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ORIGINAL RESEARCH

FAM129B promoted tumor invasion and proliferation via facilitating the phosphorylation of FAK signaling and associated with adverse clinical outcome of non-small cell lung cancer patients

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Background: Family with sequence similarity 129, member B (*FAM129B*), also called MINERVA, is upregulated and promotes tumor invasion in multiple types of cancer. However, the mechanism and clinicopathological significance of *FAM129B* remains unclear.

Materials and methods: Online KM-plotter tool and immunohistochemistry were used to predict the prognostic value of *FAM129B* expression in lung cancer tissues. Western blotting analysis, MTT, colony formation assay and matrigel invasion assay were performed after overexpressing or depleting *FAM129B*.

Results: In this study, using the online KM-plotter tool, we found *FAM129B* was correlated with adverse outcome in non-small cell lung cancer (NSCLC) patients ($P<0.001$). Immunohistochemistry results revealed that *FAM129B* showed negative or dim expression in normal lung tissues while presented positive cytoplasmic expression in both squamous cell lung carcinoma and lung adenocarcinoma. The positive ratio of *FAM129B* in clinical NSCLC tissue samples (77/187, 41.2%) was significantly higher than that in normal lung tissue samples (8/68, 11.8%; $P<0.001$). *FAM129B* expression associated with advanced TNM staging ($P<0.001$) and positive regional lymph node metastasis ($P<0.001$). The results of Kaplan-Meier analysis suggested that the survival time of patients with positive *FAM129B* expression was significantly shorter than those with negatively *FAM129B* expression ($P<0.001$). Proliferation and invasion assay revealed that *FAM129B* prominently facilitated tumor proliferation and invasion in NSCLC cells. Western blotting results revealed that *FAM129B* upregulated the expression of MMP2 and Cyclin D1 by enhancing the phosphorylation of FAK at Tyr 397 and Tyr 925. Incorporation of FAK inhibitor in the medium significantly downregulated the phosphorylation of FAK and subsequently attenuated increasing expression of MMP2 and Cyclin D1 induced by *FAM129B* overexpression.

Conclusion: Our results indicated that *FAM129B* may be a new prognosis predictor of NSCLC patients and impact tumor invasion and proliferation of NSCLC cells through promoting the activation of FAK signaling.

Keywords: *FAM129B*, invasion, proliferation, FAK, non-small cell lung cancer

Introduction

Family with sequence similarity 129, member B (*FAM129B*), also called MINERVA, is upregulated in several human cancers, including breast cancer, renal cancer, colon cancer, lung cancer, endometrial cancer, leukemia and central nervous system tumors.[1,2] There is a pleckstrin homology domain near the N-terminus and a proline-rich domain near the C-terminus of *FAM129B*.[3] *FAM129B* is known to inhibit TNFα-dependent apoptosis

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and to promote tumor invasion in HeLa cells.[3,4] Conrad et al demonstrated that *FAM129B* facilitated activation of the Wnt/β-catenin signaling pathway, thereby inhibiting apoptosis in melanoma cells.[5] However, in the literature, *FAM129B* served various functions depending on tissue type. In HeLa cells, silencing the *FAM129B* gene expression did not affect tumor growth,[3] whereas in gene-targeted *FAM129B*-mutant mice, wound healing was significantly delayed. In the same study, overexpression of *FAM129B* contributed to enhancing cell motility in HaCaT cells.[6] Therefore, it is likely that *FAM129B* may play diverse roles in various types of tissues or tumors.

Previous studies reported that *FAM129B* functioned as a molecule downstream of EGFR and ERK.[3,7] In melanoma cells, knockdown of *FAM129B* prominently inhibited WNT3A-mediated activation of the Wnt/β-catenin signaling pathway, indicating that *FAM129B* was an important regulator of Wnt/β-catenin signaling.[5] Focal adhesion kinase acts as a key integrator of the growth-factor pathway and the ERK/MAPK signaling pathway, thereby playing an important role in regulating tumor invasion and migration.[8–12] Moreover, FAK protein was also required for the proper regulation of Wnt/β-catenin signaling.[13] Thus, FAK may be a potential downstream regulator of *FAM129B*, remaining unknown to date.

The purpose of the present study was to investigate the expression and association with clinicopathological features of *FAM129B* in non-small-cell lung cancer (NSCLC) tissue samples. By transfecting with *FAM129B* cDNA plasmid or siRNA in NSCLC cell lines, we investigated the effect of *FAM129B* on cellular invasion, survival and cell movement.

