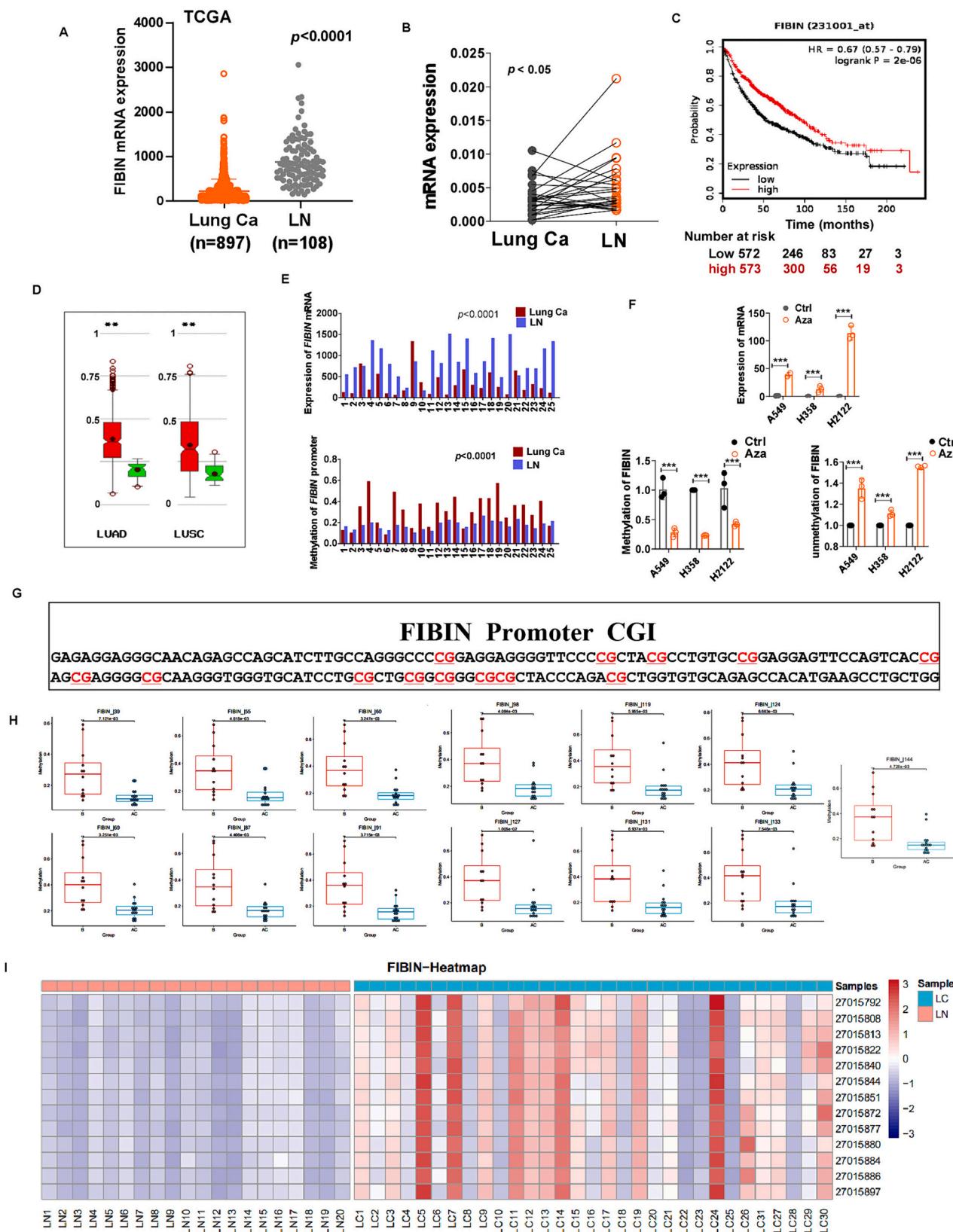


Fig. 1. Promoter hypermethylation mediates FIBIN downregulation in lung cancer.

A. Analysis of FIBIN expression in lung cancer using data from the TCGA database; B. Quantitative real-time PCR (qRT-PCR) measurement of FIBIN expression in lung cancer tissues ($n = 26$); C. Kaplan-Meier analysis of FIBIN expression levels in lung cancer patients; D. Assessment of methylation levels in the promoter region of FIBIN in both lung cancer and non-cancerous tissues, utilizing data from MethHC; E. Examination of methylation and expression changes of FIBIN in 25 paired lung cancer tissues, based on data from MethHC; F. Evaluation of FIBIN expression and methylation levels in lung cancer cells treated with Aza by qPCR and qMSP; G. Analysis of the CpG site within the promoter region of FIBIN; H-I. Methtarget evaluated the methylation status of the FIBIN in lung cancer.

2.12. Luciferase reporter assay

Cells were seeded and transiently transfected with FOPflash or TOPflash luciferase reporter plasmids, FIBIN or vector, and Renilla luciferase reporter pRL-TK. The TOPflash plasmids contain TCF/LEF binding sites, while FOPflash contains mutated TCF/LEF binding sites. Renilla was utilized as a control. Subsequently, luciferase activity was measured with a dual-luciferase reporter assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

2.13. In vivo experiments

For xenograft assay, 5×10^6 cells (either vector or FIBIN-overexpressing A549 cells) in 100 μL PBS were injected subcutaneously into nude mice (female BALB/c, 4 weeks old, $n = 3$, self-control). Over the following 19 days, the width and length of the tumor were measured every 3 days. Tumor volume analysis formula: volume = length \times width $^2 \times 0.5$.

For the metastasis assay, A549 stably expressing FIBIN and vector cells (2.5×10^6 cells, 0.1 mL PBS per mouse) were injected into the tail vein of 4-week-old BALB/c nude mice. After a period of 30 days post-injection, the animals were euthanized (when the mice's state worsened), and lung lavage was performed to isolate complete lung tissue from the mice. Subsequently, the tissue was immobilized in Bouin's fluid.

