FOXD1 Expression Is Associated with Poor Prognosis in Non-small Cell Lung Cancer

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Abstract. *Aim: Clinical microarray datasets were analyzed to search for new therapeutic targets and prognostic markers of non-small cell lung cancer (NSCLC). Materials and Methods: Microarray datasets from 90 lung cancer specimens, were analyzed with focus on the FOXD1 gene. Levels of FOXD1 mRNA were assessed in lung cancer cell lines and these levels were correlated with survival. Results: FOXD1-knockdown led to suppression of cell proliferation. Moreover, patients with high FOXD1 expression survived for a significantly shorter time than those with low FOXD1 expression. Conclusion: The expression status of FOXD1 is a novel prognostic factor and may lead to new treatment strategies for NSCLC.*

Lung cancer is the leading cause of cancer-related deaths worldwide (1). In recent years, therapies for lung cancer have significantly advanced and individualized treatment with rational targeted-therapies has led to a substantial increase in the survival of the patients. The discovery of somatic mutations in the gene encoding the epidermal growth factor receptor (EGFR) kinase provided the first indication that an aberrant oncogenic tyrosine kinase plays a role in the pathogenesis of non-small-cell lung cancer (NSCLC). Following this finding, several additional oncogenic genes in NSCLC were discovered. This information led to targeted-therapy with crizotinib, which is associated with dramatic response rates in patients with *EML4-ALK* re-arrangements as EGFR tyrosine kinase inhibitors in *EGFR* mutations (2-4). However, approximately 50% of patients with NSCLC are not suitable candidates for such a targeted approach (5). Therefore, research is in progress to identify additional and novel driver oncogenes or druggable targets.

Microarray analysis has been used to investigate many oncogenic genes. For breast cancer, the Oncotype DX Breast Cancer Assay has become one of the standards-of-care for individualized treatment in patients with early-stage breast cancer (6). For lung cancer, however, it appears unlikely that additional novel treatment targets will be identified. Nonetheless, several genes that may have a prognostic value have been investigated using microarray analysis (7-9) suggesting that the identification of novel genes with prognostic relevance in lung cancer is a valuable tool.

The *FOXD1* gene is located on chromosome 5q12 and encodes a DNA-binding protein that is 100-amino-acids long. The FOXD1 protein acts as a transcription factor and contains a forkhead domain that binds DNA as a monomer; it also contains two loops and is termed "winged helix" (10). Transcription factors that contain a forkhead domain play an essential role in kidney morphogenesis (11, 12) and in specification of the temporal retina in mammals (13). The FOXD1 protein also has a role in a wide array of biological processes, including proliferation, invasion and tumorigenesis (14). *FOXD1* is up-regulated in prostate cancer (15) and has also been associated with resistance to chemotherapy in ovarian cancer patients (16). Although these data suggest that *FOXD1* is widely implicated in various malignancies, there is no information regarding its expression in lung cancer.

In this study, gene expression profiling of 90 NSCLC samples was performed using pathway signature analysis. Herein, it is demonstrated for the first time that *FOXD1* is related to proliferation of lung cancer cells and is a prognostic marker in patients with NSCLC.

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Key Words: *FOXD1*, non-small cell lung cancer, microarray, pathway signature, PTEN.

Materials and Methods

Cell culture. NCI-H520, NCI-H522, NCI-H358 (American Type Culture Collection, Manassas, VA, USA) cells were maintained in RPMI1640 supplemented with 10% bovine calf serum and 1% penicillin/streptomycin. All cell lines were grown at 37˚C in a humidified atmosphere with 5% CO_2.

Patient population. Ninety primary lung cancer specimens were collected from patients undergoing surgery at the Keio University Hospital. All patients were admitted to Keio University Hospital. Written informed consent was obtained before enrollment. The study was approved by the Institutional Review Board of Keio University School of Medicine (Institutional Review Board #16-90-1). Table I lists the clinical data of the NSCLC patients enrolled in this study.

RNA isolation from tissue and cells. Matched normal lung tissues were also obtained from an adjacent area. Total RNA was prepared using the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) after treatment with TRIzol (Invitrogen Corp. Carlsbad, CA, USA).