Materials and methods

Patients and clinical specimens

This study was performed with the approval of the local institutional review board of China Medical University. Each of the patients signed informed consent. The tissue samples of 187 patients (111 males and 76 females) were collected from patients who underwent radical surgical excision at the First Affiliated Hospital of China Medical University from 2009 to 2012. No neoadjuvant radiotherapy or chemotherapy was given to these patients prior to surgery. After surgery, all patients received standard chemotherapy according to the NCCN guideline. Sixty-eight of the 187 patients had samples of the corresponding noncancerous tissues. Complete follow-up data were obtained from all 187 lung cancer patients. The survival time was calculated from the day of surgery to the day of death due to recurrence or metastasis or to the end of follow-up. The median age was 60 years old (range 29–83 years). Of the 187 patients, 87 patients were older than 60 years. Histological diagnosis and grading were done according to the fourth edition of WHO classification of tumors of the lung.[14] There were 76 squamous cell lung carcinomas and 111 lung adenocarcinomas in our cohort. Of the 187 cases, 71 tumors were well differentiated and 116 were classified as moderately or poorly differentiated. Tumor staging was performed according to the seventh edition of the American Joint Committee on Cancer TNM Staging System for Lung Cancer.[15] The tumors included 138 stages I–II and 49 stage III. Lymph node metastases were present in 82 of the 187 patients.

Immunohistochemistry

Using the streptavidin-peroxidase method, immunohistochemistry staining was performed according to the manufacturer's instructions (Ultrasensitive; MaiXin, Fuzhou, China). The sections were incubated with anti-*FAM129B* antibodies (mouse anti-human; dilution, 1:200; HPA024312, Sigma-Aldrich, St Louis, MO, USA) at 4°C overnight, followed by biotinylated goat anti-mouse IgG secondary antibodies. The slides were scored by two investigators who were blinded to the clinical data. The scores were obtained by evaluating the staining intensity and percentage of positive cells in representative areas. We used the following strategy to assess the slides: intensity, 0 (no signal), 1 (weak), 2 (moderate) or 3 (high); percentage of cells, 1 (1%–25%), 2 (26%–50%), 3 (51%–75%) or 4 (76%–100%). We multiplied the scores of staining intensity and percentage to obtain a final score (range 0–12). When tumors had scores ≥4, they were defined as positive for *FAM129B* expression. When tumors had scores <4, they were defined as negative for *FAM129B* expression.

Online analysis of overall survival in NSCLC patients

The KM Plotter Online Tool (http://www.kmplot.com) was used to evaluate the prognostic value of *FAM129B*, based on the information on mRNA expression levels from 1,145 NSCLC patients. The details of clinical features of specimens were described in a previous paper.[16]

Cell culture

The cell lines used in the present study, including NCI-A549 (A549), NCI-H460 (H460) and NCI-H1299 (H1299) were obtained from the Shanghai Cell Bank (Shanghai, China). All the three cell lines were derived from NSCLC. The details of cell characteristics can be found on www.atcc.org. All the three cell lines were cultured in RPMI 1640 (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% FBS (Thermo Fisher Scientific), 100 IU/mL penicillin (Sigma-Aldrich) and 100 μg/mL streptomycin (Sigma-Aldrich). The cells were grown in sterile culture dishes and passaged every 2 days after using 0.25% trypsin (Thermo Fisher Scientific).

Western blotting

Total protein was quantified using the Bradford method.[17] In total, 50 μg of total cellular protein samples was separated by 10% SDS-PAGE and immunoblotted with the following primary antibodies: *FAM129B* (1:200; HPA024312, Sigma-Aldrich); GAPDH (1:5,000; Sigma-Aldrich); MMP2, MMP9, cyclin A2, cyclin B1, cyclin D1, cyclin E1, p-FAK (Tyr 397), p-FAK (Tyr 576), p-FAK (Tyr 925), FAK (1:1,000; Cell Signaling Technology, Danvers, MA, USA). Immunoreactive bands were visualized using enhanced chemiluminescence (ECL, Pierce, Rockford, IL, USA).

Plasmid transfection and siRNA treatment

The plasmids (pCMV6-ddk-myc and pCMV6-ddk-myc-*FAM129B*) were purchased from Origene (Rockville, MD, USA). *FAM129B*-siRNA (sc-92820) and scrambled-siRNA (sc-37007) were purchased from Santa Cruz Biotechnology. Transfection was performed according to the manufacturer's instructions using the Lipofectamine 3000 reagent (Thermo Fisher Scientific). Cells were transfected with 3 μg of indicated plasmid or 10 μL of indicated siRNA. Forty-eight hours after plasmid/siRNA transfection, cells were used for functional assays and the remaining cells were collected for western blot analysis.

Matrigel invasion assay

Cell invasion assays were performed with 20 μL Matrigel (1:3 dilution; BD Biosciences, San Jose, CA, USA) coated on the upper surface of 24-well Transwell chambers with 8 μm pores (Costar, Cambridge, MA, USA). After incubation for 18 hours, the cells that had passed through the filter were fixed with 4% paraformaldehyde and stained with hematoxylin. Ten randomly selected fields at 400× magnification were counted under a microscope. Each experiment was carried out in triplicate.

Colony formation assay

After the indicated transfection, cells were plated on 6 cm cell culture dishes (1,000 per dish for H460 and A549 cell lines) for 12 days. The cells were then fixed with methyl alcohol for 30 minutes and stained with Giemsa. The numbers of colonies with more than 50 cells were counted. All experiments were carried out in triplicate.

