

Original Article

FAM129A promotes invasion and proliferation by activating FAK signaling pathway in non-small cell lung cancer

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Abstract: Family with sequence similarity 129, member A (FAM129A), also called Niban or C1orf24, was initially identified from a rat model of hereditary renal carcinoma. FAM129A inhibited apoptosis and promoted migration and proliferation in human cancers. However, little is known about the downstream signaling during tumor progression. Our data showed that FAM129A played an oncogenic role in non-small cell lung carcinoma (NSCLC), which up-regulated the protein levels of MMP2 and Cyclin D1 through activating the FAK signaling pathway. Treatment by FAK inhibitor counteracted the increase of MMP2 and Cyclin D1 expression following by FAM129A transfection through attenuating the phosphorylation of FAK. Results of immunohistochemistry revealed that the expression of FAM129A was significantly associated with larger tumor size ($P=0.036$), advanced TNM stage ($P<0.001$), and lymph node metastasis ($P=0.001$). Subsequent Kaplan-Meier analysis indicated that patients with FAM129A expression presented with poorer clinical outcome ($P=0.001$). Taken together, our results suggested that FAM129A may promote tumor proliferation and invasion of NSCLC through facilitating the phosphorylation of FAK and upregulated MMP2 and Cyclin D1. Overexpression of FAM129A may be a prognostic predictor in NSCLC patients.

Keywords: FAM129A, non-small cell lung cancer, FAK signaling, invasion, proliferation

Introduction

Family with sequence similarity 129, member A (FAM129A), also called Niban or C1orf24, was initially identified from a rat model of hereditary renal carcinoma [1, 2]. Subsequent studies showed that FAM129A overexpression inhibited apoptosis and promoted migration and proliferation in human cancers [3-7]. FAM129A expression was controlled by microRNA-106b [6]. The increase in FAM129A phosphorylation, which was induced by UV irradiation, was mediated by the AKT/MDM2/p53 signaling pathway, which has anti-apoptotic effects [7]. However, little is known about the downstream signaling pathway during tumor progression to date.

Several studies have reported that FAM129A was overexpressed in thyroid carcinomas and head and neck squamous cell carcinomas but

decreased in the corresponding normal epithelium [8, 9]. To our knowledge, no published literature has addressed the prognostic value of FAM129A in human cancers, especially human lung cancer.

The purpose of this study was to identify the downstream signaling pathways of FAM129A that regulate carcinogenesis and progression in human non-small cell lung carcinoma (NSCLC), as well as the prognostic value of FAM129A expression in clinical NSCLC tissue samples.

Materials and methods

Cell culture

The cell lines A549 and H460 (from the Shanghai Cell Bank, Shanghai, China) were used in the present study. Cells were cultured

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in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 100 IU/ml penicillin (Sigma, St. Louis, MO, USA), and 100 µg/ml streptomycin (Sigma, St. Louis, MO, USA).

Western blotting

Total protein was quantified using the Bradford method [10]. Fifty micrograms of total cellular protein samples was separated on 10% SDS-PAGE and immunoblotted with the following primary antibodies: FAM129A (1:200, Abcam, ab64903, Cambridge, MA, USA), GAPDH (1:5000, Sigma, St. Louis, MO, USA), Cyclin A2, Cyclin B1, Cyclin D1, Cyclin E1, MMP2, MMP9, p-FAK (Tyr397), p-FAK (Tyr576), p-FAK (Tyr925), and FAK (1:1000; Cell Signaling Technology, Danvers, MA, USA) antibodies. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL, Pierce, Rockford, IL, USA).

Plasmid transfection and small interfering RNA treatment

The plasmids used in the present study (pCMV6-ddk-myc and pCMV6-ddk-myc-FAM129A) were purchased from Origene (Rockville, MD, USA). FAM129A-siRNA (sc-78648) and NC-siRNA (sc-37007) were purchased from Santa Cruz Biotechnology. Transfection was carried out using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

MTT

Cells were seeded in 96-well plates at approximately 3000 cells per well 24 h after transfection. After the cells were incubated for the indicated times, 20 µl of MTT (thiazolyl blue) solution (5 mg/ml) was added to each well and incubated at 37°C for 4 h. After the medium was removed, the resulting MTT formazan was solubilized in 150 µl of DMSO. The absorbance was read at a test wavelength of 490 nm, and samples were assayed in triplicate.

