

Family with sequence similarity 83, member B is a predictor of poor prognosis and a potential therapeutic target for lung adenocarcinoma expressing wild-type epidermal growth factor receptor

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Abstract. Lung adenocarcinoma (ADC) patients with tumors that harbor no targetable driver gene mutation, such as epidermal growth factor receptor (*EGFR*) gene mutations, have unfavorable prognosis, and thus, novel therapeutic targets are required. Family with sequence similarity 83, member B (*FAM83B*) is a biomarker for squamous cell lung cancer. *FAM83B* has also recently been shown to serve an important role in the *EGFR* signaling pathway. In the present study, the molecular and clinical impact of *FAM83B* in lung ADC was investigated. Matched tumor and adjacent normal tissue samples were obtained from 216 patients who underwent complete lung resection for primary lung ADC and were examined for *FAM83B* expression using cDNA microarray analysis. The associations between *FAM83B* expression and clinicopathological parameters, including patient survival, were examined. *FAM83B* was highly expressed in tumors from males, smokers and in tumors with wild-type *EGFR*. Multivariate analyses further confirmed that wild-type *EGFR* tumors were significantly positively associated with *FAM83B* expression. In survival analysis, *FAM83B* expression was associated with poor outcomes in disease-free survival and overall survival, particularly when stratified against tumors with wild-type *EGFR*. Furthermore, *FAM83B* knockdown

was performed to investigate its phenotypic effect on lung ADC cell lines. Gene silencing by *FAM83B* RNA interference induced growth suppression in the HLC-1 and H1975 lung ADC cell lines. *FAM83B* may be involved in lung ADC tumor proliferation and can be a predictor of poor survival. *FAM83B* is also a potential novel therapeutic target for ADC with wild-type *EGFR*.

Introduction

Cancers are among the leading causes of mortality worldwide, with 8.2 million cancer-related deaths in 2014. Among different cancers, lung cancer has the highest mortality rate with 1.59 million deaths; this is more than twice the number of hepatocellular carcinoma deaths, which is the second most fatal form of cancer (1). Lung cancer patients have poor prognosis and at initial hospital visit are often diagnosed at an advanced stage, beyond the possibility of surgical intervention (2).

Cytotoxic chemotherapeutic agents are usually administered for advanced lung cancer therapy and are chosen according to histological subtype. Recently, molecular targeted agents and immune checkpoint inhibitors have been developed and have been in clinical use since the 2000s (3). In non-small cell lung cancer, including adenocarcinoma (ADC), which represents 40% of all lung cancers, several types of driver gene mutation that promote oncogenic transformation and tumor growth by aberrant activation of proliferation signaling pathways have been identified (4). These driver genes are proposed as novel candidates for molecular targeted therapy. Epidermal growth factor receptor (*EGFR*) activating mutations lead to auto-phosphorylation and promote *EGFR*/*KRAS*/*MEK*/*ERK* signaling (5). Aberrant anaplastic lymphoma kinase (*ALK*) protein, produced by an *ALK* fusion gene, drives *MEK*/*ERK* and *PI3K*/*Akt* pathways (6). *EGFR*-tyrosine kinase inhibitor (*TKI*) and an *ALK* inhibitor against the above two aberrant

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signals prevent tumor progression with a high response rate of 56.0-70.0%, while cytotoxic chemotherapy or immune checkpoint inhibition are successful only in 19.0-34.1% of cases (7-11). Thus, these precision medicines based on genetic or molecular features of lung ADC can provide therapy with a high response rate and fewer adverse effects (7-11). However, 29.2-40.0% of lung ADC patients have no targetable genetic features (4,12,13). There is an urgent need to discover novel biomarkers and therapeutic targets, and studies are ongoing to establish targeted therapy for rare driver gene mutations of malignancy (4).

We have identified and reported family with sequence similarity 83, member B (*FAM83B*) as a novel diagnostic and prognostic marker for lung squamous cell cancer (SqCC) (14). Comprehensive gene expression analysis using cDNA microarray analysis and immunohistochemistry showed lung SqCC expressed higher levels of *FAM83B* compared with lung ADC or adjacent normal lung tissue, and correlated with patient prognosis (14). *FAM83B* is also overexpressed in several other types of cancer, such as breast, ovary, bladder, and lung, and is associated with tumor proliferation (15). Additionally, induction of *FAM83B* in human mammary epithelial cells resulted in neoplastic growth by increasing mitogen-activated protein kinase (MAPK) signaling, while induction in human breast cancer cell lines resulted in TKI resistance (15). Aberrant *EGFR* signals and downstream signaling play an important role in targeted therapy for lung ADC; therefore, we assumed that *FAM83B* also correlates with tumor oncogenesis and growth in lung ADC. Here, we show an association between *FAM83B* expression in lung ADC and demographics and clinicopathological features.

Materials and methods

Ethics statement. This study was conducted with approval of the Ethics Committee of Fukushima Medical University (approval no. 2775). The participants' human rights and welfare were defended in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

Case selection. We identified 216 patients who underwent lung resection at Fukushima Medical University between January 2008 and June 2015 and who were pathologically diagnosed as primary lung ADC. *FAM83B* mRNA levels were determined in matched lung tumor and adjacent normal lung tissue and compared with clinical features and prognosis. The data collected were; age at surgery, sex, smoking history, presence of activating *EGFR* mutation in the tumor, histological ADC subtype, tumor size, lymph node metastasis, distant metastasis, pleural invasion, lymphovascular invasion, vascular invasion, date of surgery, date of recurrence, last confirmed survival date, and date of death. Disease-free survival (DFS) was defined as the time from surgery to the first recurrence or death. Overall survival (OS) was defined as the time from surgery to death. To ensure a sufficient observation period, prognostic analyses were performed for patients who underwent complete resection up to December 2013, and who were followed up for 5 years. Patients who had an additional advanced malignant history within 5 years before lung resection, died of postoperative complications, or who were

followed up for less than 12 months were excluded. In total, 126 patients were analyzed.