2.14. Cytoplasmic, membrane and nuclear extraction

Cytoplasmic, membrane and nuclear extracts were separated and extracted using the Subcellular Protein Fractionation kit (#78840, Thermo Fisher Scientific) following the manufacturer's protocol. All samples and extracts were frozen and stored at -80°C .

2.15. iTRAQ proteomics and Co-immunoprecipitation (Co-IP) assay

iTRAQ proteomics analyses were performed as described previously [24]. For the Co-IP assay, cells were collected, re-suspended in RIPA buffer, lysed by sonication, and quantified with a BCA protein assay kit. Subsequently, non-specific binding was cleared using A-Sepharose magnetic beads for 30 min at 4°C . Pre-cleared cell lysate was incubated with immunoprecipitating antibody overnight (or added GFP-tagged magnetic beads). Immune complexes were isolated using A-Sepharose magnetic bead slurry. The beads were washed, and the resulting supernatant was used for Western blotting.

2.16. Western blot

Western blot assays were conducted as reported previously [25]. The following primary antibodies were used in this study: FIBIN (#122621, Abcam, MA, USA), total β -catenin (sc-7963, Santa Cruz, CA, USA), Non-phospho (Active) β -catenin (#19807s, Cell Signaling Technology, MA, USA), GFP (sc-9996, Santa Cruz, CA, USA), ANXA2 (Cat No. 60051-1-Ig, Proteintech, China), GSK-3 β (sc-377213, Santa Cruz, CA, USA), Na/K ATPase (ab76020, Abcam, MA, USA), PCNA (sc-56, Santa Cruz, CA, USA), β -tubulin (WL01931, Wanleibio Inc., Liaoning, China), β -actin (sc-8432, Santa Cruz, CA, USA). Na/K ATPase, β -tubulin, and PCNA were utilized as the control of membrane, cytoplasmic and nuclear, respectively.

2.17. Statistical analysis

Statistical differences in our data were analyzed using SPSS VERSION 20.0 (Chicago, IL, USA) and Prism (version 8.0, GraphPad Inc., CA, USA). Data obeying normal distribution and homogeneity of variance between two groups were compared using unpaired t-tests (for paired samples, a paired t-test was employed). Categorical variables

Table 1

Clinicopathological features of TCGA lung adenocarcinoma patients.

Clinicopathological features	Numbers	Expression of FIBIN		<i>P</i> value
		High	Low	
Gender				0.105
Male	210	95	115	
Female	246	130	116	
Tumor Grade				0.547
T1	154	82	72	
T2	244	118	126	
T3	40	18	22	
T4	18	7	11	
Lymph Nodes Metastasis				0.477
N0	301	149	152	
N1	85	43	42	
N2	68	31	37	
N3	2	2	0	
Distant Metastasis				0.017
M0	314	159	155	
M1	21	4	17	
Unknown	121	62	59	
TNM stage				0.039
I	249	128	121	
II	109	56	53	
III	77	37	40	
IV	21	4	17	

were assessed using χ^2 test. The statistical significance was considered as *p*-values < 0.05.

3. Results

3.1. FIBIN is downregulated in lung cancer and associated with patient survival

The expression of FIBIN was found to be downregulated in lung cancer based on the analysis of the TCGA database (Fig. 1A). Subsequently, we conducted tissue validation and observed significantly higher levels of FIBIN mRNA in matched adjacent tissues compared to lung cancer tissues ($P < 0.05$, Fig. 1B). Kaplan-Meier survival analysis revealed a positive correlation between FIBIN expression and survival times in lung cancer patients (Fig. 1C). Moreover, our analysis of clinical data from the TCGA database demonstrated an association between FIBIN expression and pathological features. Specifically, we found that distant metastasis ($P < 0.05$) and TNM stage ($P < 0.05$) were related to FIBIN expression in lung adenocarcinoma (Table 1).

Based on these findings, it is suggested that FIBIN may serve as a potential tumor suppressor gene and predictive biomarker for disease progression.

3.2. Promoter hypermethylation of FIBIN leads to its downregulation in lung cancer

Previous reports have shown that aberrant methylation of promoters affects gene expression [23,26,27]. Thus, we analyzed the methylation status of FIBIN based on the data from the MethHC database. The results revealed a significantly higher methylation level of FIBIN in both lung adenocarcinoma and lung squamous cell carcinoma compared to the control (Fig. 1D). Analysis of the methylation status and expression of FIBIN in paired lung cancer tissues and paracancerous tissues ($n = 25$) from an online database showed an increased methylation status and a decreased expression level of FIBIN in lung cancer (Fig. 1E). Treatment of lung cancer cells (A549, H358 and H2122) with Aza resulted in the restoration of FIBIN expression, reduction in methylation levels, and an increase in unmethylated alleles. This suggested that the downregulation of FIBIN was at least partially regulated by promoter hypermethylation (Fig. 1F), indicating a potential negative correlation between the methylation status and expression level of FIBIN.

