Fibroblast Growth Factor 11 (FGF11) Promotes Non-small Cell Lung Cancer (NSCLC) Progression by Regulating Hypoxia Signaling Pathway

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Research Article

Keywords: NSCLC, FGF11, HIF-1α, hypoxia, cell proliferation

Posted Date: April 19th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-414555/v1

Version of Record: A version of this preprint was published at Journal of Translational Medicine on August 17th, 2021. See the published version at https://doi.org/10.1186/s12967-021-03018-7.

Abstract

Background: Recently, accumulating studies highlight the critical regulatory roles of fibroblast growth factors (FGF), and a series of FGF, participated in the progression of multiple human cancers, including non-small cell lung cancer (NSCLC).

Methods: Gene transcriptome analysis was used to identify the differential expression of FGF11 in NSCLC tumor tissues, GSE75037 and GSE81089 database analysis was performed on NSCLC tumor tissues and adjacent normal tissues to validate the expression of FGF11. Then, we selected 100 cases of NSCLC tumor tissues and 30 cases of matched adjacent normal tissues to confirm the mRNA and protein level of FGF11 by qRT-PCR and immunohistochemistry. Bioinformatics analysis and dual luciferase reporter analysis was also performed to examine the direct regulatory of FGF11 by miR-525-5p. CCK-8 and transwell assay was also performed to detect the cell proliferation, migration and invasion. Signal pathway analysis was also investigated the effect of FGF11 on NSCLC cell proliferation was associated with the hypoxia signaling pathway. The role of FGF11 in NSCLC tumor growth was further explored by in vivo study.

Results: FGF11 was overexpressed in NSCLC tumor tissues and tumor cell lines, the high expression of FGF11 was closely associated with poor overall survival of NSCLC patients. In vitro loss- and gain- of function experiments demonstrated that FGF11 knockdown inhibited, whereas FGF11 overexpression promoted the proliferation, migration and invasion of NSCLC cells. The dual luciferase reporter assay confirmed that FGF11 was downregulated by miR-525-5p, and the effect of FGF11 on cell proliferation, migration and invasion could be interfered by miR-525-5p. We further found that FGF11 had significant correlation with hypoxia signaling pathway activation, meanwhile regulating HIF-1α. Further experiments implicated that the oncogenic role of FGF11 could be blocked via interfering of HIF-1α in NSCLC cells. Moreover, knockdown of FGF11 suppressed NSCLC tumor growth whereas overexpression of FGF11 promoted tumor growth in vivo.

Conclusions: FGF11 might be functioned as an oncogene in tumor development, the findings of our study revealed a novel regulatory mechanism of FGF11 involved in hypoxia signaling pathway, which offers novel strategies for the treatment of NSCLC.

Background

Globally, cancer incidence and mortality are increasing worldwide, according to the *GLOBOCAN* 2018 estimate in 185 countries and 36 cancers, lung cancer is the most prevalent common type of cancer and occupy the leading cause of mortality [1]. Lung cancer is usually subtyped into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which accounts for approximately 20% and 80%, respectively [2, 3]. Despite the diagnosis and treatment has been made in great advance, the management of NSCLC still remains one of the most serious medical challenges[4, 5]. Therefore, to better

understand the potential carcinogenetic mechanisms and exploring novel therapeutic target is of crucial importance to NSCLC patients.

Fibroblast growth factor (FGF) family members play important roles in a variety of tumors, including lung cancer, which activate through multiple ways, such as promoting cell proliferation and epithelial-mesenchymal transition (EMT) [6-8]. Previous report have showed that downregulation of fibroblast growth factor 5 inhibited proliferation and invasion of human non-small cell lung cancer cells [9]. Fibroblast growth factor 11 (FGF11) as one member of FGF, however, the molecular mechanism study of FGF11 is still less understood till now [10, 11].

microRNAs (miRNAs) are small non-coding RNAs which function in mRNA silencing and post-transcriptional modulation of target gene expression [12, 13]. The dysregulation of miRNAs has been associated with increasing number of human diseases, especially in cancer progression, including gastric cancer [14], breast cancer [15], lung cancer [16], colorectal cancer [17], et al. However, the regulation of FGF by miRNAs still underappreciated.

At present, the prognostic role of FGF11 was only reported in rare cancers, such as nasopharyngeal carcinoma [18], prostate cancer [19], etc., while its role in other diseases have few investigated [20, 21]. Previous report also clarified that copy number variations (CNVs) of FGF11 gene was closely correlated with lung cancer [22], which implicated its critical roles in lung cancer. Considering the priority of hypoxia induced factors-1 alpha (HIF-1α) in Hypoxia signaling pathways is a key pathogenic element in varieties of tumors, including lung cancer [23-26], however, FGF11 could be induced by lack of oxygen (Hypoxia), and, in turn, promote the function of HIF-1α [21]. Nevertheless, in cancer development, whether FGF11 exerts its role by promoting HIF-1α remains need to be further studied.

Through the GEPIA analysis of FGF11 expression in multitude cancer types, we predicted that FGF11 was abnormal high expressed in lung cancer tumor tissues, which associated with poor prognosis. Several miRNA databases predicted that miR-525-5p has a pseudo binding with FGF11, which implicated that FGF11 might be regulated by miR-525-5p. GSEA analysis also showed in lung tumor tissues, the expression of FGF11 has a significant correlation with hypoxia pathway activation. Therefore, in this research, the molecular mechanism of FGF11 regulated by miR-525-5p and hypoxia pathway will be investigated through the in vitro and in vivo experiment for treatment of NSCLC.

Material And Methods

Patients and clinical follow up

100 cases of NSCLC tumor tissues and 30 cases of matched adjacent normal lung tissues were obtained from patients who were newly diagnosed with NSCLC by histopathology and undergone surgery at Tongji Hospital. All selected patients did not receive any other treatment before surgical operation. After surgy, the sample of tissues were snap-frozen in liquid nitrogen. The use of human tissue samples was approved by the Ethics Committee of Tongji Hospital, all patients and/or their relatives have signed the

informed consents. To monitor the patient survival, all selected patients were clinically followed up for four years, however, if patients failed to follow up or died of accidents were excluded. Patients were also grouped into high and low level (n=50) groups based on the medium level of FGF11 expression in NSCLC tissues.

Cell culture

Two human NSCLC cell lines (A549, NCI-H460), and human normal lung epithelial cell line (BEAS-2B) were purchased from ATCC (Manassas, VA, USA). All cells cultured in RPMI 1640 medium (Gibco, USA) which supplemented with 10% FBS (Gibco, USA), 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C, 5% CO_2 incubator.

RNA extraction

Total RNA was purified from NSCLC tumor cell lines and tissues using RNeasy mini kit (Qiagen, USA), then the isolated RNA was transcribed into cDNA using the PrimeScript RT reagent kit (Cat# RR037A, Takara, China). miRNA was transcribed using the miRNA RT primer which designed by Guangzhou RiboBio, Co., Ltd.