Comprehensive gene expression analysis. Matched tumor and adjacent normal lung tissue samples, 7 mm³ in size, were excised from surgical specimens and frozen in liquid nitrogen. Frozen samples were processed for total RNA extraction using ISOGEN (Nippon Gene, Tokyo, Japan). As a control, common reference RNA was prepared by mixing equal amounts of total RNA extracted from 22 human cell lines to reduce cell type-specific expression bias (16). Synthetic 80mer polynucleotide probes representing 14,400 human transcripts (MicroDiagnostic, Tokyo, Japan) were arrayed using a custom arrayer. Labeled cDNA was synthesized from 5 μ g of sample RNA using SuperScript II (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and Cyanine 5-dUTP (PerkinElmer, Inc., Waltham, MA, USA), while Cyanine 3-dUTP (PerkinElmer, Inc.)-labeled cDNA was synthesized from 5 μ g of human common reference RNA. Hybridization was performed using a Labeling and Hybridization kit (Microdiagnostic). Signals were measured using a GenePix 4000B Scanner (Axon Instruments; Molecular Devices, LLC, Sunnyvale, CA, USA), and then processed into primary expression ratios (ratio of Cyanine-5 intensity of each sample to the Cyanine-3 intensity of human common reference RNA). Each ratio was normalized by multiplication with normalization factors using GenePix Pro 3.0 software (Axon Instruments; Molecular Devices, LLC). The primary expression ratios were converted into log₂ fold changes (designated log ratios). An expression ratio of 1 (i.e., log ratio of 0) was assigned to spots that exhibited fluorescence intensities under detection limits, and we included these in the calculation of signal averages. Data were processed using Microsoft Excel software (Microsoft Corporation, Redmond, WA, USA) and the MDI gene expression analysis software package (MicroDiagnostic, Inc., Tokyo, Japan). mRNA expression data related to *FAM83B* were extracted for this study.

Preparation of cell lines. HLC-1 cells were purchased from Riken Cell Bank (Saitama, Japan). NCI-H2347, NCI-H1975 and MCF-7 cells were purchased from American Type Culture Collection (Manassas, VA, USA). HLC-1 cells were grown in Ham's F12 medium (087-08335; Wako Pure Chemical Industries, Ltd., Osaka, Japan). NCI-H2347 and NCI-H1975 cells were grown in RPMI-1640 medium (R8758; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), MCF-7 cells were grown in DMEM medium (Wako Pure Chemical Industries, Ltd.). All media contained 10% fetal bovine serum (Nichirei Biosciences, Tokyo, Japan) and 1% penicillin-streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂.

siRNA preparation. siRNAs against *FAM83B* were purchased (Hs_FAM83B_8 and Hs_FAM83B_9 FlexiTube siRNA; Qiagen GmbH, Hilden, Germany). Sequences of siRNAs were: Hs_FAM83B_8 (siRNA-8): 5'-CAGGAACGAGTTTCA GACTTT-3', and Hs_FAM83B_9 (siRNA-9): 5'-TCCCGTTAT TTGACAACCTCAA-3'.

As a negative control, AllStars negative control siRNA (Qiagen GmbH,) was used. As a positive control, AllStars Hs Cell Death siRNA (Qiagen GmbH,) was used.

siRNA transfection efficiency assay. For 96-well siRNA transfections, 0.3 μ l of Lipofectamine RNAiMAX (Thermo Fisher Scientific, Inc.) in 10 μ l of serum-free Opti-MEM (Thermo Fisher scientific, Inc.) was added to preplated siRNAs in each well and incubated for 5 min at room temperature. MCF7, HLC-1, H2347, or H1975 cells were added at 1.0×10^4 to each well. After incubation for 72 h, 10 μ l of Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) was added and absorbance at 450 nm measured 4 h later using a Multiskan GO (Thermo Fisher scientific, Inc.). Each test was replicated three times.

RNA interference and cell proliferation assay. According to a transfection efficiency test, RNA interference experiments (RNAi) were performed using siRNA-9. siRNA-9 (final concentration of 2.5 nM) and 7.5- μ l Lipofectamine RNAiMAX were mixed in 100 μ l of serum-free Opti-MEM in a microcentrifuge tube, then added within 20 min to cells in 6-well plates. RNAi was performed using 1×10^5 cells/well for HLC-1, and 5×10^4 cells/well for H1975 and replicated three times. To determine the silencing effects of siRNA against *FAM83B*, cell numbers were counted after transfection using Clone select imager (Molecular devices Japan, Tokyo, Japan).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from cell lines using TRIzol reagent (Thermo Fisher Scientific, Inc.) and a PureLink RNA Mini Kit (Thermo Fisher Scientific, Inc.) according to the manufacturers' instructions. RNA quantity was assessed using a Nanodrop UV-Vis Spectrophotometer (Thermo Fisher Scientific, Inc.), and samples with a 260/280 nm absorbance ratio of 1.8 or larger were adopted as eligible for RT-PCR. Relative mRNA expression was determined by RT-PCR. One-step RT-qPCR using a Taqman RNA-to-CT 1-Step kit (Thermo Fisher Scientific, Inc.) was performed according to the manufacturer's instructions. To detect *FAM83B* mRNA, Taqman gene expression assays (Hs00289694_m1; Thermo Fisher Scientific, Inc.) were used. As an endogenous control, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, Hs02758991_g1; Thermo Fisher Scientific, Inc.) was used. Forty cycles of amplification were performed for each triplicated sample. The $\Delta\Delta$ Cq method was applied for quantitative evaluation (17). Cycle quantification (Cq) values were calculated by Step One Plus software version 2.3 (Thermo Fisher Scientific, Inc.). Δ Cq was defined as the difference between *FAM83B* Cq and *GAPDH* Cq, and $\Delta\Delta$ Cq was defined as the ratio to the endogenous control sample. Signals undetected after 40 cycles were considered to have an expression of zero.