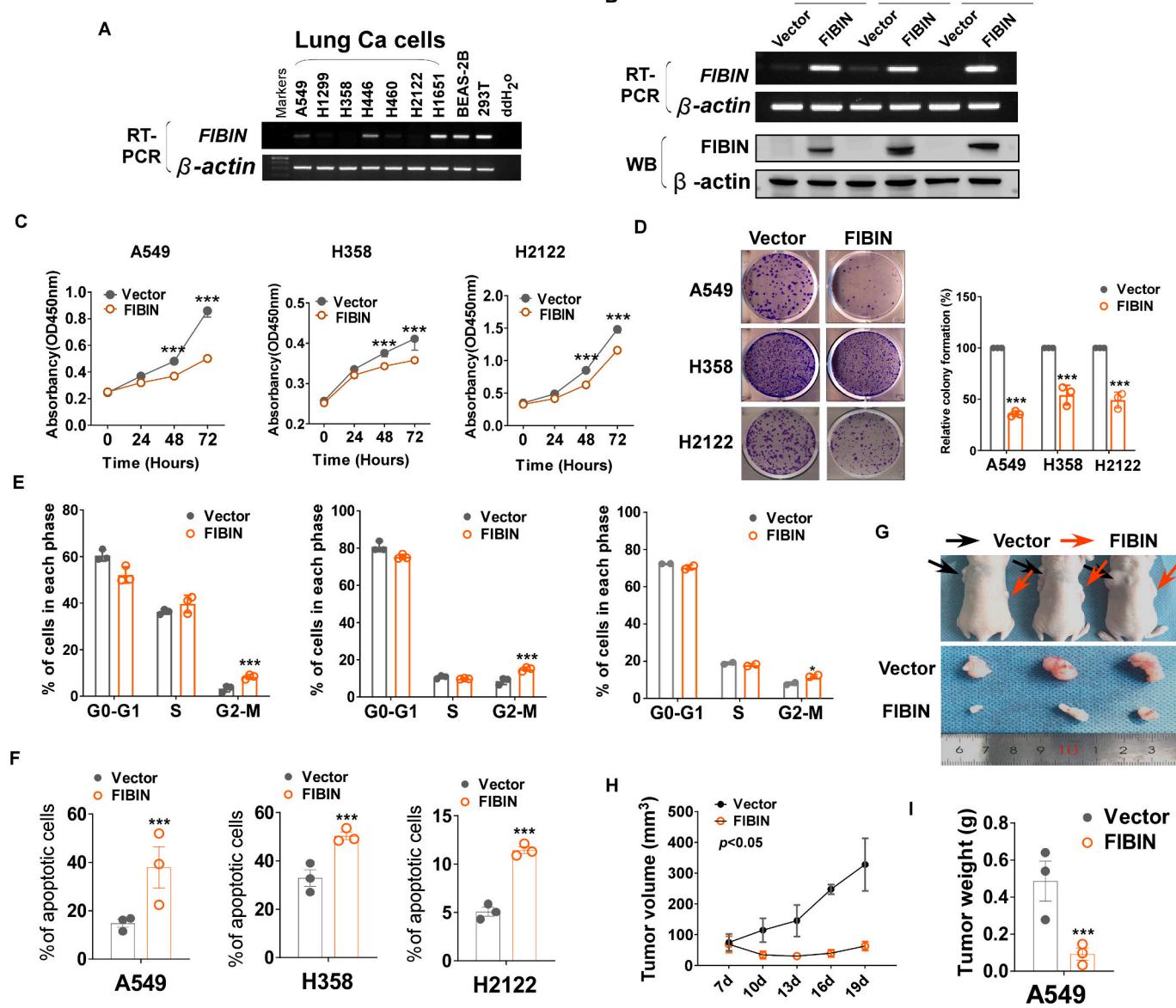


Fig. 2. FIBIN inhibited the proliferation of lung cancer cells.

A. The expression levels of FIBIN in lung cancer cells and normal cell lines; B. FIBIN stably transfected cells were verified by Western blotting and RT-PCR; C-D. The effects of FIBIN on proliferation were assessed by CCK8 and colony formation assay.

E. Histogram statistics on the impact of FIBIN on the cell cycle (From left to right, the cell lines were A549, H358 and H2122); F. Histogram statistics on the impact of FIBIN on the apoptosis; G. Xenografts before and after resection in control and FIBIN groups ($n = 3$); H-I. The volume and weight of tumors derived from nude mice.

To further verify the methylation extent of FIBIN in lung cancer, Methtarget was performed on lung cancer tissues. The analysis identified 13 CpG sites in the promoter region of FIBIN (Fig. 1G), all of which exhibited significantly elevated levels of methylation compared to controls (Fig. 1H-I). These results revealed that the CGI within the FIBIN promoter was hypermethylated in lung cancer, contributing to its downregulated expression.

3.3. FIBIN exerted anti-proliferation ability in lung cancer

To investigate the role of FIBIN in lung cancer cells, we examined the level of FIBIN in cell lines. Downregulation or silence of mRNA expression of FIBIN could be observed in almost all these lung cancer cell lines as compared with BEAS-2B and 293 T (immortalized normal cell lines) by RT-PCR (Fig. 2A). Subsequently, FIBIN was ectopically expressed in FIBIN-decreased A549, H358 and H2122 cell lines through

stable transfection. The expression of FIBIN in stably transfected was verified according to Western blotting and RT-PCR (Fig. 2B). As shown in Fig. 2C-D, overexpression of FIBIN led to a marked reduction in lung cancer cell growth as demonstrated by CCK-8 and colony formation assays. Furthermore, it was found that FIBIN promoted cell cycle arrest at the G2/M phase (Fig. 2E, Supplementary Fig. S1A) and induce apoptosis to suppress proliferation in lung cancer cells (Fig. 2F, Supplementary Fig. S1B-C). We further evaluated the anti-proliferative capacity of FIBIN *in vivo* with a tumor xenograft model. The volume and weight of tumors derived from the FIBIN-overexpressed group markedly declined compared to those derived from the control group (Fig. 2G-I, $n = 3$). Accordingly, elevated nuclear fragmentation and reduced expression of Ki67 were observed by H&E staining and IHC in the FIBIN-overexpressed group (Supplementary Fig. S1D). These findings revealed that FIBIN suppresses lung cancer cell proliferation *in vitro* and tumor growth *in vivo*.