QRT-PCR analysis

QRT-PCR reactions were performed on ABI Prism 7500 real time PCR instrument (Applied Biosystems, CA, USA) using the SYBR Green PCR Master Mix (Takara). The relative expression of target gene was determined by $2^{-\Delta\Delta Ct}$ method. GAPDH and U6 was chosen as the internal control genes for mRNA and miRNA. The primer sequences of FGF11, HIF-1α and GAPDH were listed as following: FGF11 forward: 5'-GGCATCGTCACCAAACTGTT-3'; reverse: 5'- GCAGTCCCTCAGCATTCATG-3'; HIF-1α forward: 5'-GACAGCCTCACCAAACAGAG -3'; reverse: 5'- GTAGCTGCATGATCGTCTGG -3'; GAPDH forward: 5'-CTGACTTCAACAGCGACACC-3'; reverse: 5'- CTGACTTCAACAGCGACACC-3'; U6 forward: 5'-GCTTCGAGGCAGGTTACATG-3'; reverse: 5'- GCAACACACAACATCTCCCA-3'.

Cell transfection

FGF11 overexpressed lentivirus plasmid, FGF11-shRNA lentivirus plasmid and relevant sh-Negative Control (sh-NC) vector or empty vector were chemically synthesized from GenePharma Co. Ltd. (Shanghai, China). 1×10^5 cells were seeded in a 24-well plate, when cell growth reached at 50~60% confluence, cells were used for infecting the lentivirus plasmids.

MiR-525-5p mimic and inhibitor was purchased from Guangzhou RiboBio (Guangzhou, China). The miRNA mimic/inhibitor was transfected into tumor cells upon their confluence reaching 40%-50% at a working concentration of 50nM. Cells were used for analysis after 48-72h culture.

Dual luciferase reporter assay

The fragment of FGF11 3'UTR region containing miR-525-5p binding sites (named as WT), and fragment containing site-directed mutagenesis (named as MUT) were cloned into psiCHECK-2.0 vector. Then miR-525-5p mimic or inhibitor which purchased from Guangzhou RiboBio were co-transfected into HEK293 cells with FGF11 3'UTR WT or MUT plasmids in 24 well plates. Luciferase activity was examined using the dual luciferase reporter assay kit (Promega) after 48 hours transfection.

Cell Counting Kit 8 (CCK-8) assay

Cell Counting Kit-8 (CCK-8; Dojindo, Japan) assay was used to detect the cell proliferation. NSCLC cells were seeded in a 96-well plate at a density of 3,000 cells/well, then cultured for 0d, 1d, 2d, 3d, 4d, after culture, 10μl of CCK-8 solution was added at every time point, and the plates were incubated for another 3-4 h. The absorbance at 450nm was measured with a microplate reader (Bio-Rad, CA, USA), and the value of OD450 was recorded.

Colony formation assay

After lentivirus infection of FGF11 overexpressed plasmid and/or co-transfected with miR-525-5p, the cells were cultured in a 6-well plate at a density of 1000 cells/well, after 2 weeks culture, 4% paraformaldehyde and 0.1% crystal violet (Sigma-Aldrich) were used to fix and stain the cells. The cell colonies were calculated and photographed by microscope (Olympus).

Cell migration and invasion assay

After 24h of cell infection or transfection, harvested the cell and counted the cell number. To analyze the effect of FGF11, the cell migration was determined by wound healing assay and cell invasion was analyzed by transwell assay. For wound healing assay, the cells were seeded into a 6-well plate of 2×10^5 cells, when the cell confluency reached at 70-80%, a "wound" scratch was created on cell monolayer using 100μl tip. The cells were continued culture for 48h after washed with warmed PBS, the scratch healing difference was calculated through phase inversion microscope. Meanwhile, the same cells were inoculated in transwell chamber at a density of 5×10^4 cells, the upper chamber was filled with matrigel serum-free RPMI1640 medium while the lower chamber was filled with medium containing 10% FBS. After 24 h culture, the cells were allowed to invade through membrane to lower chamber, then the cells in lower chamber were stained with 0.5% crystal violet (Sigma-Aldrich). Finally, the invading cells were photographed and recorded by using the microscope (Olympus). The experiment was repeated at least three times.

Cell apoptosis assay

Flow cytometry analysis was performed to detect NSCLC cell apoptosis. NSCLC cell lines were transfected and cultured for 2 days, then the cells were harvested and resuspended with cold PBS and centrifuged. Then cells were double stained by annexin V-fluorescein isothiocyanate (FITC) and

propidium iodide (PI), following the manufacturer's instructions. Cell apoptosis was examined using flow cytometry (BD, Biosciences, USA), then analyzed by Flow Jo software (Version 13.0, BD).

Western blot analysis

Total protein purified from NSCLC tumor tissues and cells using RIPA lysis buffer containing protease inhibitor cocktail (Invitrogen, USA), then quantified by a BCA Protein assay kit (Solarbio, Beijing, China). SDS-PAGE electrophoresis was performed by loading equivalent amounts of protein. After transferred to a PVDF membrane (Millipore, MA, USA), it was blocked with 5% skimmed milk and incubated with specific primary antibodies against FGF11 (1:1000; Cell signaling, MA, USA) and β-actin (1:2000; Cell signaling, MA, USA) for 2h or overnight at 4°C, followed by incubation with HRP-linked secondary antibody (1:3000; Cell signaling, MA, USA) at room temperature for 1 hour. The protein bands were visualized by using an enhanced chemiluminescence kit (Santa Cruz, TX, USA) and photographed on gel imager system (Bio-Rad). β-actin was considered as a loading control.

Animal tumor xenograft model

Balb/c nude mice (female, aged 6-8 weeks, weight 18-20 g) were used to perform the in vivo animal experiment, the mice were maintained under specific pathogen-free conditions, which purchased from the Slac Laboratory Animal Center (Shanghai, China). 5×10^5 A549 and/or NCI-H460 cells stably infected with FGF11, FGF11 shRNA or sh-NC lentivirus plasmid were resuspended in 100μl of PBS and subcutaneously injected into the right flank of mice, 5 mice in each group. When tumor growth, the tumors were monitored using caliper biweekly and the tumor volume was calculated using the formula: V(tumor) = 0.5 × length × width^2. Five weeks after tumor cell inoculation, all the mice were euthanized by CO_2 asphyxiation, and the xenograft tumors were excised, weighted and subjected to H&E staining and immunohistochemistry (IHC) analysis of Ki-67 protein.

All experimental procedures about the use of animal were approved by the Ethics Committee of ***, and all efforts were made to minimize animal suffering, according to the NIH Guide for the Care and Use of Laboratory Animals.

Statistical analysis

All results data were analyzed by GraphPad Prism 18.0 software (GraphPad Software, San Diego, CA, USA). Comparisons were analyzed using the appropriate Student's *t*-test, one-way analysis of variance or χ2 test. Correlation between FGF11 and HIF-1α expression in NSCLC tissues was assessed by Pearson's correlation analysis. $P<0.05$ was considered to indicate significant statistical difference.

Results

FGF11 is upregulated in human NSCLC tissues and significantly correlated with the disease progression

Bioinformatics search from TCGA database of GSE75037 and GSE81089 NSCLC transcriptome analysis, we found the expression of FGF11 was higher in NSCLC tissues than adjacent normal tissues (Figure 1A and 1B, $p<0.001$). To further validate it, we selected 100 cases of NSCLC tumor tissues and 30 cases of matched adjacent normal lung tissues at *** Hospital. After extracted total RNA, the relative expression of FGF11 was detected by qRT-PCR. As shown in Figure 1C, the mRNA expression of FGF11 was remarkably higher in NSCLC tissues compared to adjacent normal tissues ($p<0.005$). Moreover, through the immunohistochemistry analysis, the protein level of FGF11 showed much higher in lung cancer tumor tissue than normal tissue (Figure 1D, $p<0.01$).