SDS-PAGE. Cell lysates were prepared by homogenization of cells in RIPA lysis buffer (SC-24948; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), using a Polytron homogenizer (Sonifier SFX250; Emerson Electric Co., St. Louis, MO, USA) at 4°C. After centrifugation at 10,000 x g for five min at 4°C, supernatants were mixed with an equal volume of Sample buffer (2X Laemmli Sample

Buffer; Bio-Rad Laboratories, Inc., Hercules, CA, USA). 2-mercaptoethanol (Bio-Rad; 200:1) was then added and samples heated for three min at 100°C. Five micrograms of each sample were then loaded on a polyacrylamide gel (Supersep ace 5-20%; Wako Pure Chemical Industries, Ltd.) and electrophoresis was performed to separate proteins (18).

Western blotting. Proteins were transferred to a polyvinylidene difluoride membrane (Immobilon; Merck KGaA) in Towbin transfer buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS, 20% methanol) (19). The membranes were then blocked with 5% skimmed milk in PBS (0.137 M NaCl, 2.6 mM KCl, 1.8 mM KH_2PO_4 , 8.1 mM $\text{Na}_2\text{HPO}_4/12\text{H}_2\text{O}$) and incubated overnight in primary antibody solution at 4°C. Anti-FAM83B antibodies (1:1,000; PA5-28548; ThermoFisher scientific, or 1:2,000; HPA031464; Atlas Antibodies AB, Stockholm, Sweden) or an anti-GAPDH antibody (1:2,500; no. 2118; Cell Signaling Technology, Inc., Danvers, MA, USA) were used as primary antibodies. Membranes were then incubated with secondary antibody (anti-rabbit IgG, Horseradish Peroxidase-Linked species-specific whole antibody (1:20,000; GE Healthcare UK Ltd., Amersham, UK). The chemiluminescent signals were captured with the ImageQuant LAS 4000 system (GE Healthcare UK Ltd.) using ECL select Western Blotting Detection Reagent (GE Healthcare UK Ltd.) according to the manufacturer's instructions.

Statistical analysis. Statistical analyses were performed using SPSS 21.0 (IBM Corp., Armonk, NY, USA). The patient cohort was divided into two subgroups according to high or low *FAM83B* expression with the log ratio of zero as the boundary. Patients were divided into two groups according to median age. Tumor (T), Nodes (N), and Metastasis (M) (TNM) factors of lung cancer were classified according to the Union for International Cancer Control 7th edition (20). T factor was not adopted but tumor size and pleural invasion were. Continuous variables were compared by two-tailed t-tests or one-way ANOVA, and categorical variables were compared by the Chi-square test or Fisher's exact test, as appropriate. Multivariate analyses using a binary logistic regression model were performed to evaluate independent predictors of *FAM83B* expression. DFS and OS were estimated using the Kaplan-Meier method, and survival curves were compared using log-rank tests. Variables that were suitable for a Cox proportional hazards univariate model with significance were analyzed by a multivariate model to adjust for potential confounders. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

FAM83B is highly expressed in ADC with wild type-EGFR. This study included 119 male and 97 female patients, with a mean age of 68.5 years (range 26-87 years). Up to 111 (51.4%) were current or former smokers, and 118 (54.6%) had wild-type *EGFR* ADC. *FAM83B* tended to be expressed at higher levels in solid subtypes while lower *FAM83B* expression was observed in lepidic pattern tumors that were less aggressive. Mean tumor size was 2.9 cm (range 0.8-14.0). The clinicopathological characteristics of patients according to

Table I. Clinicopathological and genetic features of lung adenocarcinoma patients according to *FAM83B* expression in tumor tissue.

Variable	<i>FAM83B</i> expression		P-value
	High (n=55) (%)	Low (n=161) (%)	
Age			0.271
≤69 years old	25 (45.5)	87 (54.0)	
≥70 years old	30 (54.5)	74 (46.0)	
Sex			0.006
Male	39 (70.9)	80 (49.7)	
Female	16 (29.1)	81 (50.3)	
Smoking history			0.006
Never smoker	18 (32.7)	87 (54.0)	
Former or current smoker	37 (67.3)	74 (46.0)	
EGFR gene			<0.001
Wild-type	43 (78.2)	75 (46.6)	
Mutant	12 (21.8)	86 (53.4)	
Pathological subtype			<0.001
Lepidic	9 (16.4)	36 (22.4)	
Papillary	27 (49.1)	99 (61.5)	
Acinar	5 (9.1)	12 (7.5)	
Solid	7 (12.7)	10 (6.2)	
Other variants	7 (12.7)	4 (2.4)	
Tumor size			0.164
≤3 cm	29 (52.7)	102 (63.4)	
>3 cm	26 (47.3)	59 (36.6)	
LN metastasis			0.706
N0	46 (83.6)	131 (81.4)	
N1/N2/N3	9 (16.4)	30 (18.6)	
Distant metastasis			1.000
M0	54 (98.2)	159 (98.8)	
M1	1 (1.8)	2 (1.2)	
Pleural invasion			0.598
(-)	39 (70.9)	120 (74.5)	
(+)	16 (29.1)	39 (24.2)	
Lymphatic invasion			0.184
(-)	44 (80.0)	114 (70.8)	
(+)	11 (20.0)	47(29.2)	
Vascular invasion			0.217
(-)	37 (67.3)	122 (75.7)	
(+)	18 (32.7)	39 (24.2)	

Pathological subtype is the International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society adenocarcinoma classification. *FAM83B*, family with sequence similarity 83, member B; *EGFR*, epidermal growth factor receptor; LN, lymph node.