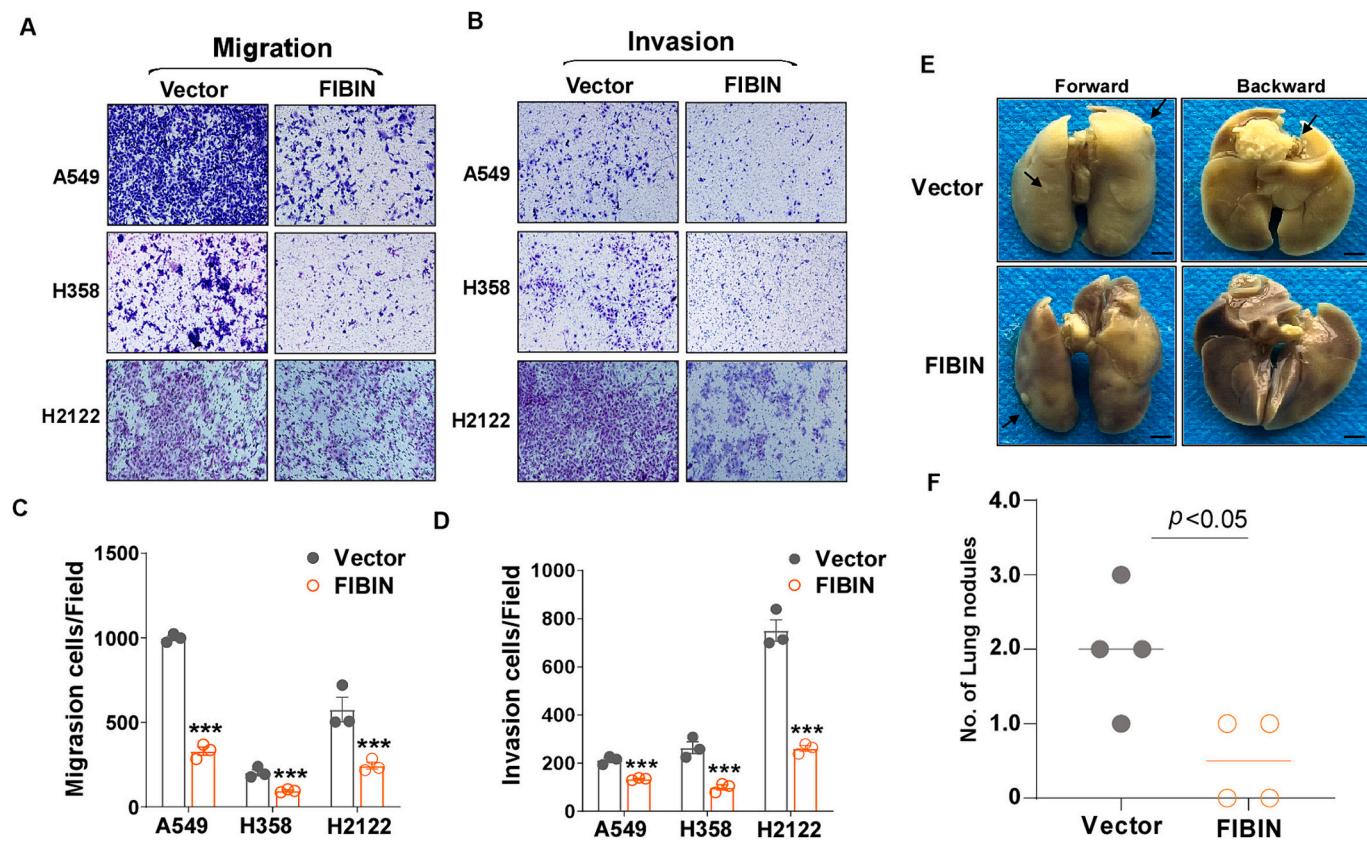


Fig. 3. FIBIN suppressed migration and invasion in lung cancer.

A. Transwell cell migration assay in control and FIBIN-overexpressed cells; B. The influence of FIBIN on the cell invasion assay; C. Histogram statistics of migration assay (**P < 0.001). D. Histogram statistics of invasion assay (**P < 0.001); E. Representative images of lung metastasis model; F. quantitative results of lung nodules.

3.4. FIBIN suppressed cell migration and invasion in lung cancer

Compared to vector cells, we found that FIBIN-overexpressed A549, H358 and H2122 cells notably inhibited the cells traversing the transwell membrane in the transwell assay (Fig. 3A-D). To assess the impact of FIBIN on lung cancer metastasis *in vivo*, A549 cells stably expressing vector or FIBIN were injected into nude mice through the tail vein. It was found that A549 cells expressing FIBIN exhibited fewer metastatic tumors within the lungs of nude mice (Fig. 3E-F). These data demonstrated that FIBIN performed an anti-cancer role in lung cancer cells *in vitro* and prevented lung metastatic potential *in vivo*.

3.5. FIBIN regulates subcellular localization and protein stability of ANXA2

To obtain more insight into the possible anti-tumor mechanism of FIBIN, 8-plex isobaric labelling for relative and absolute quantification (iTRAQ) was used to identify altered pathways in A549 cells with stable FIBIN transfection. The data showed the most significant changes in 11 up-regulated and 9 down-regulated proteins (Fig. 4A). Previous studies have reported that Annexin A2 (ANXA2) serves as a widely used biomarker and plays a significant role in the metastasis of various malignant tumors [28,29]. ANXA2 promotes epithelial-mesenchymal transition by regulating multiple signaling pathways, leading to tumorigenesis and accelerating thymic degeneration [28]. Thus, we focused on ANXA2 and confirmed it was decreased in lung cancer cells with stable FIBIN expression (Fig. 4B). Notably, transfection of FIBIN led to the decline of ANXA2 as evidenced by immunofluorescence staining, particularly in the cell membrane and cytoplasm (Fig. 4C).