In addition, to further evaluate the clinical significance of FGF11 on patient survival, we first selected 1144 cases of NSCLC patients from TCGA database, and allocated into a FGF11-high expression group (n=571; >median) and a FGF11-low expression group (n=573; <median) based on the median expression level of FGF11, then using Kaplan Meier plotter analysis, it showed that high expression of FGF11 in NSCLC was remarkedly associated with poor overall survival of patients (Fig. 1E, $p<0.05$). Meanwhile, our dataset of NSCLC patients also showed the similar tendency (Fig. 1F, $p<0.05$).

The knockdown of FGF11 inhibits NSCLC tumor cells proliferation and promote apoptosis in vitro

The highly expression of FGF11 in human NSCLC samples prompted us to explore the biological function of FGF11 in NSCLC tumorigenesis. FGF11 expression was silenced in A549 and NCI-H460 cells by infection of FGF11 shRNA (shFGF11) lentivirus plasmid. The transfection efficiency was confirmed by qRT-PCR analysis (Figure 2A, $p<0.005$) and western blot (Figure 2B). CCK-8 assay was used to examine the cell proliferation after transfection with shFGF11 in A549 and NCI-H460 cells, the result suggested that knockdown of FGF11 notably inhibit the tumor cells proliferation (Figure 2C, $p<0.005$). Moreover, as indicated by colony formation assay, FGF11 knockdown reduced the ability of tumor cells colony formation (Figure 2C, $p<0.005$). In addition, flow cytometry was used to detect the cell apoptosis, it showed that knockdown of FGF11 remarkably increased the cell apoptosis in A549 and NCI-H460 (Figure 2D, $p<0.005$).

The overexpression of FGF11 promotes NSCLC tumor cells proliferation

FGF11 expression was overexpressed in A549 and NCI-H460 cells by infection of FGF11 overexpression (FGF11-OV) lentivirus plasmid. The transfection efficiency was confirmed by qRT-PCR analysis (Figure 3A, $p<0.005$) and western blot (Figure 3B). CCK-8 assay was used to examine the cell proliferation after transfection of FGF11-OV in A549 and NCI-H460 cells, the result showed that overexpression of FGF11 notably promote the tumor cells proliferation (Figure 3C, $p<0.005$). Moreover, as indicated by colony formation assay, overexpression of FGF11 increased the ability of tumor cells colony formation (Figure 3D, $p<0.005$).

FGF11 promotes NSCLC cells migration in vitro

We then aimed to evaluate the effect of FGF11 on the migration of NSCLC tumor cells A549 and NCI-H460. Wound healing assay and transwell assay were both used to detect the cells migration. The result of wound healing assay revealed that the migratory capacities of A549 and NCI-H460 cells were significantly weakened by knockdown of FGF11 (Figure 4A, $p<0.01$), but remarkably enhanced by FGF11 overexpression (Figure 4B, $p<0.005$), whereas the transwell assay also showed the similar result, which implicated that the migrative ability of A549 and NCI-H460 cells remarkably suppressed by knockdown of FGF11 (Figure 2B, $p<0.01$), but inversely increased by FGF11 overexpression (Figure 2D, $p<0.01$).

FGF11 might be downregulated by miR-525-5p in NSCLC tumor progression

To further explore the modulation of FGF11 by miRNAs. We first found that there have 4 common miRNAs (miR-326, miR-330-5p, miR-525-5p, miR-520a-5p) predicted by databases of miRDB, Starbase and Targetscan (Figure 5A). QRT-PCR was performed to examine the different miRNAs expression in A549 and NCI-H460 cells, miR-525-5p showed the lowest expression level (Figure 5B, $p<0.005$). Dual luciferase reporter assay showed that co-transfection of miR-525-5p reduced the relative luciferase activity of FGF11 3'UTR but have no effect on mutant FGF11 3'UTR (Figure 5C, $p<0.005$). Meanwhile, western blot analysis also confirmed that miR-525-5p could significantly inhibit the expression of FGF11 in A549 and NCI-H460 cells (Figure 5D).

Furthermore, qRT-PCR was also performed to detect the expression of miR-525-5p in NSCLC samples, the result showed that the expression of miR-525-5p was significantly lower in lung cancer tumor tissues compared to normal tissues (Figure 5E, $p<0.005$), and it represent a negative correlation between miR-525-5p and FGF11 (Figure 5F, $p<0.001$).

To further examine the function of miR-525-5p on tumor cells proliferation, migration and invasion. CCK-8 assay was used to detect the cell proliferation, we found that miR-525-5p could significantly inhibit the cell proliferation, whereas overexpression of FGF11 would impair its effect in A549 and NCI-H460 cells (Figure 5G, $p<0.005$). At the same time, colony formation assay also showed the similar result (Figure 5H, $p<0.005$). The cell migration and invasion were validated by transwell assay, the result showed that miR-525-5p inhibited the cell migration (Figure 5I, $p<0.01$) and invasion (Figure 5J, $p<0.01$), whereas FGF11 overexpression could rescue its effect.

FGF11 might regulate hypoxia signaling pathway for NSCLC progression

Bioinformatics analysis using GSE81089 NSCLC RNA-seq data showed a series of differentially expressed genes. Then through the GSEA analysis, we found that there was a significantly correlation between FGF11 and hypoxia signaling pathway (Figure 6A, NES=1.85, $P<0.001$, FDR $q=0.125<0.25$). In addition, since HIF-1α is the critical regulators in hypoxia signaling, we next explored whether FGF11 regulate the expression of HIF-1α. Interestingly, we performed qRT-PCR to find that the mRNA expression of HIF-1α was reduced by knockdown of FGF11 (Figure 6B, $p<0.05$), the protein level was also has a remarkable reduction by western blot analysis (Figure 6C, $p<0.05$). However, the relative mRNA expression of HIF-1α would be increased by FGF11 overexpression in A549 and NCI-H460 cells (Figure 6D, $p<0.005$),

western blot also confirmed that the protein level of HIF-1α was increased by FGF11 overexpression (Figure 6E, $p<0.005$).

In addition, to further validate the cell proliferation, migration and invasion of HIF-1α on A549 and NCI-H460 cells. CCK-8 assay was examined to detect the cell proliferation, the result showed that overexpression of FGF11 could significantly promote A549 and NCI-H460 cells proliferation, but HIF-1α siRNA inhibited its proliferation notably and blocked the effect of FGF11 (Figure. 6F, $p<0.01$). Meanwhile, colony formation assay also got the similar result (Figure. 6G, $p<0.01$). Then, the cell migration and invasion were detected by transwell assay, it showed that overexpression of FGF11 could significantly enhance A549 and NCI-H460 cells migration, but HIF-1α siRNA inhibited its migration notably and blocked the effect of FGF11 (Figure. 6H, $p<0.01$), as well as the effect on cell invasion (Figure. 6I, $p<0.01$).

Knockdown of FGF11 inhibits NSCLC tumor growth in vivo

The role of FGF11 in NSCLC tumor growth was further explored in vivo. In consistence with the in vitro findings, knockdown of FGF11 result in a distinct reduction of A549 and NCI-H460 tumor growth compared to control group mice (Figure 7A), as well as the average tumor weight (Figure 7B, $p<0.005$). Through the immunohistochemistry analysis of Ki67 in A549 and NCI-H460 tumor mice model, the result showed that knockdown of FGF11 could significantly inhibit proliferation antigen Ki-67 (Figure 7C, $p<0.005$). In contrast, the overexpression of FGF11 could promote the tumor growth remarkably (Figure 7D), as well as the average tumor weight (Figure 7E, $p<0.005$). Moreover, the staining intensity of proliferation antigen Ki-67 in the tumor tissues was also notably increased by FGF11 overexpression (Figure 7F, $p<0.005$).