FAM83B expression in tumor tissue are summarized in Table I. Univariate analysis showed that higher *FAM83B* expression correlates with males (P=0.007), smoking history (P=0.007), and wild-type *EGFR* tumors (P<0.001). Multivariate analysis showed wild-type *EGFR* tumors correlate with *FAM83B* expression (P<0.001) (Table II).

Correlation between FAM83B expression obtained from cDNA microarray analysis and EGFR mutation in lung ADC. The mean *FAM83B* expression in adjacent normal lung tissue (n=98), wild-type *EGFR* tumors (n=118) and mutant *EGFR* tumors (n=98) was -0.190, standard deviation (SD) of 0.365, 0.877, SD of 0.689, and -0.231, SD of 0.425, respectively.

Table II. Associations between clinicopathological characteristics of lung adenocarcinoma patients and *FAM83B* expression in tumors.

Variable	Odds ratio	95% confidence interval	P-value
Univariate analysis			
≥70 years old	1.411	0.763-2.609	0.272
Male	2.468	1.277-4.769	0.007
Smoking	2.417	1.271-4.596	0.007
Wild-type <i>EGFR</i>	0.243	0.120-0.495	<0.001
Histological Subtype	2.202	0.795-6.101	0.129
Tumor size >3 cm	1.550	0.835-2.878	0.165
LN metastasis	0.854	0.377-1.934	0.706
Distant metastasis	1.472	0.131-16.560	0.754
Pleural invasion	1.201	0.607-2.373	0.599
Lymphatic invasion	0.606	0.288-1.275	0.187
Vascular invasion	1.522	0.780-2.970	0.218
Multivariate analysis			
Wild-type <i>EGFR</i>	0.243	0.120-0.495	<0.001

Where histological subtype is the comparison between predominantly non-solid and solid histological subtypes. EGFR, epidermal growth factor receptor; FAM83B, family with sequence similarity 83, member B; LN, lymph node.

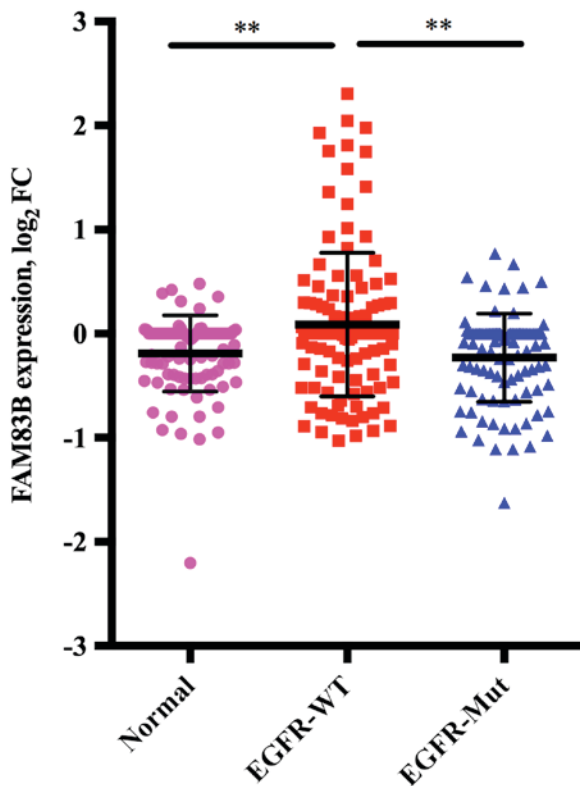


Figure 1. Expression levels of *FAM83B* obtained by cDNA microarray analysis of tumors stratified by the presence or absence of *EGFR* mutation, and adjacent normal tissue. The expression of *FAM83B* in tumors with wild-type *EGFR* was significantly higher when compared with tumors with mutant *EGFR* ($P<0.01$) or adjacent normal tissue ($P<0.01$). No significant difference in *FAM83B* expression was identified between tumors with mutant *EGFR* and adjacent normal tissue ($P=0.852$). Horizontal bars represent the mean expression levels. Data are presented as the mean \pm standard deviation. ** $P<0.01$, as indicated. EGFR, epidermal growth factor receptor; FAM83B, family with sequence similarity 83, member B; Mut, mutant; Normal, normal adjacent lung tissue; WT, wild-type.

Multiple comparison of these three groups showed that *FAM83B* expression in wild-type *EGFR* tumors was higher than in adjacent normal lung tissue ($P<0.001$) or mutant *EGFR* tumors ($P<0.001$), while there was no significant difference between mutant *EGFR* tumors and adjacent normal lung tissue ($P=0.852$) (Fig. 1).

FAM83B is a predictor of poor lung ADC prognosis, especially for ADC with wild-type *EGFR*. The *FAM83B* high expression group showed significantly shorter survival times both in DFS ($P=0.011$) and OS ($P=0.001$). Subgroup analysis showed that the *FAM83B* high expression group had shorter DFS and OS with wild-type *EGFR* tumors ($P=0.017$, $P=0.008$, Fig. 2), while no significant difference was found in patients with mutant *EGFR* tumors ($P=0.746$, $P=0.588$). Survival analysis using a Cox regression hazard model was then conducted. For DFS, univariate analysis showed that high levels of *FAM83B* expression, male sex, lymph node metastasis, pleural invasion, lymphovascular invasion, and vascular invasion were involved in poor prognosis. Multivariate analysis identified high levels of *FAM83B* expression and lymph node metastasis as independent poor prognostic factors (Table III). In OS, univariate analysis showed high levels of *FAM83B* expression, male sex, wild-type *EGFR* tumors, lymph node metastasis, pleural invasion, lymphovascular invasion, and vascular invasion as poor prognostic factors. Multivariable analysis showed high levels of *FAM83B* expression, pleural invasion, and vascular invasion were independent predictors of poor prognosis (Table III).