MTT

Cells were seeded on 96-well plates at approximately 3,000 cells per well 24 hours after transfection. After incubation for the indicated number of days, 20 μL of MTT (thiazolyl blue) solution (5 mg/mL) was added to each well and incubated at 37°C for 4 hours. After removing the medium, the resultant MTT formazan was solubilized in 150 μL of DMSO. The absorbance was read at a test wavelength of 490 nm, each carried out in triplicate.

Statistical analysis

SPSS version 22.0 for Windows (SPSS, Chicago, IL, USA) was employed for statistical analyses. The Pearson's chi-squared test was selected to evaluate possible associations between *FAM129B* and clinicopathological features. Kaplan–Meier survival analyses were performed for the 187 patient specimens. The log-rank test was carried out to compare the significance. The Mann–Whitney *U* test was chosen to evaluate the significance of image analysis results of the invasive assay, colony formation assay and MTT test. $P<0.05$ was considered to indicate statistically significant differences.

Results

FAM129B correlated with tumor development and predicted poor survival of NSCLC patients

First, we applied the KM-plotter tool to predict the impact of *FAM129B* gene expression on overall survival. As shown in Figure 1A, we showed that the *FAM129B* gene was associated with worse outcome in NSCLC patients in terms of overall survival (cut-off value: median; $P<0.001$). Next, immunohistochemistry staining was performed in 187 cancerous tissue samples and 68 non-cancerous tissue samples. *FAM129B* was negatively or dimly expressed in the normal bronchial epithelium (Figure 1B) and alveolar epithelium (Figure 1C). However, *FAM129B* was positively expressed in the cytoplasm of tumor cells in both squamous cell lung carcinoma (Figure 1D) and lung adenocarcinoma (Figure 1E). *FAM129B* tended to exhibit stronger expression in cancerous tissues than in adjacent non-cancerous tissues (Figure 1F–H). The positive ratio of *FAM129B* in lung cancer tissues (77/187, 41.2%) was significantly higher than that of adjacent noncancerous lung tissue samples (8/68, 11.8%; $P<0.001$). *FAM129B* expression significantly correlated with advanced TNM stage ($P<0.001$) and positive lymph node metastasis ($P<0.001$; Table 1). There were no significant correlations between *FAM129B* expression and other clinicopathological features, including age, gender, tumor size, histological differentiation, histological type and smoking status ($P>0.05$; Table 1). Kaplan–Meier analysis showed that the overall survival of patients with *FAM129B* expression was significantly poorer than that of patients without *FAM129B* expression ($P<0.001$; Figure 1I).