Discussion

Recent years, lung cancer has been considered as the most common incidence and highest mortality rates in Chinese cancer progression. Non-small cell lung cancer (NSCLC) occupied approximately 80% of lung cancer, which is a clinically heterogeneous and multifactorial disease, including various genetic changes. Treatment advances in targeting EGFR (epidermal growth factor receptor) and VEGF (vascular endothelial growth factor) signaling pathways promote the diagnosis and prognosis in NSCLC therapy [27-30]. In addition, fibroblast growth factor (FGF) ligand and receptor have been widely studied in a variety of tumors, including lung cancer, which activate through multiple ways [31]. For example, the downregulation of FGF5 play important roles in inhibiting cell growth and invasion of human NSCLC cells [9]. However, through TCGA database analysis, FGF11 was predicted overexpressed in NSCLC tissues than normal tissues, but its molecular mechanism study is still less understood till now. Herein, we believe to better explore FGF11-targeting therapy could be considered as a feasible methodology for the treatment of NSCLC.

In this study, we first used human NSCLC transcriptome analysis followed by qRT-PCR and western blot examination, a significantly increase of FGF11 expression was found not only in clinical NSCLC tumor tissues but also in NSCLC cell lines, suggesting its oncogenic effect. In vitro gain- and loss- of function

experiments, we found that the overexpression of FGF11 promoted, whereas knockdown of FGF11 suppressed the proliferation, migration and invasion of NSCLC cells. Similarly, NSCLC apoptosis also was inhibited by FGF11 overexpression, which could be inversed by knockdown of FGF11. Besides, the oncogenic activity of FGF11 was also verified in the nude mouse xenograft model bearing NSCLC cells.

MiR-525-5p has been investigated in multiple human cancers, including Glioma [32], Cervical Cancer [33], et al. The function of miR-525-5p might be considered as a tumor suppressor. However, there still few studies about its function in lung cancer and other human cancers. Through the bioinformatics search and experimental validation, we found miR-525-5p could downregulate FGF11 expression in NSCLC, and also play critical roles in the proliferation and migration of tumor cells.

Hypoxia has been widely investigated in cancer progression, which involved in multiple process [34, 35]. Successive advances in genetic and genomic analysis revealed that HIF-1α was a key transcriptional regulator in hypoxia signaling pathways [36]. By performing bioinformatics analysis, we revealed that FGF11 was closely correlated with hypoxia signaling, and furthermore, qRT-PCR analysis and western blot all validated that FGF11 could regulate HIF-1α expression positively. Since HIF-1α has been acted as an indispensable signaling transduction mediators by regulation of their target genes, thereby affecting cell function and fate [37-40]. Rescue experiments further suggested that knockdown of HIF-1α diminished the oncogenic role of FGF11 in NSCLC cells. We therefore speculated that FGF11 might function as an oncogene to upregulate and increase HIF-1α activity, thus leading to the progression of NSCLC.

In conclusion, to our knowledge, we were first to show that FGF11 was upregulated in NSCLC, and functional experiments indicated FGF11 acted as an oncogene in NSCLC partly through modulating hypoxia signaling pathway. We believed our findings provided promising evidence to implicate that FGF11 may have considerable potential as a therapeutic target and prognosis biomarker for NSCLC patients in the future.

Abbreviations

Fibroblast growth factor (FGF); Non-small cell lung cancer (NSCLC); hypoxia inducible factor (HIF); microRNAs (miRNAs); Cell counting kit-8 (CCK-8)

Declarations

Ethics approval and informed consents

The use of human tissues was approved by the Ethics Committee of Tongji Hospital, all patients and/or their relatives signed the informed consents.

All animal experimental procedures were approved by the Ethics Committee of Tongji Hospital.

Funding

This work was supported by Natural Science Fund of Hubei Province (2020CFB740).

Authors' contributions

Xiaowei Wu and Minjie Li: experiment studies, clinical studies, Data collection and Manuscript writing;

Yu Deng, Shun Ke, Fan Li and Yujin Wang: interpreted the patient data, Data collection and Manuscript writing;

Shuchang Zhou: Project design, data analysis, manuscript editing. All contributing authors have read and agreed to the final version of the manuscript.

Acknowledgements

The authors declare that there are no competing interests.