The expression and subcellular localization of ANXA2 are critical for

its diverse biological functions in cancer [30]. Several studies have shown that the presence of ANXA2 on the cell membrane promoted tumor cell metastasis[31–33]. Thus, we examined ANXA2 expression in the cytoplasmic, membrane and nuclear extracts. As expected, western blotting results revealed a decrease in ANXA2 expression in the cell membrane and cytoplasm following FIBIN transfection, with no significant difference observed in the nucleus (Fig. 4D). It has been reported that abnormal ubiquitination or degradation of ANXA2 in breast cancer results in its high expression [34]. ANXA2 is regulated by ubiquitin-protein ligase UBE3A in promoting the invasion of breast cancer and liver cancer [35,36]. In cells transfected with FIBIN, treatment with cycloheximide (CHX) accelerated the degradation of ANXA2 protein (Fig. 4E), while treatment with MG132 enhanced the protein expression of ANXA2 (Fig. 4F), providing consistent evidence that FIBIN could regulate the protein stability of ANXA2. Additionally, FIBIN was observed to co-localize with ANXA2 in lung cancer cells. Thus, we utilized PyMOL to predict FIBIN-ANXA2 interactions and generate a corresponding figure (Fig. 4G). The results revealed that FIBIN (the slate cartoon model) could interact with ANXA2 (the cyan cartoon model) through hydrogen bond and electrostatic interactions at specific sites, including HIS14-THR136, THR169-ARG63, GLN66-ARG284, CYS13-VAL298, ASN30-LYS152, GLU175-THR55, ARG63-LYS119, ASP60-LYS104, and ARG62-GLU96. The binding between FIBIN and ANXA2 was further verified by Co-IP (Fig. 4H). These results showed that FIBIN could reduce ANXA2 expression and interact with it.

3.6. FIBIN interacts with ANXA2 to inactivate Wnt/β-catenin signaling

As mentioned in several studies, ANXA2 could activate the wnt/β-catenin pathway [37,38]. To further verify that FIBIN restricted

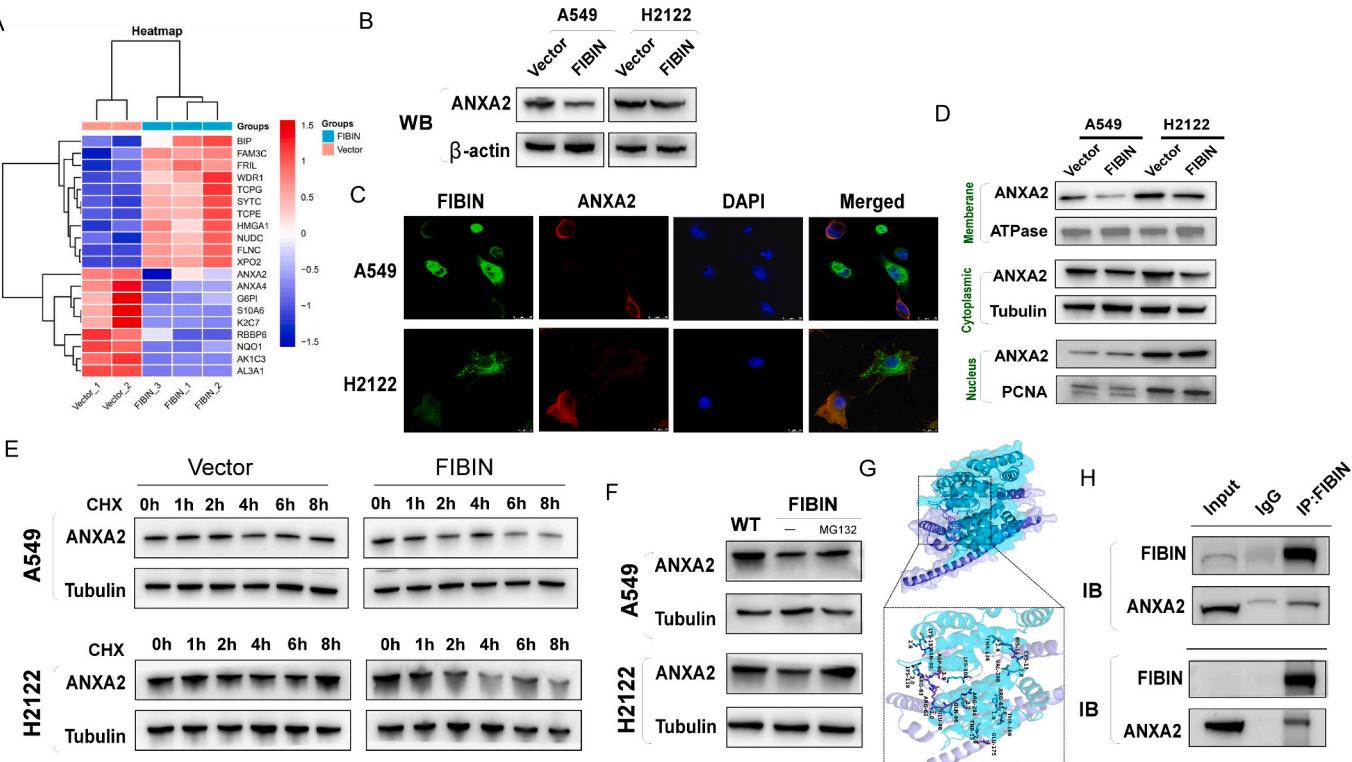


Fig. 4. FIBIN regulated the expression, subcellular localization and protein stability of ANXA2.