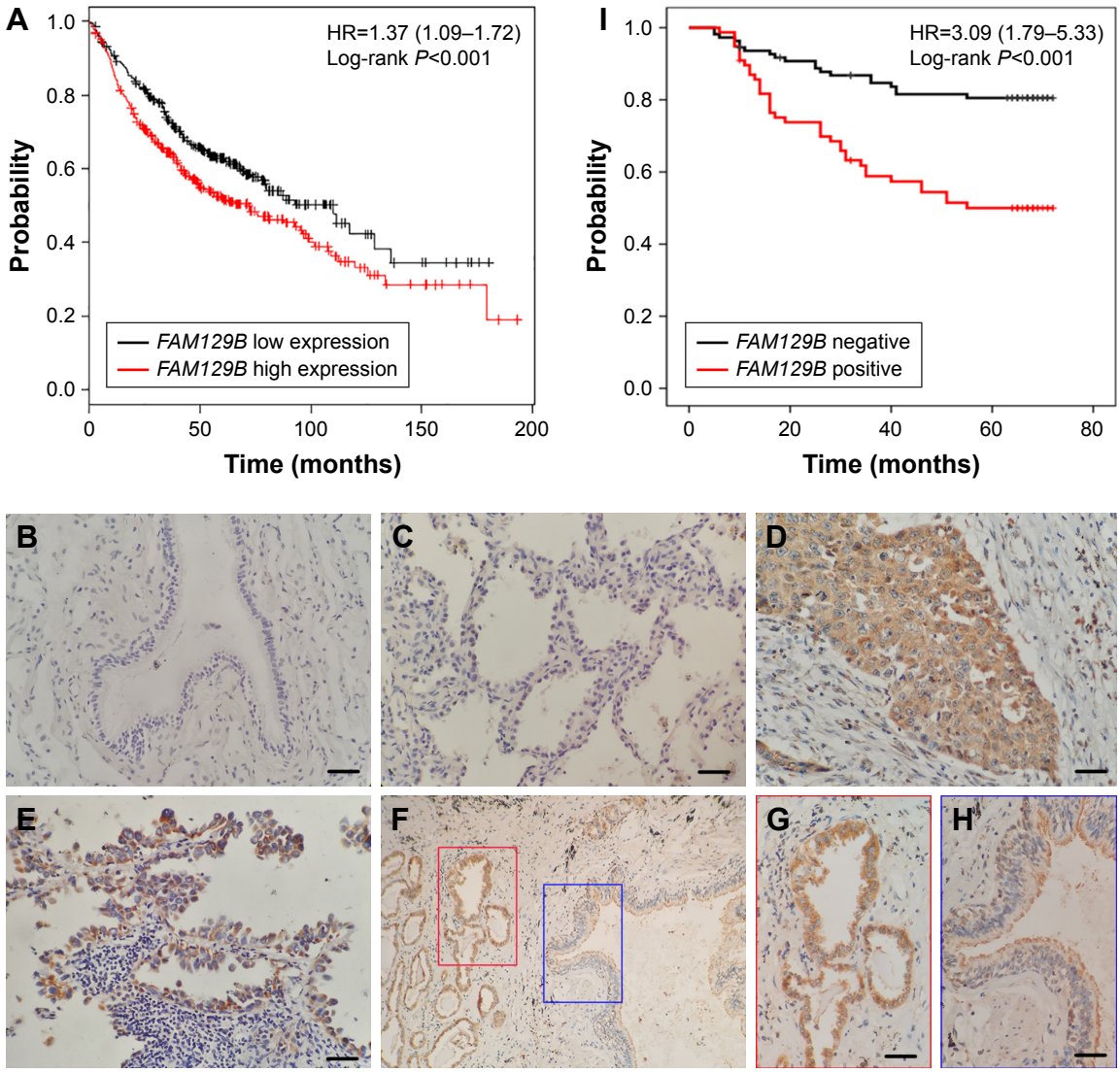


Figure 1 *FAM129B* was associated with worse prognosis of NSCLC patients.
Notes: (**A**) Results of online software survival prediction (KM plotter). The survival time of patients with higher *FAM129B* mRNA expression was significantly shorter than that of patients with lower *FAM129B* mRNA expression. Representative figures of *FAM129B* expression in normal bronchial (**B**), alveolar epithelial (**C**), squamous cell lung carcinoma (**D**) and lung adenocarcinoma (**E**). Magnification, 400×, scale bar=50 μm. *FAM129B* presented higher expression levels in NSCLC tissue samples than in corresponding noncancerous tissues (**F**, 100×; **G** and **H**, inset, 400×, scale bar=50 μm). Kaplan–Meier survival analysis revealed that patients with positive *FAM129B* expression presented poorer overall survival than did those with negative *FAM129B* expression (**I**).
Abbreviation: NSCLC, non-small-cell lung cancer.

Overexpression of exogenous *FAM129B* promoted tumor invasion and proliferation in NSCLC cells

Using western blotting, we evaluated protein levels of *FAM129B* in 3 NSCLC cell lines (H1299, A549 and H460). *FAM129B* showed relatively higher protein levels in H1299 and A549 cells than in H460 cells (Figure 2A). Subsequently, we transfected *FAM129B* cDNA plasmid into H460 cells (Figure 2B) and found that the protein levels of MMP2 and cyclin D1 were significantly elevated following overexpressing *FAM129B*, whereas other proteins including MMP9, cyclin A2, cyclin B1 and cyclin E1 presented no visible changes (Figure 2C). Subsequent Matrigel invasion assay, colony formation assay and MTT assay revealed that cellular invasion and survival were prominently enhanced by transfecting exogenous *FAM129B* plasmid (Figure 2D–H).

Knockdown of endogenous *FAM129B* downregulated the expression of MMP2 and cyclin D1, thereby inhibiting tumor invasion and proliferation in NSCLC cells

To further elucidate the function of *FAM129B*, we knocked down endogenous *FAM129B* by RNAi in A549 cells

Table 1 The association between *FAM129B* expression and clinicopathological characteristics

	FAM129B+ (n=77)	*FAM129B*– (n=110)	*P*-value
Age (years)			
<60	38	62	0.344
≥60	39	48	
Gender			
Male	42	69	0.262
Female	35	41	
Tumor size			
≤3 cm	27	54	0.057
>3 cm	50	56	
TNM staging			
I–II	46	92	**<0.001***
III_A	31	18	
Regional lymph node metastasis			
No	30	75	**<0.001***
Yes	47	35	
Histological differentiation			
Well	24	47	0.109
Moderate & Poor	53	63	
Histological type			
Squamous cell cancer	32	44	0.831
Adenocarcinoma	45	66	
Smoking history			
Never	27	37	0.839
Ever	50	73	

Note: *P-value <0.05.

(Figure 3A). Compared with the control group, *FAM129B*-specific siRNA strongly decreased protein levels of MMP2 and cyclin D1 (Figure 3B). Correspondingly, depletion of *FAM129B* significantly inhibited tumor invasion, colony formation and proliferation ability of A549 cells (Figure 3C–G).