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Figures

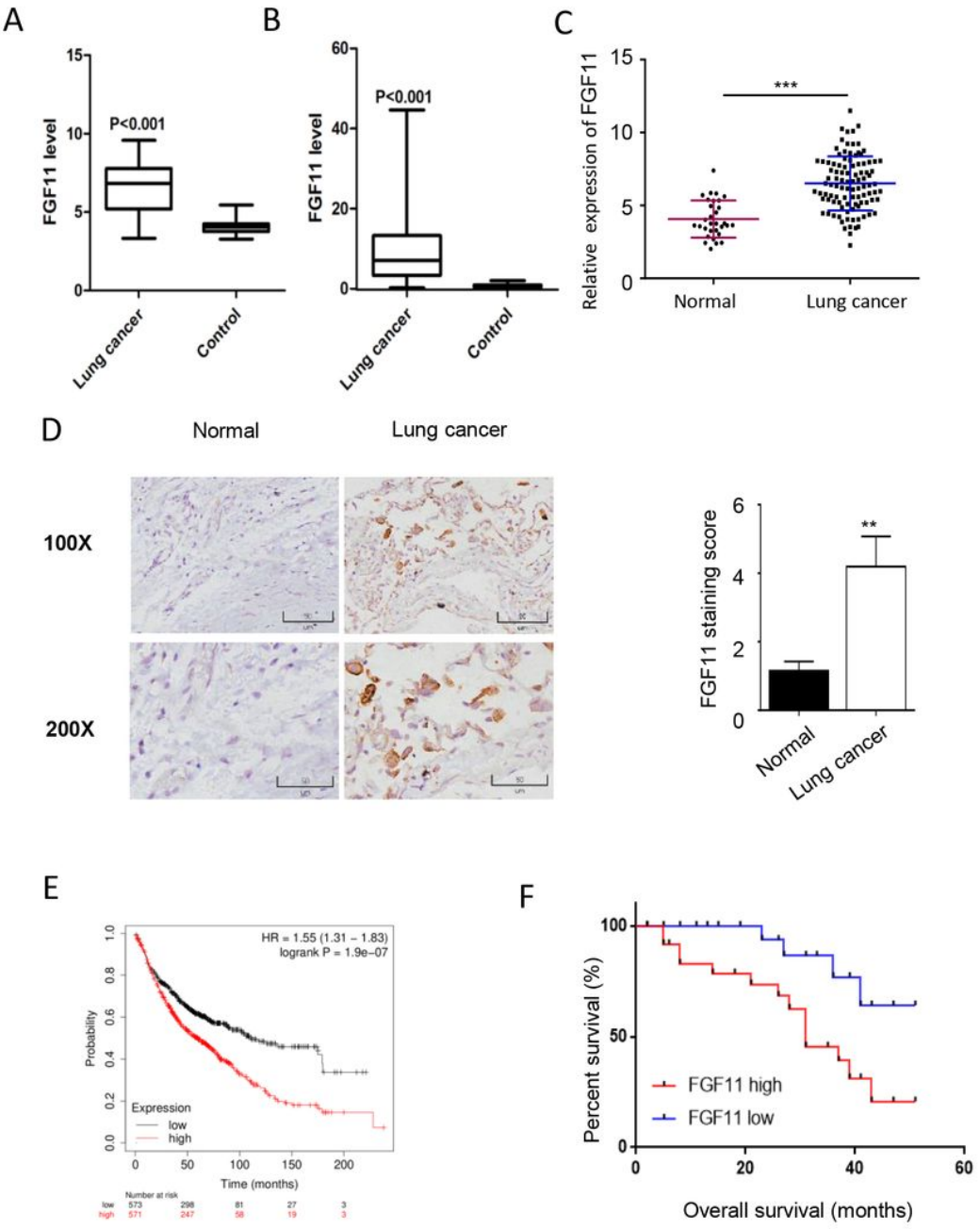

Figure 1

FGF11 is upregulated in human NSCLC tissues and cells associated with poor prognosis. (A) The relative expression of FGF11 from GSE75037 database, p<0.001. (B) The relative expression of FGF11 from GSE81089 database, p<0.001. (C) qRT-PCR analysis of FGF11 expression in 100 cases of NSCLC tissues and 30 cases of adjacent normal tissues. ***P<0.005 vs. normal tissues. (D) The examination of FGF11 protein level by immunohistochemistry in NSCLC tumor tissue compared to normal tissue. (E) The prediction of overall survival from 1144 cases of NSCLC patients from TCGA database. (F) Kaplan-Meier plotter analysis the survival curve of FGF11 higher expression group (n=50) compared to lower expression group (n=50) in NSCLC patients. Note: *P<0.05, ** P<0.01, ***P<0.005.

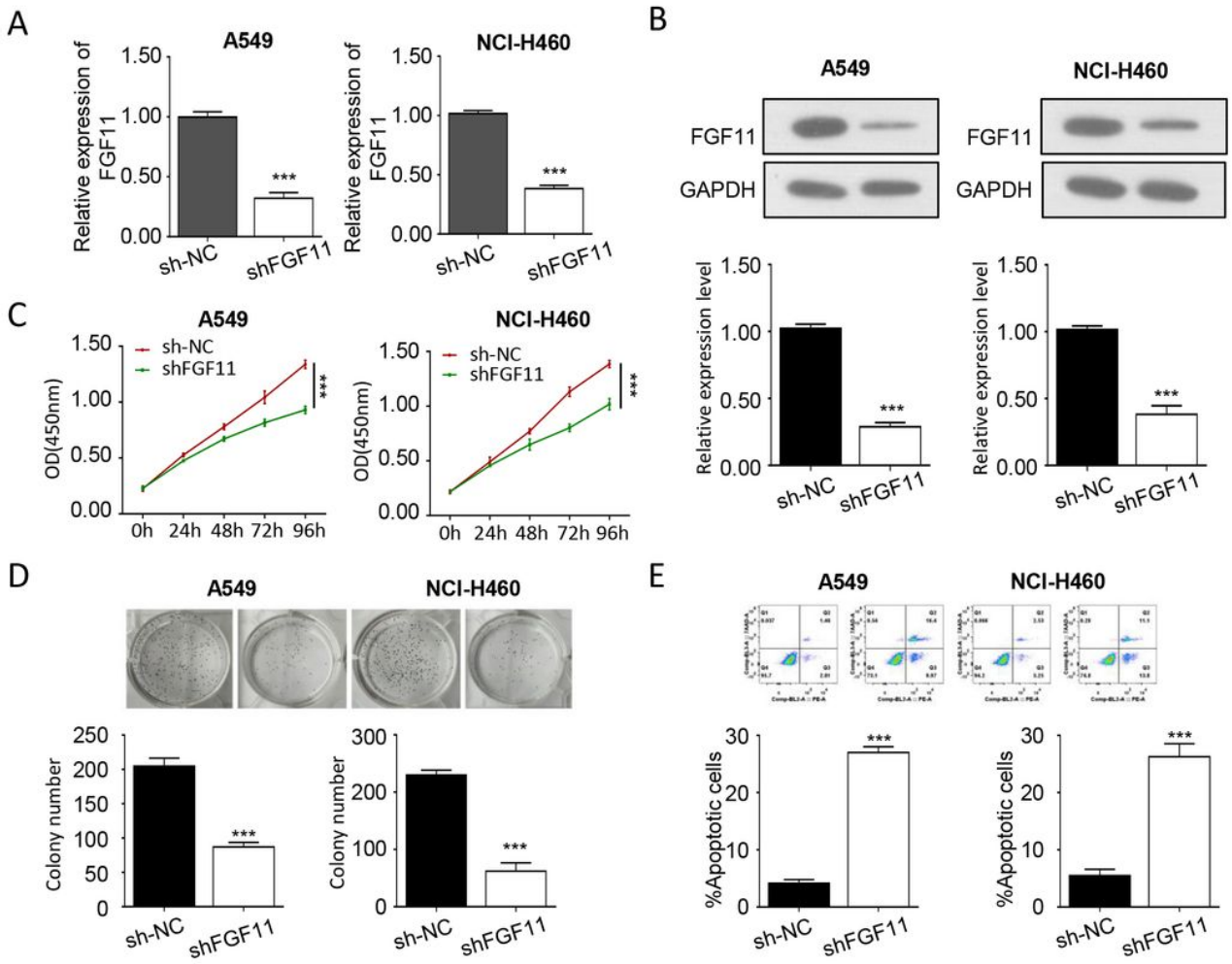

Figure 2

Knockdown of FGF11 inhibits NSCLC cell proliferation and promotes cell apoptosis in vitro. Generating FGF11 shRNA (shFGF11) lentivirus plasmid, then transfected into NSCLC tumor cells for 48-72h. (A) qRT-PCR analysis of FGF11 mRNA expression in A549 and NCI-H460 cells after transfection. (B) Western blot detected FGF11 expression levels in A549 and NCI-H460 cells. (C) The proliferation of A549 and NCI-H460 cells after transfection was detected by CCK-8 assay. (D) colony formation assay of A549 and NCI-H460 cells after transfection. (E) Flow cytometry detected the cell apoptosis after staining with Annexin-V and PI. Note: *P<0.05, ** P<0.01, ***P<0.005 vs. sh-NC or empty vector-transfected cells.