A. iTRAQ analysis was performed to identify altered pathways in A549 cells with stable FIBIN transfection; B. The impact of FIBIN on the expression of ANXA2 in A549 and H2122 cells; C. Immunofluorescence was utilized to measure the effects of FIBIN on ANXA2; D. Membrane, cytoplasmic and nuclear expression of ANXA2 were detected by Western blot. Na/K ATPase, β-tubulin and PCNA were used as control; E-F. The protein stability of ANXA2 was assessed; G. PyMOL was employed to predict the interactions between FIBIN (lower) and ANXA2 (upper); H. The binding between FIBIN and ANXA2 were analyzed by Co-IP assay in FIBIN-overexpressed A549 and H2122 cells.

ANXA2-mediated wnt/β-catenin signaling, the influence of FIBIN on Wnt signaling activity was detected using the TOP/FOP-Flash luciferase reporter system. The results revealed that attenuated Wnt activity was observed in FIBIN-overexpressed cells (Fig. 5A). Additionally, a reduction in total β-catenin and activated (non-phospho) β-catenin was detected in FIBIN-overexpressed cells, as confirmed by Western blotting and IF (Fig. 5B-C). Furthermore, the expression of the crucial biomarkers associated with cancer stemness, including SOX2, STAT3, NANOG, KLF4, CD44, BMI, OCT4, SOX3 and ABCG2 were decreased by FIBIN (Fig. 5D). These findings indicate that FIBIN suppressed Wnt/β-catenin signaling activation.

Previous studies have reported that ANXA2 can bind to GSK3β, interfering with the generation of GSK3β/β-catenin complex and influencing the subcellular localization of β-catenin in HCC [39]. Thus, the association among ANXA2, FIBIN, GSK3β and β-catenin were further evaluated. The results revealed that ANXA2 could bind with GSK3β (Fig. 5E). Overexpression of FIBIN in A549 and H2122 cells resulted in a decreased binding of ANXA2/GSK3β and an increase in GSK3β/β-catenin interactions (Fig. 5E). Additionally, a diminished binding of GSK3β/β-catenin was noticed in the above FIBIN-overexpressed cells after transfection with ANXA2 as determined by Co-IP assays (Fig. 5F). Moreover, ANXA2 overexpression also partially reversed the inhibition of migration and invasion abilities by FIBIN (Fig. 6A-B, Supplementary Fig. S2). These observations revealed that FIBIN inactivated the Wnt/β-catenin signaling pathway by regulating ANXA2 to facilitate GSK3β interaction with β-catenin (Fig. 6C).

4. Discussion

In this paper, we first reported the successful exploitation of T1 advanced-stage lung cancer samples to identify a novel tumor

suppressor gene, FIBIN. Our findings indicated that DNA hypermethylation of the FIBIN promoter may be a key mechanism for its loss of function during metastatic lung cancer progression. We also observed that the expression of FIBIN could be restored in lung cancer cells following demethylation treatment. Furthermore, our findings ascribe that FIBIN may play a previously unrecognized crucial role in metastatic lung cancer progression as a tumor suppressor gene, exerting its effect by inhibiting migration and invasion while promoting apoptosis and cycle arrest.

These results prompted further investigation into the potential molecular mechanism underlying the function of FIBIN as a metastasis suppressor. Our iTRAQ analysis revealed a reduction in the level of ANXA2, a member of the annexin family, upon overexpression of FIBIN. It is well-established that ANXA2 plays a critical role in various biological processes, including biochemical activation of plasminogen, endocytosis, autophagy, exocytosis, and cell-cell communications [30]. Elevated expression of ANXA2 has been closely associated with invasion and metastasis in multiple cancers such as lung [40], gastric [41], cholangiocarcinoma [42], glioblastoma [43], cervical [44], colorectal [45], esophageal [29] and breast cancer [46]. Subsequently, the negative correlation between FIBIN and ANXA2 was verified by western blot and immunofluorescence. These findings suggested that FIBIN regulated ANXA2 to inhibit metastasis in lung cancer.

Furthermore, it was observed that FIBIN co-localized with ANXA2 and decreased the distribution of ANXA2 in both the plasma membrane and cytoplasm. Co-IP and PyMOL results further evidenced that FIBIN could interact with ANXA2. Previous studies have indicated that when located in the cytoplasm and plasma membrane, ANXA2 played a role in regulating actin cytoskeleton dynamics, endocytosis and exocytosis, cell-cell adhesion, cell polarity and endosome formation [47]. The localization of ANXA2 on the cell surface has been shown to enhance