Colony formation assay

After the indicated transfection, cells were seeded into 6-cm cell culture dishes (1000 cells 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 number of colonies with more than 50 cells was counted. All experiments were carried out in triplicate.

Matrigel invasion assay

Cell invasion assays were performed with 20 µl matrigel (1:3 dilution; BD Bioscience, 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 h, 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 sample was assayed in triplicate.

Patient study

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. The patients did not receive neoadjuvant radiotherapy or chemotherapy before surgery. In addition, after surgery, all patients received standard chemotherapy according to the NCCN guidelines. Sixty-eight of the 187 cases provided samples of the corresponding noncancerous tissues. Complete follow-up data were obtained from all 187 lung cancer cases. The survival time was defined as the time from the day of surgery to the day of death due to recurrence or metastasis or the end of follow-up. The median age in 187 patients was 60 years old (range from 29 years old to 83 years old). Of the 187 patients, 87 patients were older than 60 years. Histological diagnosis and grading were made according to the 4th edition of the World Health Organization (WHO) classification of tumors of the lung [11]. There were 76 squamous cell lung carcinoma patients and 111 lung adenocarcinoma patients 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 (AJCC) TNM Staging System for Lung Cancer [12]. The tumors included 138 stage I-II cases and 49 stage III cases. Lymph node metastases were present in 82 of the 187 cases.

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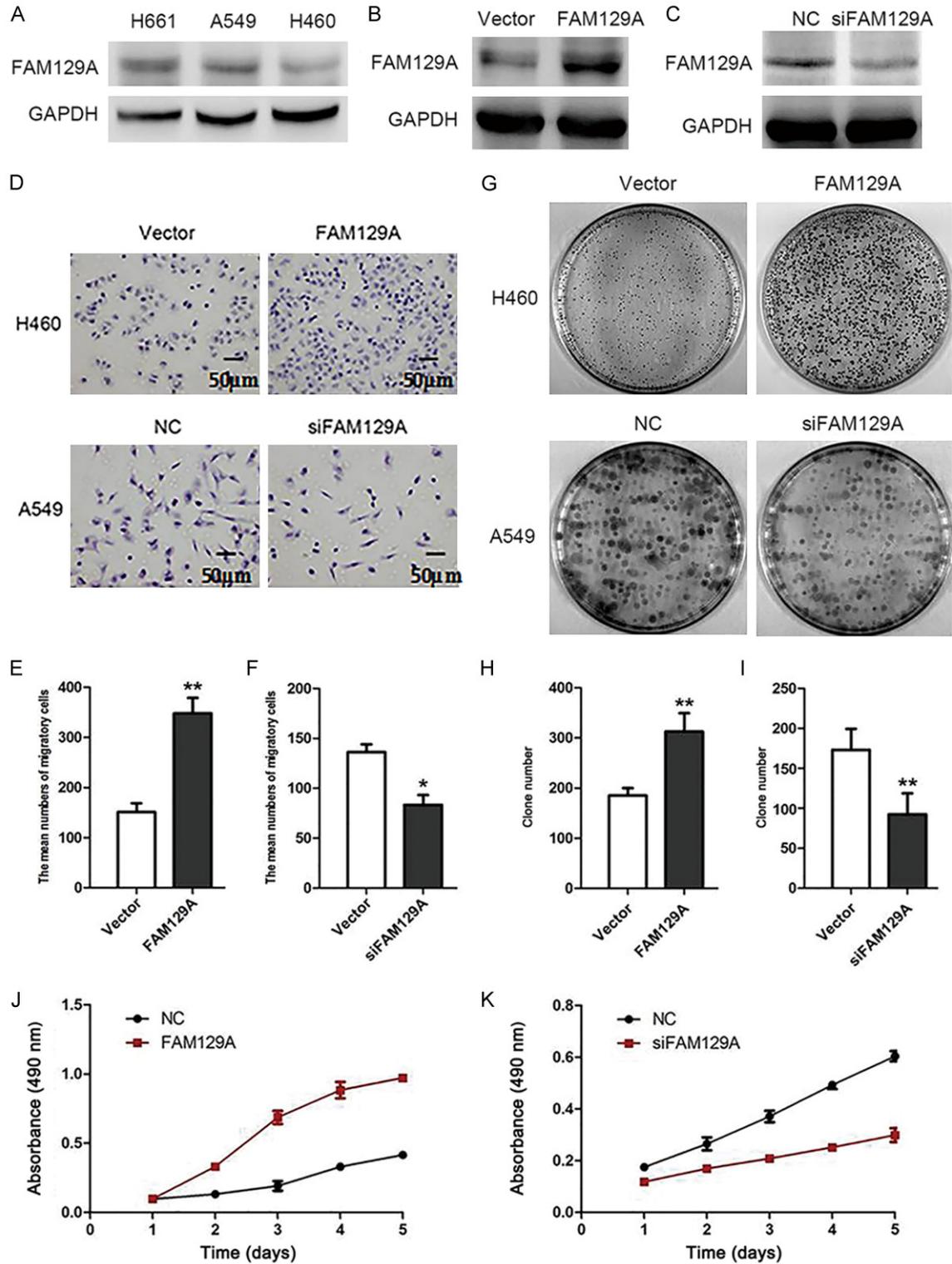