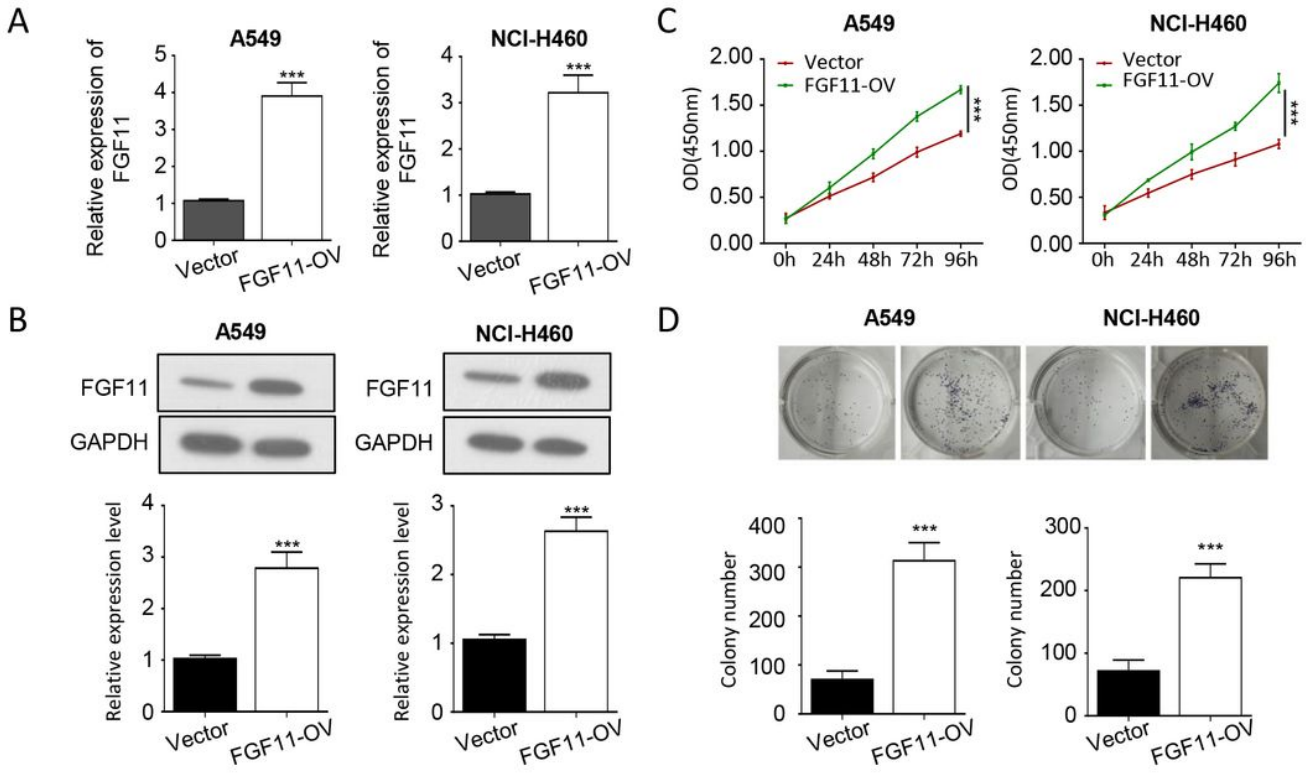

Figure 3

The overexpression of FGF11 promotes NSCLC cell proliferation. Generating FGF11 overexpressed lentivirus plasmid, then transfected into NSCLC tumor cells for 48-72h. (A) qRT-PCR analysis of FGF11 mRNA expression in A549 and NCI-H460 cells. (B) Western blot detected FGF11 expression in A549 and NCI-H460 cells. (C) CCK-8 assay detected the proliferation of A549 and NCI-H460 cells. (D) Colony formation assay detected the cell clones in A549 and NCI-H460. The statistical results data were represented via mean ± standard derivate (SD). *P<0.05, ** P<0.01, ***P<0.005 vs. NC or empty vector-transfected cells.

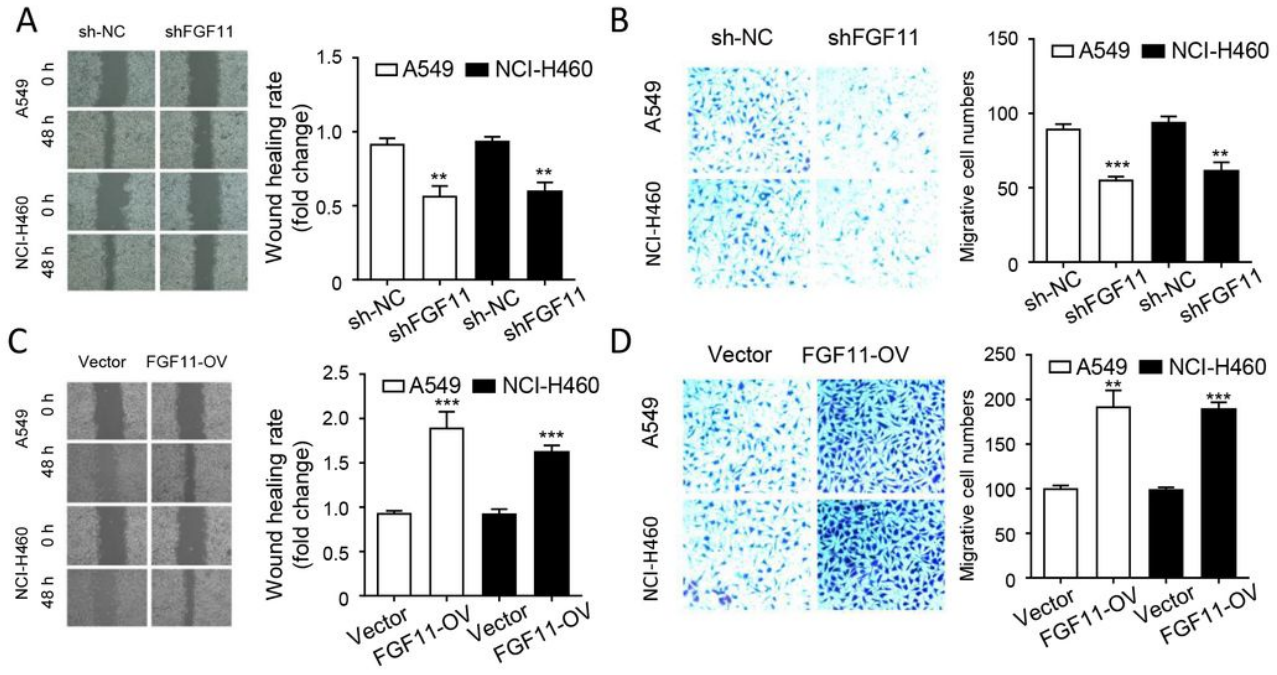

Figure 4

FGF11 promotes NSCLC tumor migration in vitro. FGF11 shRNA or FGF11 overexpressed lentivirus plasmid were transfected into NSCLC tumor cells for 48-72h. (A) Wound healing assay detected the migration of A549 and NCI-H460 cells after transfected with FGF11 shRNA. (B) Transwell assay detected the migration of A549 and NCI-H460 cells after transfected with FGF11 shRNA. (C) Wound healing assay detected the migration of A549 and NCI-H460 cells after transfected with FGF11 overexpression plasmid. (D) Transwell assay detected the migration of A549 and NCI-H460 cells after transfected with FGF11 overexpression plasmid. The statistical results data were represented via mean ± standard derivate (SD). *P<0.05, ** P<0.01, ***P<0.005 vs. sh-NC or empty vector-transfected cells.