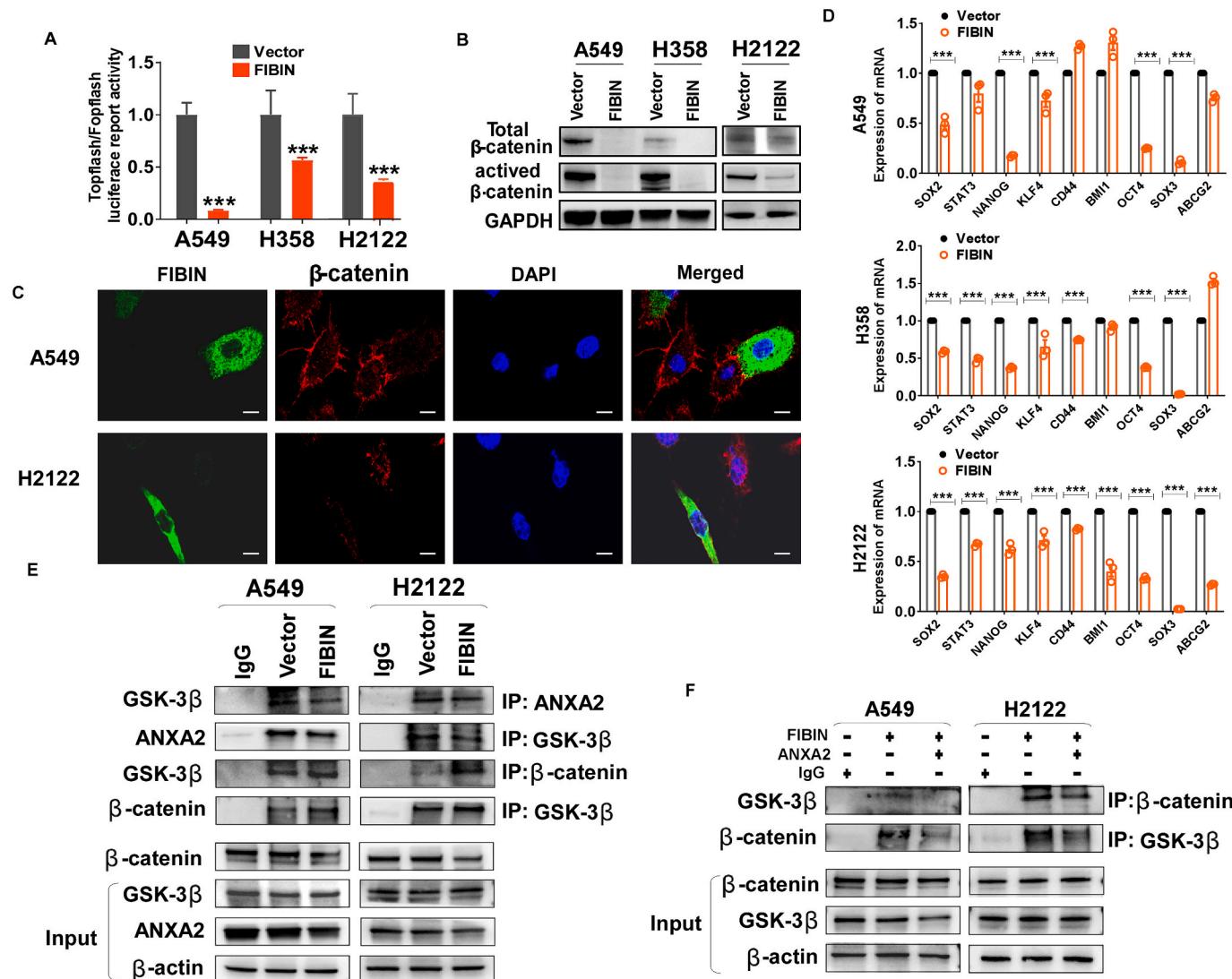


Fig. 5. FIBIN suppress wnt/β-catenin pathway activity by targeting ANXA2.

A. The luciferase reporter system was utilized to evaluate Wnt signaling activity; B. The effects of FIBIN on the wnt/β-catenin pathway; C. Immunofluorescence was utilized to detect the effects of FIBIN on β-catenin; D. qRT-PCR was employed to assess the influence of FIBIN on cell stemness. E. Co-IP assay revealed the interaction of GSK3β with ANXA2 and GSK3β with β-catenin in FIBIN-overexpressed A549 and H2122 cells; F. The interaction between GSK-3β and β-catenin was evaluated by Co-IP assay in FIBIN-overexpressed cells transfected with ANXA2.

invasion and metastasis of pancreatic ductal adenocarcinoma (PDA) [33]. Additionally, ANXA2 acts as a receptor for plasminogen (PLG) and tissue plasminogen activator (tPA), converting PLG into plasmin on the cancer cell surface. Plasmin is known to degrade extracellular matrix (ECM) and promote tumor cell metastasis [30,32]. Consistent with expectations, declined expression of ANXA2 was observed in both the plasma membrane and cytoplasm through component protein extraction, suggesting that FIBIN impacted the subcellular localization of ANXA2. It is possible that FIBIN may diminish the expression of ANXA2 on the cell surface to inhibit plasmin generation.

Furthermore, the reduced expression of ANXA2 in the cytoplasm may also serve as one of the mechanisms that mediate cancer inhibitory signaling. Previous studies have revealed that ANXA2 could bind with GSK3β[39], interfere with the production of the GSK3β/ β-catenin complex[48,49] and activate the wnt/β-catenin pathway to promote carcinoma progression [37,38]. Based on this, we hypothesized that ANXA2 may function to protect β-catenin from negative regulation by GSK3β through its interaction with GSK3β. FIBIN was found to regulate ANXA2, thereby reducing the binding activity between GSK3β and ANXA2, ultimately resulting in an increased interaction between GSK3β

and β-catenin. This promotes the degradation of β-catenin, thereby inhibiting the metastasis of lung cancer. Our results further demonstrated above hypothesis. Meanwhile, the overexpression of ANXA2 attenuated the binding of GSK3β and β-catenin by promoting the interaction between ANXA2 and GSK3β. This impedes the phosphorylation and ubiquitination of β-catenin, resulting in its accumulation in the cytoplasm. Subsequently, β-catenin translocates into the nucleus where it binds to LEF/TCF transcription factors and activates the transcription of downstream target genes. Additionally, the restoration of ANXA2 expression partially reversed the inhibitory effect of FIBIN on migration and invasion ability. This indicated that FIBIN repressed lung cancer metastasis via its effects on ANXA2. It has been reported that ANXA2 could be regulated by ubiquitination. The downregulated E3 ubiquitin ligase RBBP6 identified in the differential proteomic analysis may also play a role in regulating the protein stability of ANXA2.