Figure 1. FAM129A promoted tumor invasion and proliferation of NSCLC cells. Protein levels of FAM129A in H661, A549 and H460 cells (A). After transfection with a FAM129A cDNA plasmid in H460 cells (B) and transfection with FAM129A siRNA in A549 cells (C), western blotting analysis was used to evaluate the efficiency of transfection. Matrigel invasion assays (D-F, magnification, 400×; E, F, y-axis: the mean number of migratory cells), colony formation assays (G-I; H-I, y-axis: clone number) and MTT assays [J-K, x-axis: y-axis: time (days), y-axis: absorbance (490 nm)] were employed after transfection with the FAM129A plasmid in H460 cells and siRNA in A549 cells. * $P < 0.05$; ** $P < 0.01$.

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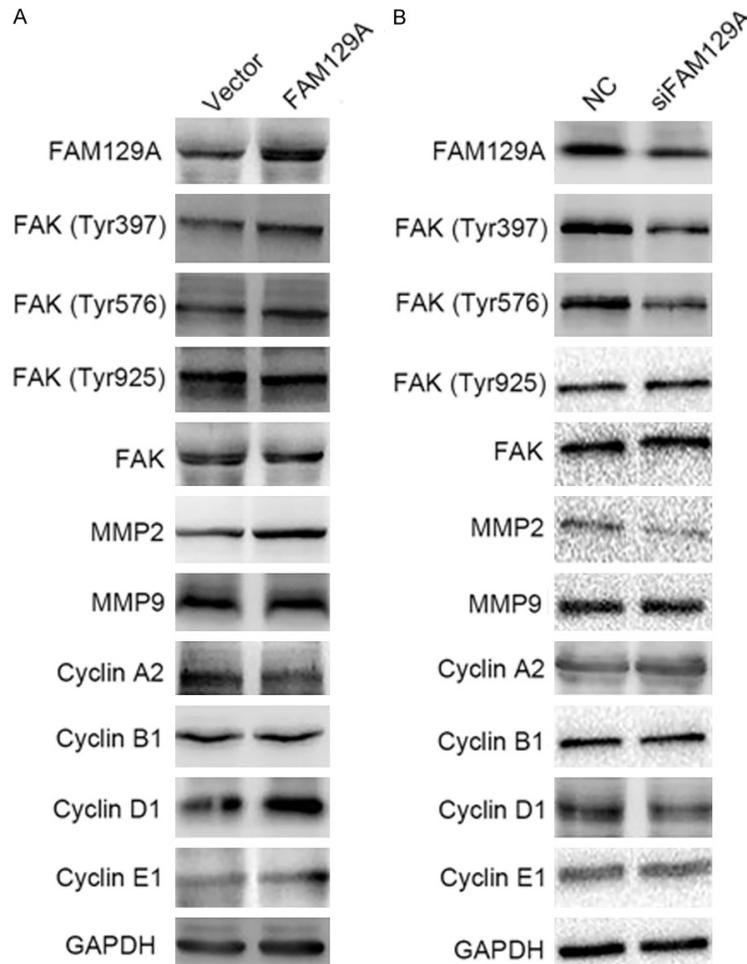