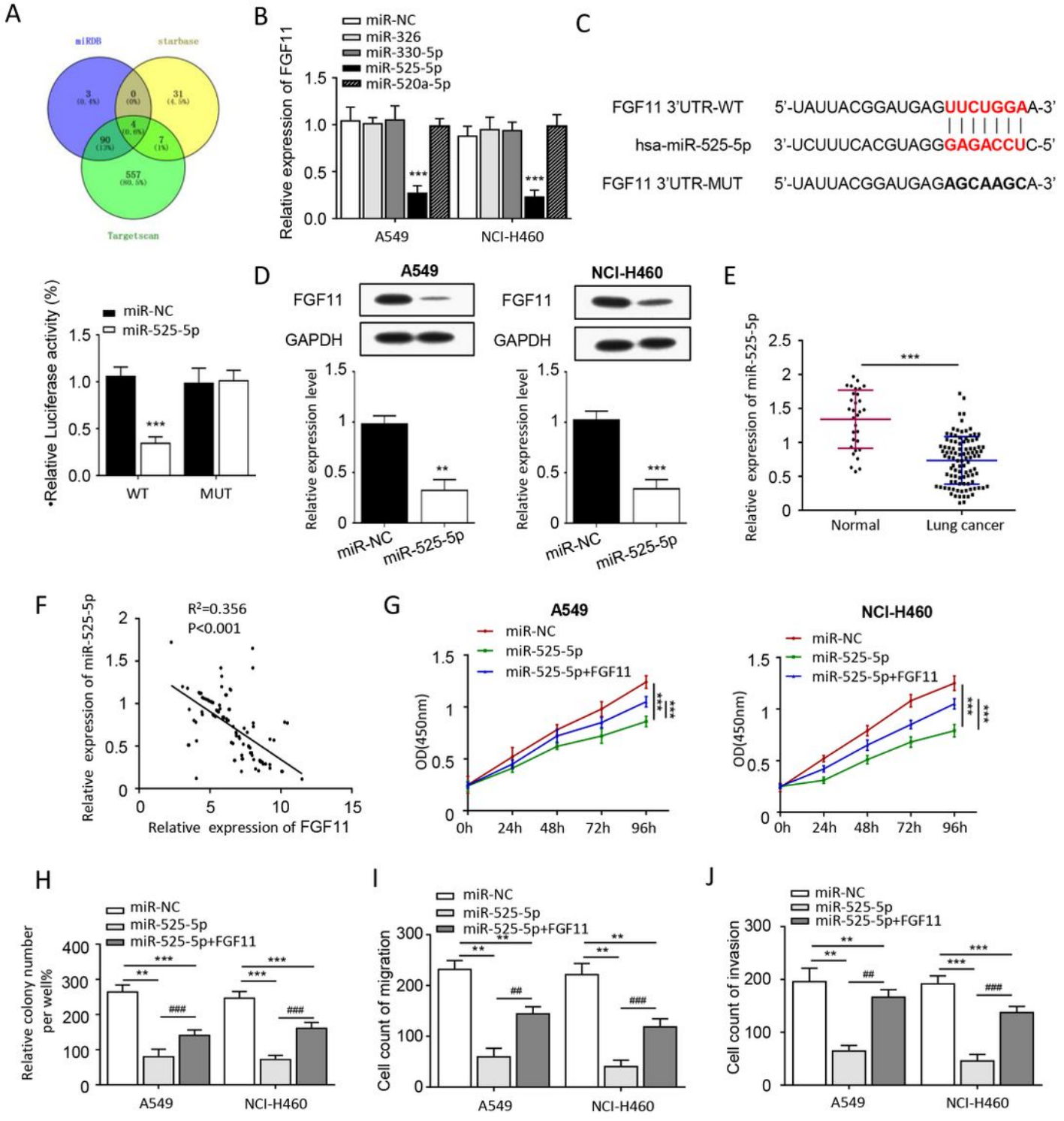

Figure 5

miR-525-5p downregulate FGF11 expression and inhibit NSCLC cells proliferation and migration. (A) Bioinformatics search of miRNAs that targeting FGF11 by miRDB, Starbase and Targetscan databases. (B) qRT-PCR detected the different miRNAs expression in A549 and NCI-H460 cells. (C) dual-luciferase reporter assay detected the luciferase activity of miR-525-5p on FGF11 3'UTR. (D) western blot detected the expression of FGF11 after transfected with miR-525-5p. (E) qRT-PCR examined the mRNA expression

in NSCLC tumor tissues compared to normal tissues. (F) The correlation of miR-525-5p and FGF11 by Spearman analysis. (G) CCK-8 assay detected the proliferation of A549 and NCI-H460 cells after transfected with miR-525-5p and/or FGF11. (H) Colony formation assay detected the clone of A549 and NCI-H460 cells after transfected with miR-525-5p and/or FGF11. (H) Wound healing assay detected the migration of A549 and NCI-H460 cells after transfected with miR-525-5p and/or FGF11. (H) Transwell assay detected the invasive ability of A549 and NCI-H460 cells after transfected with miR-525-5p and/or FGF11. The statistical results data were represented via mean ± standard derivate (SD). *P<0.05, ** P<0.01, ***P<0.005 vs. sh-NC or empty vector-transfected cells.