During the development of malignant tumors, changes in DNA methylation may occur prior to pathological alterations. The detection of these epigenetic markers may be utilized for early diagnosis and screening of tumors, monitoring recurrence, evaluating treatment efficacy, and predicting prognosis, which holds significant potential for

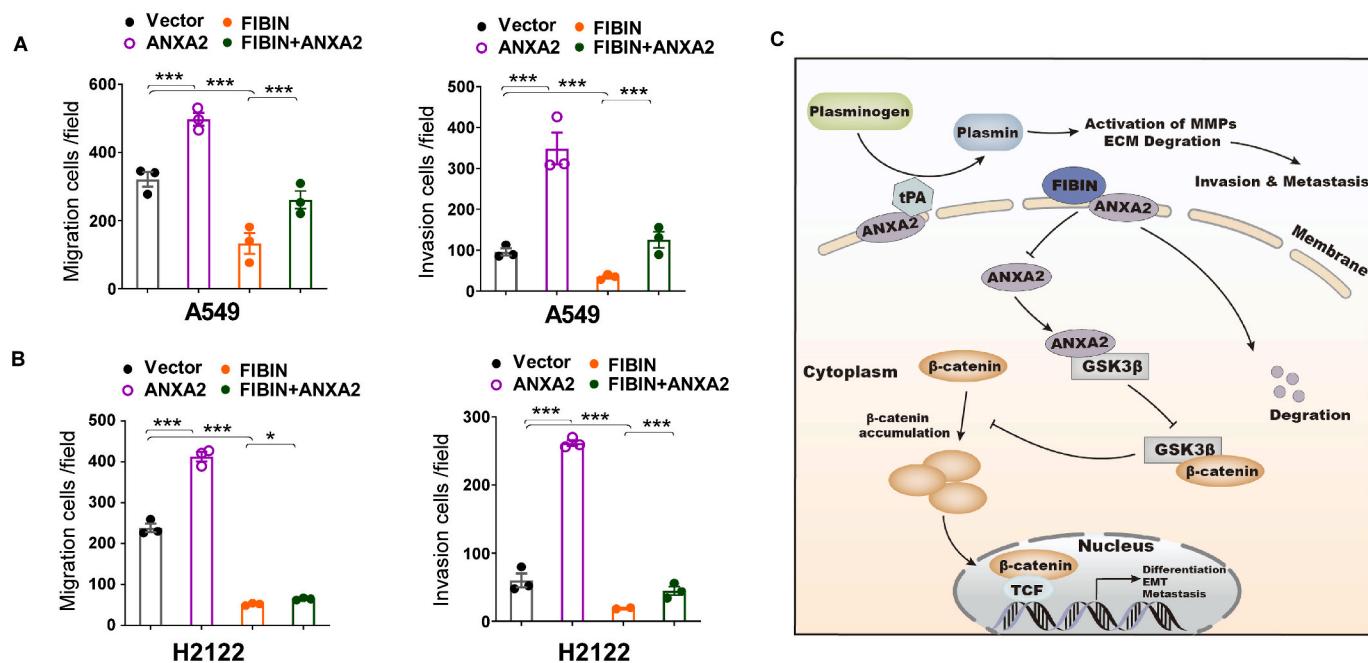


Fig. 6. ANXA2 partially reversed the inhibition of migration and invasion abilities by FIBIN.

A-B. The effects of restoring ANXA2 expression on migratory and invasive abilities of FIBIN-overexpressed lung cancer cells; C. Diagram for FIBIN modulated ANXA2 to antagonist Wnt/β-catenin signaling.

application. Therefore, FIBIN, as a novel methylation marker, has a great prospect in the diagnosis, progression prediction and treatment of early lung cancer patients. However, our study had certain limitations. Firstly, expanding the number and variety of clinical samples is crucial for evaluating the efficacy of FIBIN as a novel methylation marker for clinical application in NSCLC. Secondly, this study did not include a comparative analysis of FIBIN methylation levels in patients with T1 advanced-stage lung cancer, patients with T1 stage lung cancer, and individuals without cancer. Therefore, further improvements are needed to enhance the feasibility of using FIBIN as a predictor for early metastasis in lung cancer. Third, it should be noted that the scope of application may be limited due to the selection of A549, H2122 and H358 cells which are lung adenocarcinoma cell lines. Therefore, further validation is necessary to elucidate the biological function of FIBIN in other subtypes of non-small cell lung cancer as well as their associated mechanisms.

5. Conclusion

In summary, our observations provide support for the hypothesis that FIBIN functions as a tumor suppressor and is downregulated in lung cancer due to CpG island hypermethylation. FIBIN may exert an inhibitory effect on metastasis through the inactivation of the ANXA2/GSK3β/β-catenin axis. The combined application of FIBIN expression and methylation status is essential for the early screening of non-small cell lung cancer. It may have clinical utility as a predictive biomarker for progression or metastasis in early-stage lung cancer patients.

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Ethics approval and consent to participate

All protocols involving patient samples in this study were approved by the Institutional Ethics Committee of the First Affiliated Hospital of Chongqing Medical University and the Cancer Hospital of Chongqing University. The Animal Ethics Committee of Laboratory Animal Center, Chongqing Medical University approved this study.

Consent for publication

All authors have read this manuscript and approved for the submission.

CRedit authorship contribution statement

Mingyu Peng: Writing – original draft, Validation, Investigation, Formal analysis, Conceptualization. **Li Yang:** Writing – original draft, Validation, Investigation, Formal analysis, Conceptualization. **Jiaxin Liao:** Writing – review & editing, Visualization, Methodology. **Xin Le:** Writing – review & editing, Visualization, Methodology. **Fengsheng Dai:** Writing – review & editing, Visualization, Methodology. **Ran Sun:** Writing – review & editing, Visualization, Methodology. **Fan Wu:** Formal analysis. **Yu Jiang:** Formal analysis. **Rui Tian:** Formal analysis. **Bianfei Shao:** Resources. **Li Zhou:** Resources. **Mingjun Wu:** Project administration, Funding acquisition, Conceptualization. **Shuliang Guo:** Project administration, Funding acquisition, Conceptualization. **Ting-xiu Xiang:** Project administration, Funding acquisition, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

Not applicable.

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.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cellsig.2024.111197>.

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