Involvement of FAM83B in cell proliferation in several types of lung cancer cell line. Cell lines derived from lung ADC, including HLC-1, H2347 (both wild-type for *EGFR*) and H1975 (mutant *EGFR*), and a positive control breast cancer cell line, MCF7 (15) were prepared. RT-qPCR showed high levels

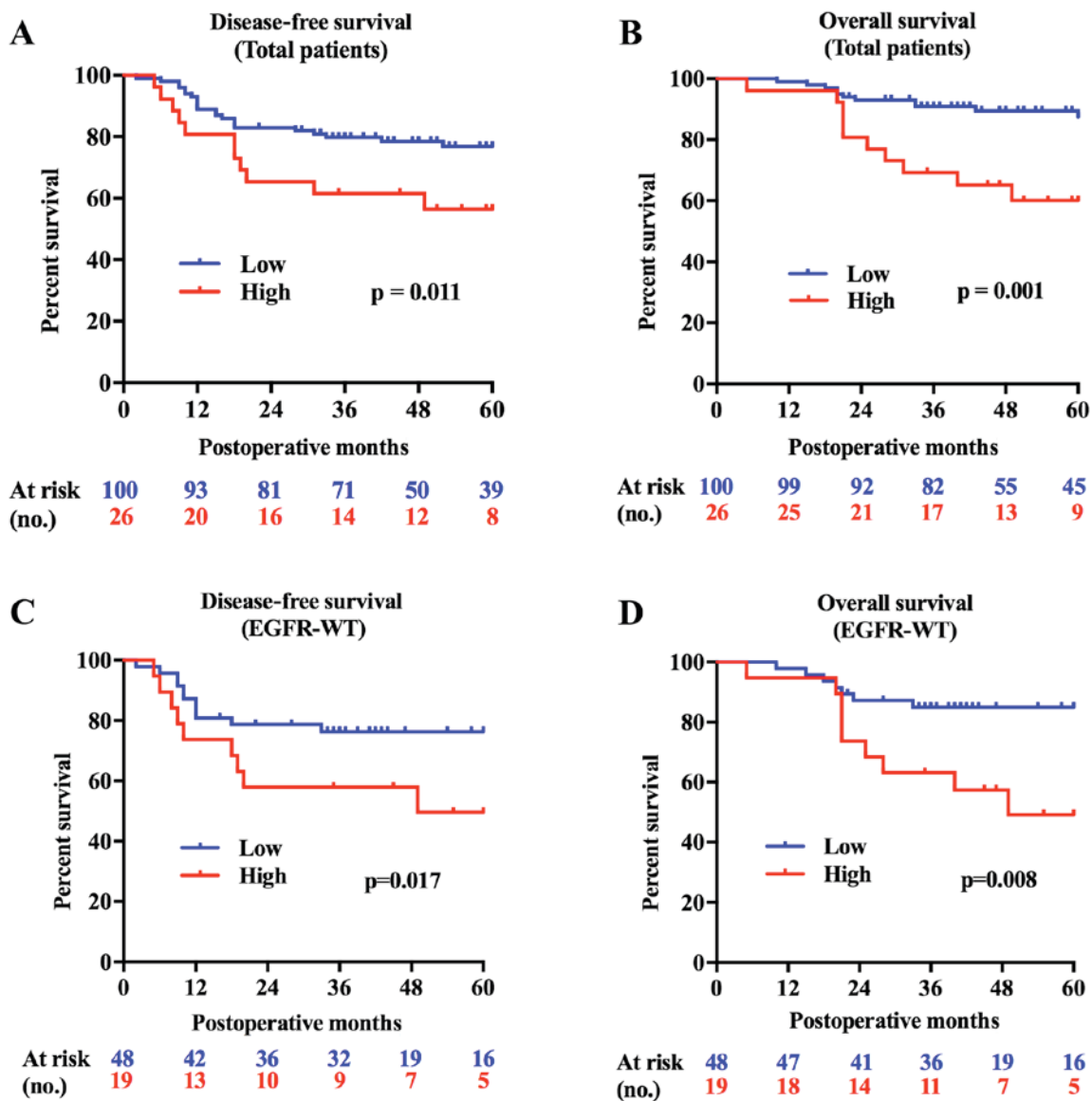


Figure 2. Kaplan-Meier curves of patient survival following lung resection. (A) DFS and (B) OS of all eligible participants. (C) DFS and (D) OS of the patients stratified by tumors with EGFR-WT. DFS, disease-free survival; OS, overall survival; EGFR-WT, wild-type epidermal growth factor receptor.

of *FAM83B* expression in HLC-1 and H2347 cells but scarcely detectable levels in H1975 cells (Fig. 3A). Immunoblot analysis showed levels of *FAM83B* that were consistent with the HLC-1, MCF7, and H1975 RT-qPCR results (Fig. 3B). In MCF7 cells, *FAM83B* knockdown with siRNA-8 or siRNA-9 caused inhibition of cell proliferation (siRNA-8; $P=0.393$, siRNA-9; $P=0.061$ at 6 days after knockdown), with siRNA-9 having the stronger anti-proliferative effect (Fig. 4E-G); therefore, we performed subsequent knockdown experiments using siRNA-9. siRNA transfection efficacy assays (Fig. 4A-D) indicated *FAM83B* RNAi should be performed in HLC-1 and H1975 cells. Depletion of *FAM83B* expression and suppression of cell proliferation were confirmed in HLC-1 and even in H1975 cells, which expressed low levels of *FAM83B* (Fig. 3C-H).