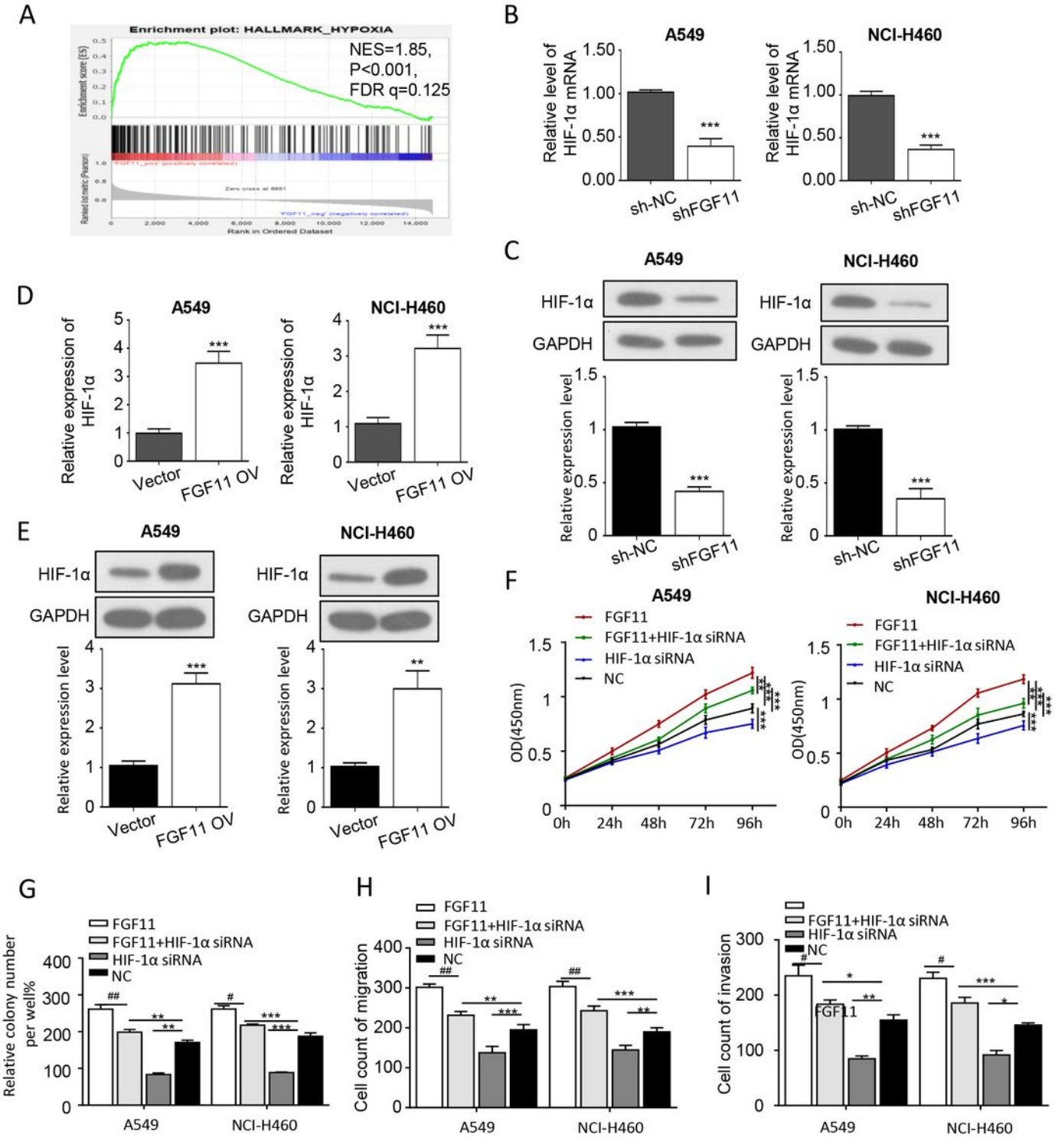

Figure 6

FGF11 regulate HIF-1α in hypoxia signaling pathways for NSCLC progression. (A) Bioinformatics analysis predicted FGF11 was closely correlated with hypoxia signaling pathway using GSE81089 NSCLC RNA-seq data. (B) qRT-PCR detected HIF-1α mRNA expression after knockdown of FGF11 in A549 and NCI-H460 cells. (C) western blot detected HIF-1α expression after knockdown of FGF11 in A549 and NCI-H460 cells. (D) qRT-PCR detected HIF-1α mRNA expression after overexpression of FGF11 in A549 and NCI-H460 cells. (E) western blot detected HIF-1α expression after overexpression of FGF11 in A549 and NCI-H460 cells. (F) CCK-8 assay detected the cell proliferation of A549 and NCI-H460 after transfected with FGF11 and/or HIF-1α siRNA. (G) Colony formation assay detected the cell clone of A549 and NCI-H460 after transfected with FGF11 and/or HIF-1α siRNA. (H) wound healing assay detected the migration of A549 and NCI-H460 after transfected with FGF11 and/or HIF-1α siRNA. (I) transwell assay detected the invasion of A549 and NCI-H460 after transfected with FGF11 and/or HIF-1α siRNA. The statistical results data were represented via mean ± standard derivate (SD). *P<0.05, ** P<0.01, ***P<0.005 vs. sh-NC or empty vector-transfected cells.

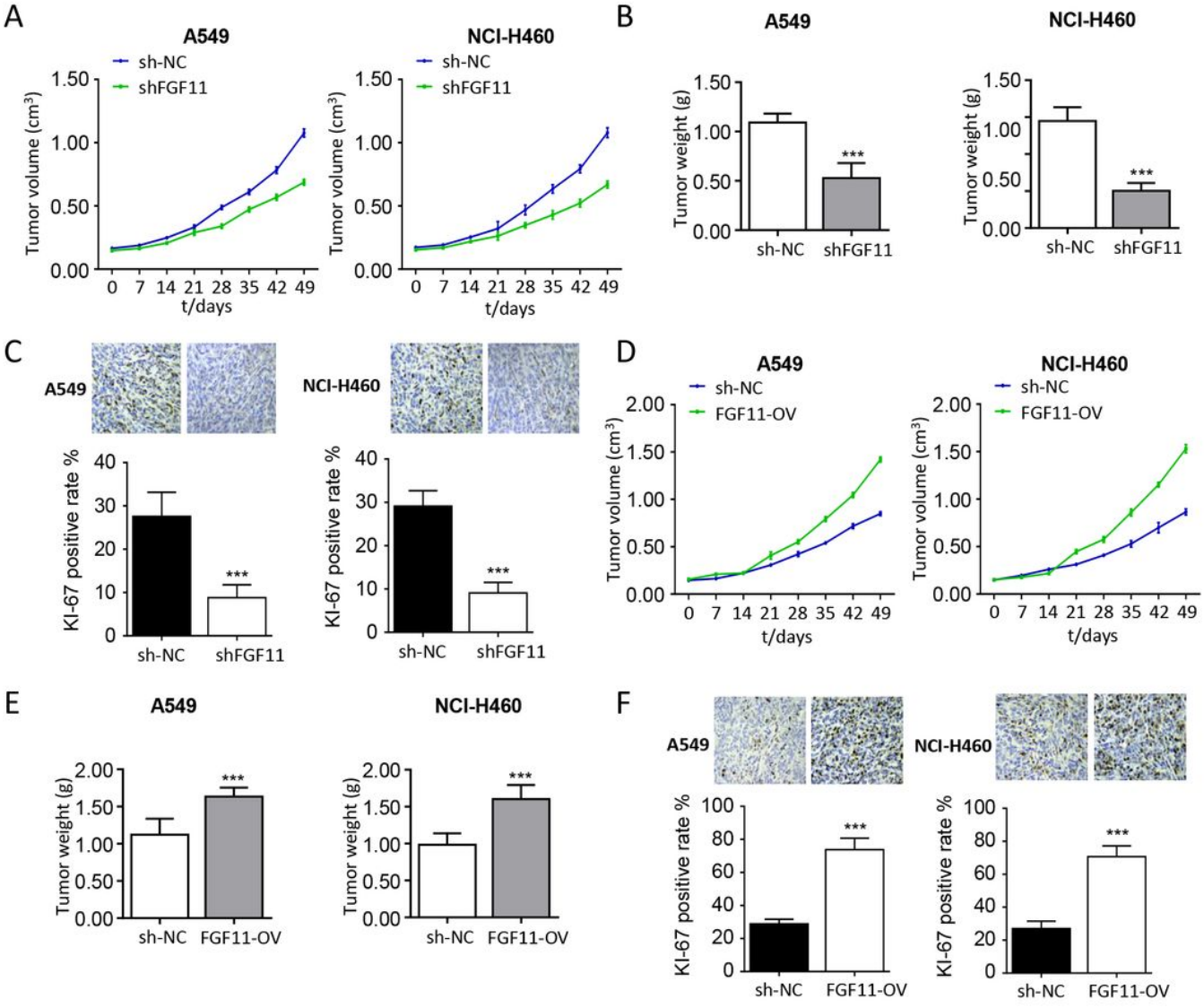

Figure 7

Knockdown of FGF11 inhibits NSCLC tumor growth in vivo. FGF11 shRNA and overexpression lentivirus plasmid were transfected into A549 and NCI-H460, then inoculated into balb/c nude mice (n=5 mice/group). (A) The tumor growth curve recorded in FGF11 shRNA tumor mice model compared to control group. (B) The quantification of tumor weights in FGF11 shRNA tumor mice model compared to control group. (C) Immunohistochemistry (IHC) analysis of Ki-67 expression levels in FGF11 shRNA tumor mice tissue compared to control group. (D) The tumor growth curve recorded in FGF11 overexpressed tumor mice model compared to control group. (E) The quantification of tumor weights in FGF11 overexpressed tumor mice model compared to control group. (F) Immunohistochemistry (IHC) analysis of Ki-67 expression levels in FGF11 overexpressed tumor mice tissue compared to control group. The statistical results data were represented via mean ± standard derivate (SD). *P<0.05, ** P<0.01, ***P<0.005 vs. control group.