FAM129B upregulated the expression of MMP2 and cyclin D1 by facilitating the phosphorylation of FAK at Tyr 397 and Tyr 925

We also investigated the levels of phosphorylated FAK at Tyr 397, Tyr 576 and Tyr 925. Compared with the protein levels of FAK protein, the phosphorylated FAK at Tyr 397 and Tyr 925 were prominently upregulated after overexpression of *FAM129B* (Figure 4A), while they were downregulated after transfection with *FAM129B* siRNA (Figure 4B). Subsequently, PF562271, a specific inhibitor of FAK, was added into the medium of H460 cells with or without *FAM129B* overexpression. Compared with the controls, the elevated expression of FAK (Tyr 397) and FAK (Tyr 925) caused by *FAM129B* overexpression was significantly inhibited by PF562271 (Figure 4C). Interestingly, the increase of MMP2 and cyclin D1 protein levels caused by *FAM129B* overexpression was also reversed by FAK inhibitor incorporation (Figure 4C). Similar tendencies were also observed in Transwell assay, colony formation assay and MTT assay (Figure 4D–H).

Discussion

Our results showed that *FAM129B* played an oncogenic role in NSCLC cells, upregulating the protein levels of MMP2 and cyclin D1 through promoting the activation of FAK by inducing its phosphorylation at Tyr 397 and Tyr 925. Furthermore, the expression of *FAM129B* significantly correlated with advanced TNM stage and lymph node metastasis, and it also predicted poor prognosis of NSCLC patients.

Previous studies demonstrated that *FAM129B* localized to the cytoplasm during an active ERK1/ERK2 MAPK cascade, while it was localized to cell junctions upon inhibition of the MAPK cascade and upon growth to confluence in HeLa cells and mouse embryonic fibroblasts.[3] In our cohort of clinical NSCLC tissue samples, *FAM129B* was localized in the cytoplasm, and no membranous expression was seen. Moreover, we showed that *FAM129B* played an oncogenic role in NSCLCs and correlated with tumor development. It is likely that the expression pattern of *FAM129B* may be tissue specific, therefore requiring more investigation.

In the present study, the expression of *FAM129B* in both online database (mRNA) and tissue samples (protein) showed significant associations with poor prognosis in NSCLC patients. To our knowledge, there has been no published study addressing the prognostic value of *FAM129B*. Therefore, *FAM129B* may be a useful factor for risk stratification and treatment decisions. There is a limitation in the present study: we could not perform an analysis of progression-free survival because of the limited information regarding chemotherapy and/or radiotherapy after surgery. Further studies are required to clarify the prognostic value of *FAM129B*.

Previous studies showed that *FAM129B* promoted tumor invasion and cell motility, but not growth.[3,5–7] Since MMPs and cyclins played crucial roles in regulating invasion and growth, respectively, we analyzed the expression of multiple MMPs and cyclins after overexpression or depletion of *FAM129B*.[18–21] Our results revealed that *FAM129B* facilitated both tumor invasion and tumor proliferation through upregulation of the expression of MMP2 and cyclin D1 in NSCLC