Discussion

In this study, we focused on comprehensive gene expression analysis of tumors from resected lungs of Japanese ADC

patients, and we showed that *FAM83B* expression was higher in tumors with wild-type *EGFR* compared with tumors with mutant *EGFR*, and that *FAM83B* expression was associated with proliferation in cell lines. Furthermore, *FAM83B* expression was identified as a biomarker of poor prognosis from patient clinical outcomes.

FAM83B was first reported by Cipriano and colleagues (15) to be correlated with anchorage-independent growth in breast cancer cell lines using a validation-based insertion mutagenesis method. *FAM83B* is a member of the *FAM83* family, which includes eight members (*FAM83A* to *H*) characterized by a domain of unknown function 1669 (DUF1669), which contains an N terminal phospholipase D-like motif. DUF1669 is considered to be involved in tumor proliferation and oncogenesis (15). *FAM83B* expression is elevated relative to normal associated tissues in several types of cancer, such as breast, ovary, cervical, testis, thyroid, bladder, lymphoid and lung (15). In lung cancer, analysis of *FAM83B* expression confirmed that *FAM83B* expression was significantly elevated in tumor

Table III. Univariate and multivariate predictors of DFS and OS.

A, DFS			
Variable	Unfavorable/favorable	Hazard ratio (95% confidence interval)	P-value
Univariate analysis			
FAM83B	High/low	2.415 (1.195-4.881)	0.014
Age	≥70/<70	1.183 (0.604-2.318)	0.624
Sex	Male/female	2.502 (1.196-5.233)	0.015
EGFR gene	Wild-type/mutant	0.595 (0.298-1.189)	0.142
Pack-year	>5/≤5	1.455 (0.735-2.881)	0.282
Tumor size	>3 cm/≤3 cm	1.073 (0.537-2.142)	0.843
N	N1+N2+N3/N0	3.852 (1.867-7.948)	<0.001
pl	Positive/negative	2.599 (1.299-5.197)	0.007
ly	Positive/negative	2.347 (1.173-4.696)	0.016
v	Positive/negative	2.929 (1.477-5.811)	0.002
Multivariate analysis			
FAM83B	High/low	2.286 (1.129-4.631)	0.022
N	N1+N2+N3/N0	3.699 (1.788-7.655)	<0.001
B, OS			
Variable	Unfavorable/favorable	Hazard ratio (95% confidence interval)	P-value
Univariate analysis			
FAM83B	High/low	3.814 (1.619-8.989)	0.002
Age	≥70/<70	1.010 (0.429-2.380)	0.981
Sex	Male/female	3.241 (1.187-8.854)	0.022
EGFR gene	Wild-type/mutant	0.297 (0.108-0.813)	0.018
Pack-year	>5/≤5	2.080 (0.839-5.154)	0.114
Tumor size	>3 cm/≤3 cm	1.297 (0.547-3.079)	0.555
N	N1+N2+N3/N0	4.342 (1.798-10.483)	0.001
pl	Positive/negative	5.313 (2.234-12.634)	<0.001
ly	Positive/negative	2.577 (1.085-6.117)	0.032
v	Positive/negative	3.338 (1.414-7.877)	0.006
Multivariate analysis			
FAM83B	High/low	3.723 (1.568-8.842)	0.003
pl	Positive/negative	5.098 (2.119-12.264)	<0.001
v	Positive/negative	2.529 (1.066-6.001)	0.035

N, lymph node metastasis according to Union for International Cancer Control 7th edition (20); pl, pleural invasion; lv, lymphovascular invasion; v, vascular invasion; DFS, disease-free survival; EGFR, epidermal growth factor receptor; FAM83B, family with sequence similarity 83, member B; N, nodes; OS, overall survival.

specimens relative to normal tissues (15). It was also reported that *FAM83B* mRNA levels were significantly higher in SqCC than in normal lung or ADC (14). It is a novel finding that *FAM83B* is more highly expressed in lung ADCs containing wild-type *EGFR* compared with ADCs carrying mutant *EGFR*. Interestingly, in breast cancer, *FAM83B* is expressed at higher levels in tumors without estrogen and progesterone receptors or human *EGFR-2*, compared with receptor-positive tumors (21). Furthermore, *FAM83H*, the paralog of *FAM83B*, is overexpressed in androgen independent-prostate

cancer (22). These findings could indicate that *FAM83B* has an oncogenic role without aberrant signals from driver gene mutations or overexpressed hormone receptors. The function of *FAM83B* remains unclear; however, a bifunctional interaction mechanism with responses to EGF was proposed. EGF signals to multiple growth and survival pathways, including the RAS/RAF/MEK/ERK and the PI3K/AKT pathways. *FAM83B* may interfere with the binding of 14-3-3 protein to CRAF, thereby promoting membrane localization of CRAF, and promoting downstream signals to MAPK (15,23,24).