Figure 2. FAM129A upregulated the expression of MMP2, Cyclin D1 and the phosphorylation of FAK. After overexpression (A) or depletion of (B) FAM129A (129A), the levels of FAM129A, p-FAK (Tyr397), p-FAK (Tyr576), p-FAK (Tyr925), FAK, MMP2, MMP9, Cyclin A2, Cyclin B1, Cyclin D1 and Cyclin E1 were tested using western blotting. GAPDH was used as a control.

Immunohistochemistry

Immunohistochemistry staining was performed using the streptavidin-peroxidase method according to the manufacturer's instructions (Ultrasensitive; MaiXin, Fuzhou, China). The sections were incubated with an anti-FAM129A antibody (mouse anti-human; dilution, 1:200; Abcam, ab64903, Cambridge, MA, USA) at 4°C overnight, followed by the biotinylated goat anti-mouse IgG secondary antibody. 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 the staining intensity and percentage to obtain a final score (range 0-12). When tumors had a score ≥ 4 , they had positive FAM129A expression. When tumors had a score < 4 , they were defined as negative.

Statistical analysis

SPSS version 22.0 for Windows (SPSS, Chicago, IL, USA) was employed for statistical analyses. Pearson's chi-square test was selected to evaluate possible associations between FAM129A and clinicopathological features. Kaplan-Meier survival analyses were performed in 187 specimens. Log-rank tests were carried out to compare the significance. Mann-Whitney U tests were chosen to evaluate the significance of the image analysis results of the invasive assay, colony formation assay and MTT test. $P < 0.05$ was defined as a significant difference.

Results

FAM129A facilitates tumor invasion and proliferation in NSCLC cells

We evaluated the protein levels of FAM129A in H661, A549 and H460 cells (**Figure 1A**). We selected H460 cells, which had low FAM129A expression, to perform cDNA transfection and selected A549 cells, which had high FAM129A expression, to perform RNAi (**Figure 1B, 1C**). Subsequent matrigel invasion assays revealed that FAM129A upregulation strongly promoted tumor invasion in H460 cells ($P < 0.01$), whereas depletion of FAM129A significantly inhibited tumor invasion in A549 cells ($P < 0.05$; **Figure 1D-F**). Moreover, colony formation assays and MTT assays showed that transfection of FAM129A cDNA significantly facilitated tumor