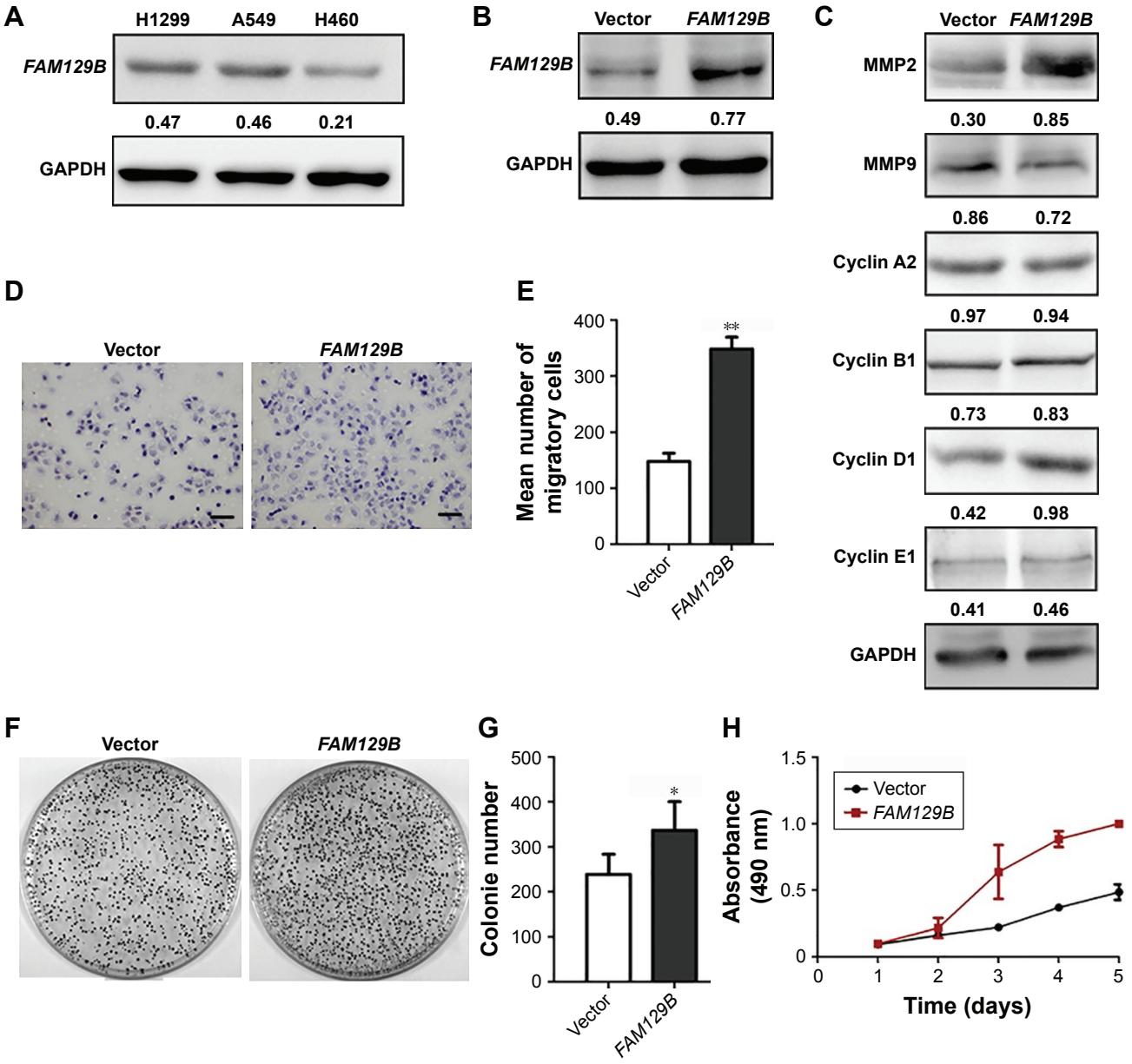


Figure 2 Overexpression of exogenous *FAM129B* upregulated the expression of MMP2 and cyclin D1 and promoted tumor invasion and proliferation of H460 cells.
Notes: (**A**) Western blotting showed that *FAM129B* was highly expressed in H1299 cells and A549 cells and weakly expressed in H460 cells. After overexpressing *FAM129B* in H460 cells (**B**), MMP2 and cyclin D1 were markedly elevated, whereas other proteins including MMP9, cyclin A2, cyclin B1 and cyclin E1 showed no visible changes (**C**). Matrigel assay (**D** and **E**, 400×, scale bar=50 μm), colony formation assay (**F** and **G**) and MTT (**H**) revealed that tumor invasion and proliferation were enhanced by overexpression of *FAM129B*. *$P<0.05$; **$P<0.01$.

cells. Previous studies were primarily based on experiments in cell lines of melanoma, uterine cervix cancer, glioblastoma and human skin.[3,5–7] Despite the fact that one NSCLC cell line was chosen, we postulate that the difference of results between the previous studies and ours was due to different type of cancers or cell lines.

Previous studies focused on the suggestion that *FAM129B* was activated by ERK or that EGFR signaling induced phosphorylation, thereby promoting downstream proteins such as H-Ras and β-catenin.[3,5,7] In our study, we found that *FAM129B* alone could activate FAK signaling. Previous studies showed that both MMP2 and cyclin D1 could be regulated by FAK signaling, thereby contributing to the effect of FAK signaling on cell migration/invasion or cell cycle progression.[22–24] As FAK was also involved in regulating ERK signaling, EGFR signaling and Wnt/β-catenin signaling, our results may shed new light on the downstream signaling of *FAM129B*; certainly, further studies are needed to further clarify these issues.[8–13]

In conclusion, our data suggested that *FAM129B* significantly correlated with advanced TNM staging and positive lymph node metastasis and that it predicted adverse prognosis