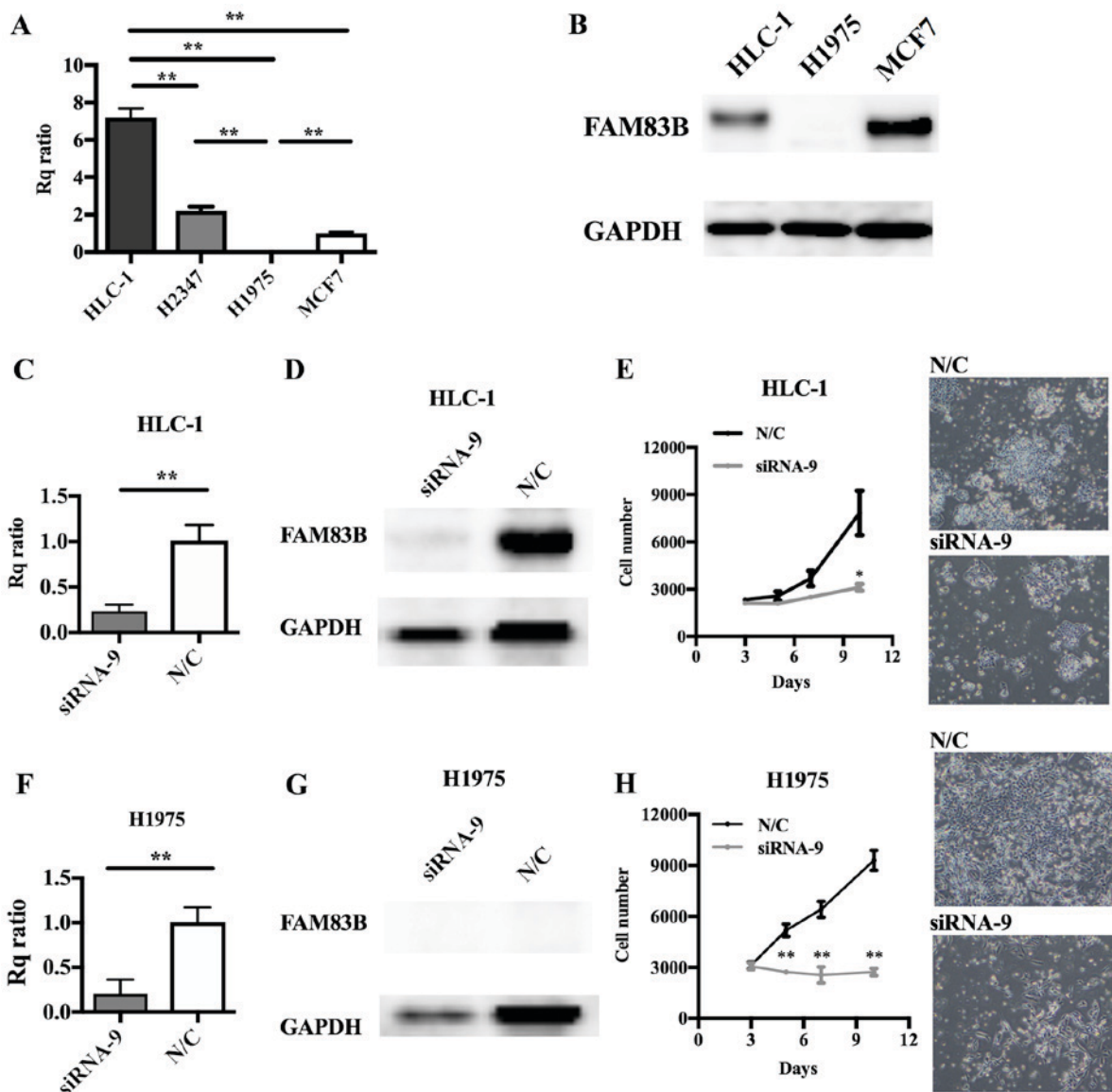


Figure 3. Effect of *FAM83B* on the proliferation of lung cancer cell lines. (A) *FAM83B* mRNA and (B) protein levels were examined by RT-qPCR and western blotting, respectively, in MCF7, H1975, H2347 and HLC1 cells. The Rq ratios for RT-qPCR to MCF7 cells were as follows: H1975, 0.0061; H2347, 2.2103; and HLC1, 7.1968. (C-H) RNAi was performed in HLC-1 and H1975 cells. Depletion of *FAM83B* mRNA and protein was confirmed by (C and F) RT-qPCR and (D and G) western blotting, respectively. Rq ratios to negative control were as follows: HLC-1, 0.3519; and H1975, 0.1675 for RT-qPCR. ** $P < 0.01$, as indicated. Cell proliferation assay in (E) HLC-1 and (H) H1975 cells following RNAi. Cell numbers were counted over time. Cell growth was significantly suppressed in HLC-1 and H1975 cells. Magnification, $\times 40$. Data are presented as the mean \pm standard deviation. * $P < 0.05$ and ** $P < 0.01$ vs. N/C. FAM83B, family with sequence similarity 83, member B; Rq ratio, relative quantification ratio; N/C, negative control; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; RNAi, RNA interference.

Furthermore, *FAM83B* may also bind to p85 and p110 subunits of PI3K to promote PI3K/AKT signaling (25), which promotes oncogenic transformation through phospholipase D activation (26), and shows resistance against EGFR-TKI (15,24,27). Our knockdown study of *FAM83B* showed that its expression was associated with tumor proliferation in cell lines expressing high levels of *FAM83B*; however, H1975 cells showed that knockdown of low levels of *FAM83B* also inhibited proliferation. Of all the *FAM83* members, only *FAM83H* knockout mice, which live for only 2 weeks, have been reported, and *FAM83H* is proposed to play a role in the maintenance of cell homeostasis (22,28). Other *FAM83* family members, including *FAM83B*, may also be required at certain levels, even in normal cells.

FAM83 family members are usually associated with poor cancer prognosis (21). Our study of lung ADC also showed that *FAM83B* correlates with poor prognosis; however, our previous study reported high *FAM83B* expression to be a biomarker for good DFS prognosis in lung SqCC (14). Snijders *et al* conducted a meta-analysis of several databases (29-31) and suggested that lung ADC expresses relatively high levels of *FAM83A*, *D*, *E*, *F*, and *H*, but *FAM83A*, *B*, *D*, and *F* correlate with prognosis. In lung SqCC, all *FAM83* members except *FAM83E* are highly expressed, but only *FAM83A* correlates with poor prognosis (21). These findings indicate *FAM83B* has separate roles among cancers or tumor subtypes.