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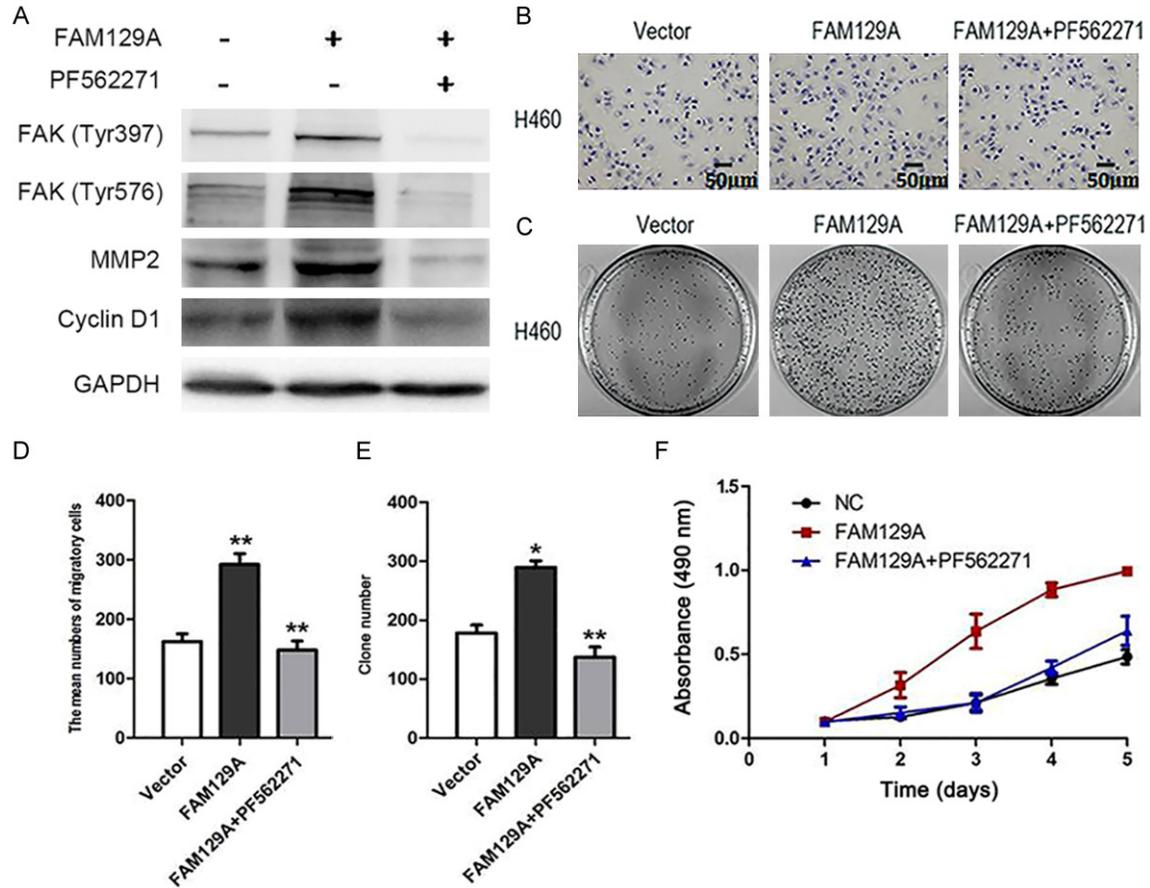


Figure 3. FAM129A upregulated the expression of MMP2 and Cyclin D1 by facilitating the phosphorylation of FAK at Tyr397 and Tyr576. The upregulation of MMP2 and Cyclin D1 caused by FAM129A plasmid transfection was counteracted by addition of PF562271, an inhibitor of FAK signaling (A). The increased tumor invasion (B, and D, magnification, 400 \times ; D, y-axis: the mean number of migratory cells) and proliferation [C, E and F; E, y-axis: clone number; F, x-axis: time (days), y-axis: absorbance (490 nm)] by FAM129A overexpression were also reversed after adding a FAK inhibitor. * $P < 0.05$, ** $P < 0.01$.

proliferation in H460 cells ($P < 0.01$), while FAM129A RNAi dramatically decreased tumor proliferation in A549 cells ($P < 0.01$; **Figure 1G-K**).

FAM129A upregulated the levels of MMP2, Cyclin D1 and phosphorylated FAK

Since MMPs and cyclins play important role in regulating tumor invasion and proliferation, we next evaluated their protein levels. We found that MMP2 and Cyclin D1 were significantly upregulated by FAM129A cDNA transfection, whereas these proteins were downregulated after FAM129A knockdown. Other proteins, including MMP9, Cyclin A2, Cyclin B1 and Cyclin E1, showed no significant changes. Meanwhile, we also found that p-FAK (Tyr397) and p-FAK (Tyr576) were increased by FAM129A overex-

pression and were inhibited by FAM129A depletion. Total FAK and p-FAK (Tyr925) showed no significant changes (**Figure 2A, 2B**).