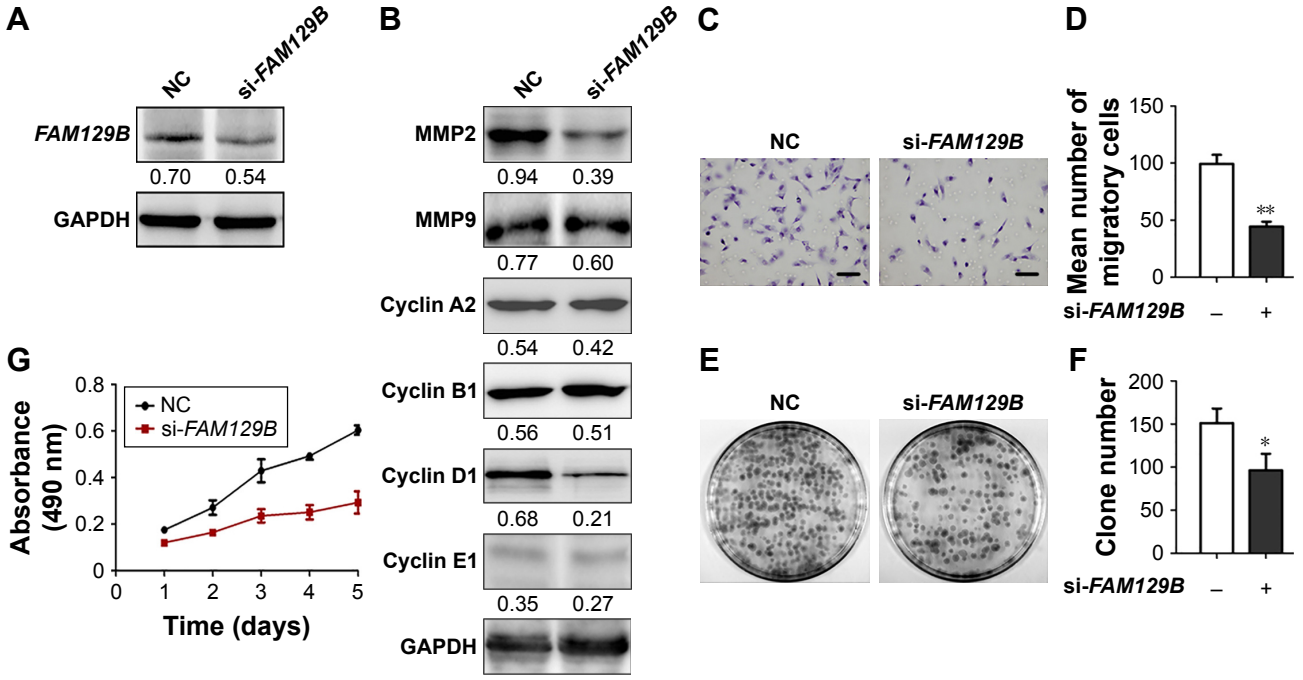


Figure 3 Knockdown of endogenous *FAM129B* downregulated expression of MMP2 and cyclin D1 and inhibited tumor invasion and proliferation in A549 cells.
Notes: After depleting the expression of *FAM129B* (**A**), protein levels of MMP2 and cyclin D1 were prominently downregulated, while other proteins such as MMP9, cyclin A2, cyclin B1 and cyclin E1 showed no significant changes (**B**). Matrigel assay (**C** and **D**, 400×, scale bar=50 μm), colony formation assay (**E** and **F**) and MTT (**G**) revealed that tumor invasion and proliferation were enhanced by overexpression of *FAM129B*. *$P<0.05$; **$P<0.01$.
Abbreviation: NC, negative control.