FAM83 family members, including *FAM83B*, are more highly expressed in lung cancer than in normal lung tissue, and

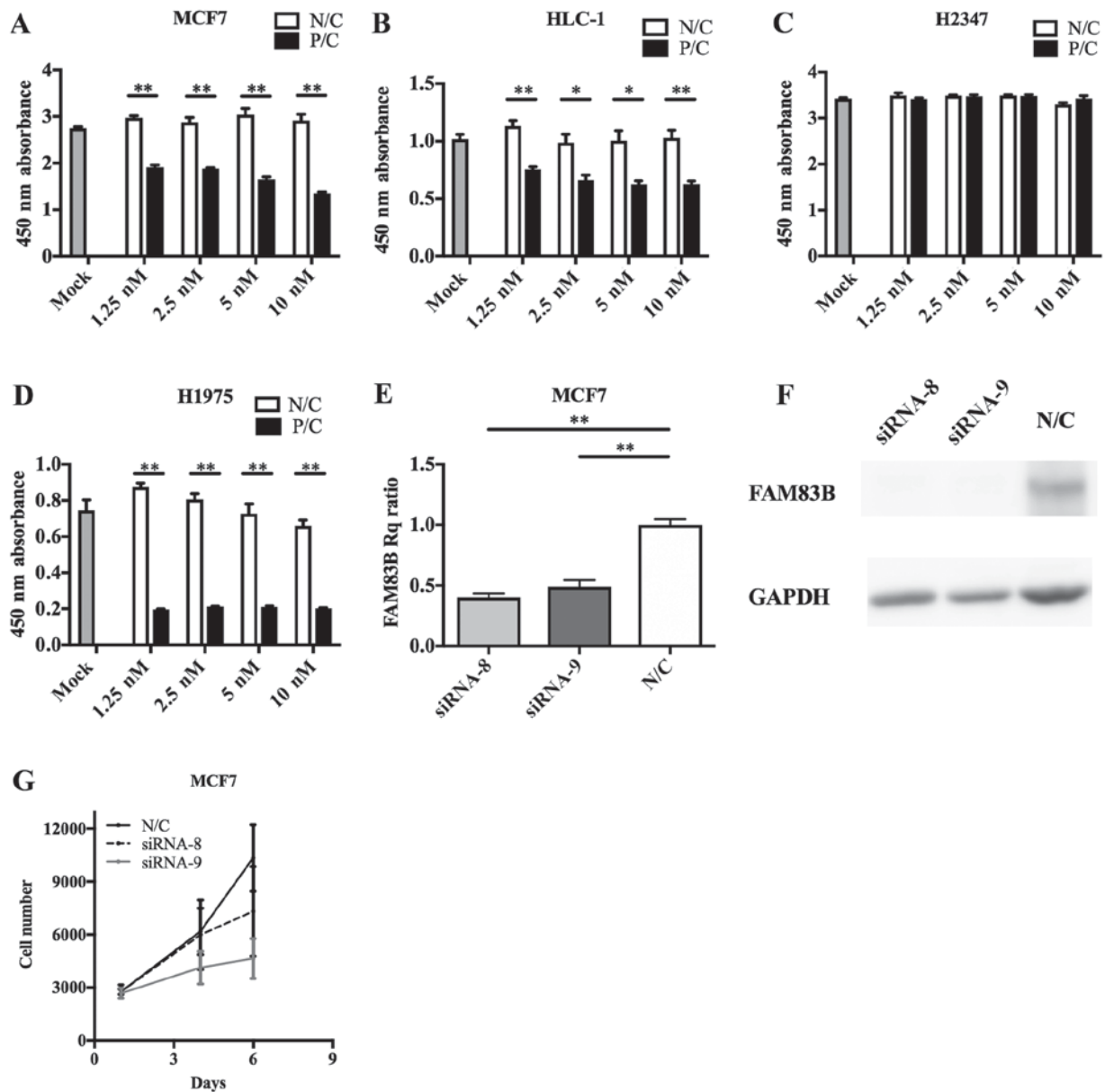


Figure 4. *FAM83B* siRNA transfection efficiency and RNA interference in cultured cells. Transfection of *FAM83B* siRNA significantly suppressed the cell growth of (A) MCF7, (B) HLC-1 and (D) H1975 cells, though not that of (C) H2347 cells. (E) Reverse transcription-quantitative polymerase chain reaction revealed that siRNA-8 and siRNA-9 reduced the expression of *FAM83B*. (F) Immunoblotting analysis showed that *FAM83B* signals were markedly depleted in MCF7 cells transfected with siRNA-8 or siRNA-9. (G) siRNA-8 and siRNA-9 suppressed cell growth, particularly that of siRNA-9. Data are presented as the mean \pm standard deviation. * $P < 0.05$ and ** $P < 0.01$, as indicated. *FAM83B*, family with sequence similarity 83, member B; siRNA, small interfering RNA; Rq, quantification relative to negative control; P/C, positive control; N/C, negative control.

their expression is reflected in the T factor of TNM classification (20,29,32). Our study did not show a correlation between tumor size and *FAM83B* expression. This contradiction was caused by the fact that the T factor includes not only tumor size but pleural invasion. Both tumor size and pleural invasion had no significant effect on *FAM83B* expression.

FAM83B RNAi suppressed proliferation of human lung cancer cell lines. This result indicates that *FAM83B* could be a potential therapeutic target against EGFR-WT malignancies, which account for 47-88.7% of lung ADC (4,29) and which are rarely indicated for molecular targeted therapies. Limitations of this study include lack of evaluation of advanced or recurrent cancer cohorts because the patient cohort was derived from operable lung ADC cases biased to early stages. Moreover, this

study did not examine other driver mutations of lung ADC, such as KRAS, ALK fusions. Subdivision based on other genomic or molecular features could further explain the function of *FAM83B* in lung ADC. Further investigation is required.

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