FAM129A upregulated the expression of MMP2 and Cyclin D1 by promoting the phosphorylation of the FAK signaling pathway

To clarify whether FAM129A upregulated the expression of MMP2 and Cyclin D1 through the FAK signaling pathway, we added PF562271, an inhibitor of the FAK signaling pathway, into the medium with or without transfection with the FAM129A cDNA plasmid. Western blotting results showed that the elevation of the protein levels of MMP2 and Cyclin D1, as well as the levels of p-FAK (Tyr397) and p-FAK (Tyr576) after FAM129A overexpression, were attenuated by PF562271 incorporation (**Figure 3A**).

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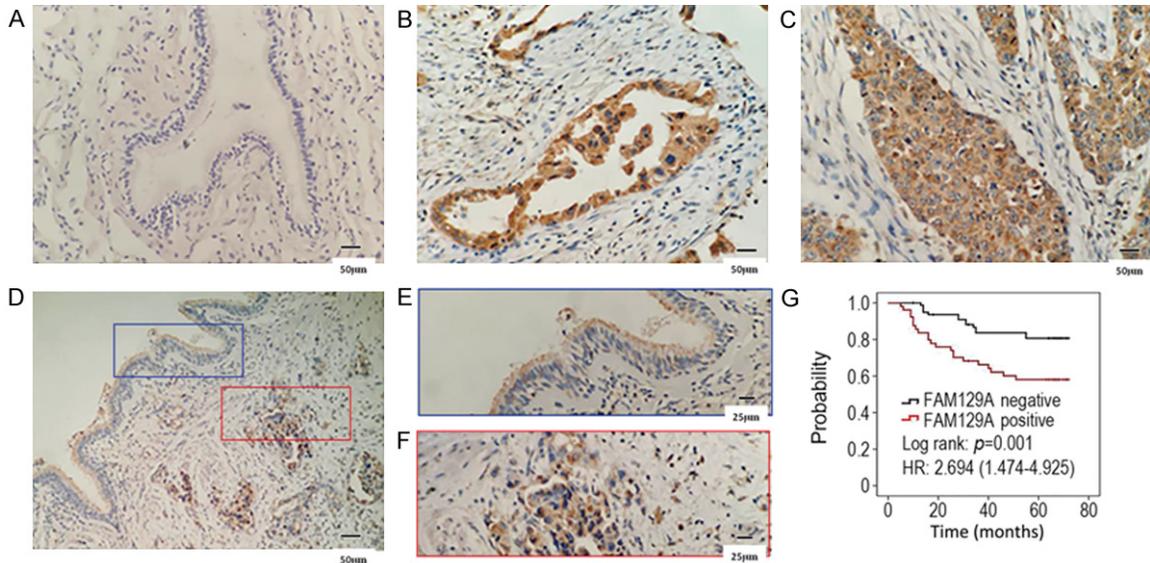


Figure 4. FAM129A expression in NSCLC tissue samples. FAM129A was negatively or weakly expressed in normal bronchial epithelium (400 \times , A) and alveoli (400 \times , B), whereas it showed strong positive expression in the cytoplasm of lung adenocarcinoma (400 \times , C) and squamous cell lung carcinoma (400 \times , D). The expression of FAM129A was higher in NSCLC samples than in the corresponding noncancerous tissues (100 \times , E; 400 \times , inset, F and G). Kaplan-Meier analysis showed that the overall survival of NSCLC patients with FAM129A overexpression was significantly shorter than those without FAM129A expression [G, x-axis: time (months), y-axis: probability].

Correspondingly, the increase in the number of migratory cells and the colony formation caused by FAM129A transfection were also attenuated by the FAK inhibitor (Figure 3B-F).

FAM129A was upregulated in NSCLC samples, significantly correlated with tumor progression and indicated poor overall survival

Using immunohistochemistry, we observed that FAM129A was positively expressed in 55.6% (104/187) of the NSCLC samples. FAM129A showed strong to medium staining in the cytoplasm, whereas it only presented weak or negative expression in normal bronchial epithelium and alveoli (Figure 4A-F). Positive FAM129A expression was significantly associated with large tumor size ($P=0.036$), advanced TNM staging ($P<0.001$) and positive lymph node metastasis ($P=0.001$). FAM129A showed no significant correlation with other clinical pathological features, including age, gender, histological differentiation, histological type and smoking status (Table 1). Kaplan-Meier analysis showed that the overall survival of patients with FAM129B expression was significantly poorer than those without FAM129B expression ($P=0.001$; Figure 4G).