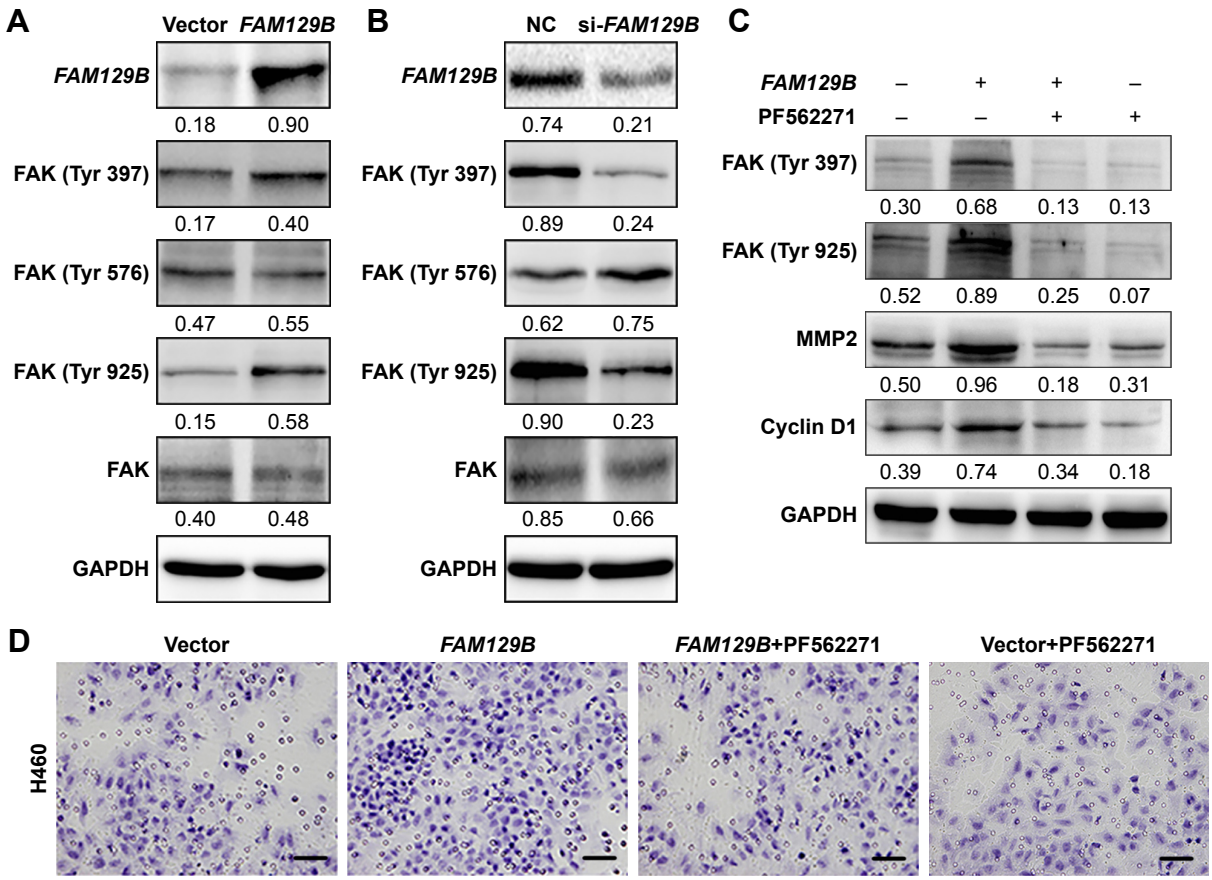


Figure 4 (*Continued*)

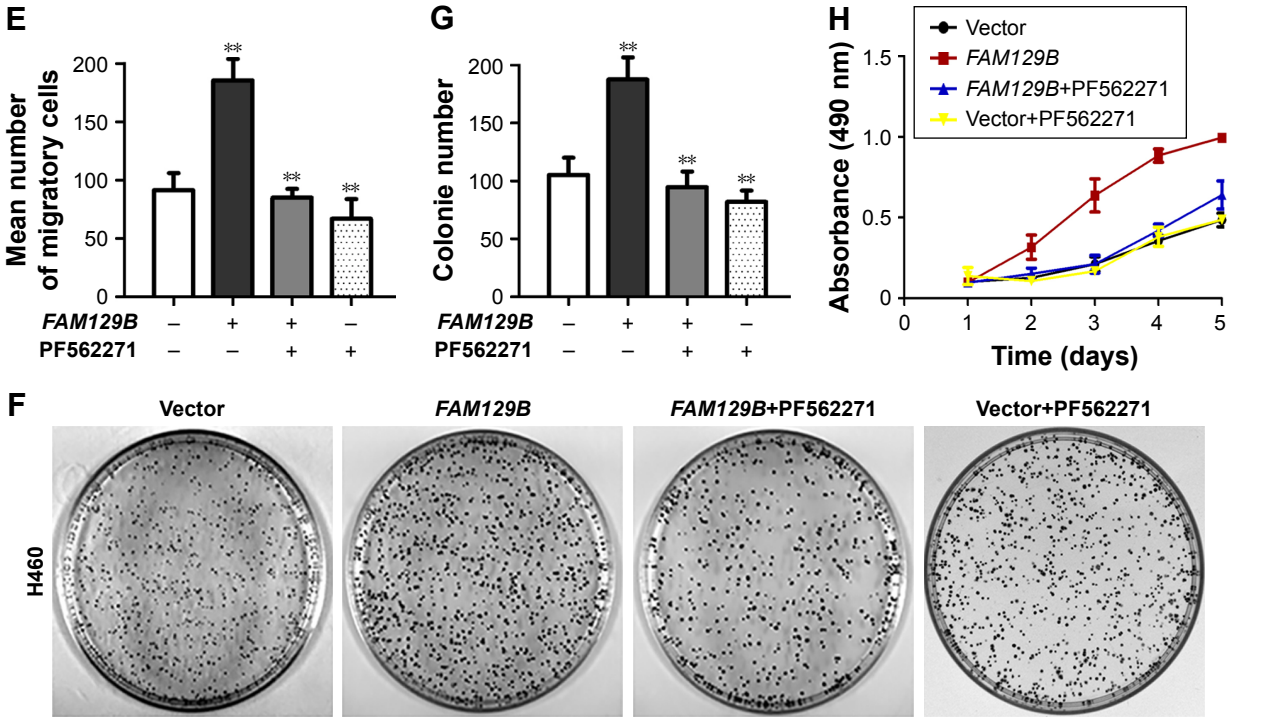


Figure 4 *FAM129B* facilitated tumor invasion and proliferation by promoting the phosphorylation of FAK at Tyr 397 and Tyr 925.
Notes: The phosphorylation of FAK (Tyr 397 and Tyr 925) was increased after overexpression of *FAM129B* in H460 and was decreased following depleting *FAM129B* in A549 (**A** and **B**). The elevation of MMP2 and cyclin D1 protein levels induced by *FAM129B* overexpression was reversed by incorporating FAK inhibitor (PF562271; **C**). The upregulation of tumor invasion (**D** and **E**, 400×, scale bar=50 μm), colony formation (**F** and **G**) and proliferation (**H**) caused by *FAM129B* overexpression were also attenuated by FAK inhibitor incorporation. $^{**}P<0.01$.

in NSCLC patients. *FAM129B* appeared to be an upstream regulator of the FAK signaling pathway, promoting the invasion and proliferation of NSCLC cells by facilitating the phosphorylation of FAK.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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