Discussion

The present study revealed that FAM129A promoted tumor invasion and proliferation by upregulating the expression of MMP2 and Cyclin D1, which was due to enhanced phosphorylation of FAK at Tyr 397 and Tyr 576. Overexpression of FAM129A was associated with tumor progression and predicted poor survival of NSCLC patients.

Previous studies have demonstrated that overexpression of FAM129A promoted migration and proliferation in several human cancers [3-7]. The results of the present study were similar to previous studies showing that FAM129A facilitated tumor invasion and proliferation in NSCLC cells. Ji et al. demonstrated that following induction of ultraviolet irradiation, FAM129A was phosphorylated in an AKT signaling-dependent manner, which thereby regulated the MDM2/p53 interaction and inhibited apoptosis [7]. Moreover, FAM129A was recently identified to be a new downstream molecule of ATF4 which indicated that it may play a role in regulating cancer progression and treatment resistance [29420561]. However, no studies have addressed the downstream signaling pathway of FAM129A, especially in circumstances with-

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Table 1. Association between FAM129A expression and clinicopathological characteristics

	FAM129A+ (n=104)	FAM129A- (n=83)	P
Age			
≤60 years	57	43	0.683
>60 years	47	40	
Gender			
Male	66	45	0.201
Female	38	38	
Tumor size			
≤3 cm	38	43	0.036
>3 cm	66	40	
TNM staging			
I-II	65	73	<0.001
III _A	39	10	
Regional lymph node metastasis			
No	47	58	0.001
Yes	57	25	
Histological differentiation			
Well	39	32	0.883
Moderate & Poor	65	51	
Histological type			
Squamous cell cancer	40	36	0.497
Adenocarcinoma	64	47	
Smoking history			
Never	33	31	0.421
Ever	71	52	

out environmental stress. Our results indicated that FAM129A may promote tumor progression by upregulating the expression of MMP2 and Cyclin D1. More importantly, we also found that the effect of FAM129A on MMP2 and Cyclin D1 was due to enhanced phosphorylation of FAK, the key protein of the FAK signaling pathway. As is known to us all, FAK plays a crucial role in the regulation of cell cycle progression and tumor invasion which induces the expression of multiple cyclins and MMPs including Cyclin D1 and MMP2 [11739801, 15557280, 17185517, 23-807215]. Previous studies and ours indicated that FAM129A may contribute to the regulation of tumor proliferation and invasion through the FAK signaling pathway.

Additionally, we employed immunohistochemical analysis to investigate the clinicopathological significance of FAM129A. The present study suggested that positive FAM129A expression was significantly correlated with large tumor size, advanced TNM staging and positive lymph

node metastasis. Several previous studies have demonstrated that FAM129A was upregulated in thyroid carcinomas and head and neck squamous cell carcinomas but was decreased in the corresponding normal epithelium [8, 9]. However, to our knowledge, no studies have investigated the clinicopathological significance in NSCLC. Therefore, FAM129A may be a useful factor for risk stratification and a potential therapeutic target for lung cancer treatment. One limitation of the present study was the lack of data on progression-free survival because of the limited information on chemotherapy and/or radiotherapy after surgery. Further studies are required to elucidate the prognostic value of FAM129A.

Conclusion

Our data suggested that FAM129A was significantly correlated with large tumor size, advanced TNM stage, and positive lymph node metastasis and predicted adverse clinical outcomes of NSCLC patients. FAM129A appears to be an upstream regulator of FAK signaling pathway and promotes the invasion and proliferation of NSCLC cells by facilitating the phosphorylation of FAK and upregulating MMP2 and Cyclin D1.

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Disclosure of conflict of interest

None.

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