Microarray analysis. GeneChip Human Genome 2.0 Arrays (Affymetrix, Inc., Santa Clara, CA, USA) were used to obtain the expression profiles of samples. Labeled cRNA was prepared using standard protocols (Affymetrix). Signal intensities of probe sets were normalized using the Affymetrix Power Tools RMA method present in the Resolver software (Rosetta Inpharmatics, Seattle, WA, USA) and log ratio values to the average of normal samples were calculated for each sample using the Resolver software.

TaqMan quantitative polymerase chain reaction (PCR) assay. Reverse transcription was performed on 1 μg of total RNA from each sample. TaqMan quantitative PCR assays were performed using an Applied Biosystems Prism 7000 Sequence Detection System (Life Technologies, Carlsbad, CA, USA). For TaqMan quantitative PCR, a TaqMan probe of human *FOXD1* (Life Technologies) was used; human *GAPDH* (Life Technologies) was used to normalize for the amount of cDNA in each assay.

siRNA transfection. Cells were transfected with a final concentration of 20 nM of *FOXD1* siRNA or negative control siRNA (Life Technologies) using the SilentFect reagent (Bio-Rad, Inc., Hercules, CA, USA) according to the manufacturer's protocol. Knockdown of *FOXD1* expression was confirmed using quantitative reverse transcription-PCR.

Cell counting. Cells were re-suspended in PBS and counted using a TC10 automated cell counter (Bio-Rad, Inc.) by loading 10 μl of each cell resuspension. Cells were counted three times and the average was obtained.

Colony formation assay. Anchorage-independent growth assays were performed in six-well cell culture plates seeded with 1,000 cells per well as previously described (17). After 10-14 days, colonies were counted.

Statistical analysis. All statistical analyses were performed with the STATA version 12.1 software. Differences between groups were tested with a two-sided *t*-test or Fisher's test. Survival curves were plotted using the Kaplan–Meier method and compared using the log-rank test. Survival data were evaluated using a Cox proportional hazards model. Independent prognostic factors were determined by univariate or multivariate analysis. *p*-Values of ≤0.05 were considered statistically significant.

Table I. *Patients' characteristics*.

Patients, n	90
Age (years)	
Median	67.5
Range	36-83
Gender	
Male	57 (63.3%)
Female	33 (36.7%)
Histology	
Ad	51 (56.7%)
Sq	24 (26.7%)
LCNEC	4 (4.4%)
Others	11 (12.2%)
Smoking (B.I.)	
Median	600
Range	0-3000
Stage	
I	68 (75.6%)
II	15 (16.7%)
III	6 (6.7%)
IV	1 (1.1%)

Ad, Adenocarcinoma; Sq, squamous carcinoma; LCNEC, large-cell neuroendocrine carcinoma; B.I., Brinkman index.

Results

Pathway signature analysis identified novel candidate genes. To identify potential therapeutic targets and/or prognostic markers specific for a subset of lung cancers with de-regulation of a specific signaling pathway, tumors were sub-grouped according to mRNA expression and their biological significance was characterized using pathway signature analysis. Eight gene clusters (1-8) and three sample groups (A-C) were found (Figure 1A). The expression of "Cluster 7" genes in "group C" was higher than both the average expression in all tumor samples and the average expression in the matched normal samples (Figure 1B). Next, mRNA expression profiling of lung tumors with the pathway signature analysis method was performed as described elsewhere (18). Each pathway signature score was calculated according to previous data, including loss of signatures for PTEN (19), PI3K inhibition (20) and mTOR inhibition (20). Interestingly, "Cluster 7" genes were correlated with loss of the PTEN signature (R=0.93) and showed inverse correlation with PI3K inhibitor (R=–0.82) and mTOR inhibitor signatures (R=–0.82) (Figure 1C). Among the "Cluster 7" genes, three genes, namely *FOXD1* (forkhead box D1), *MARK1* (MAP/microtubule affinity-regulating kinase 1) and *MSI1* (musashi RNA-binding

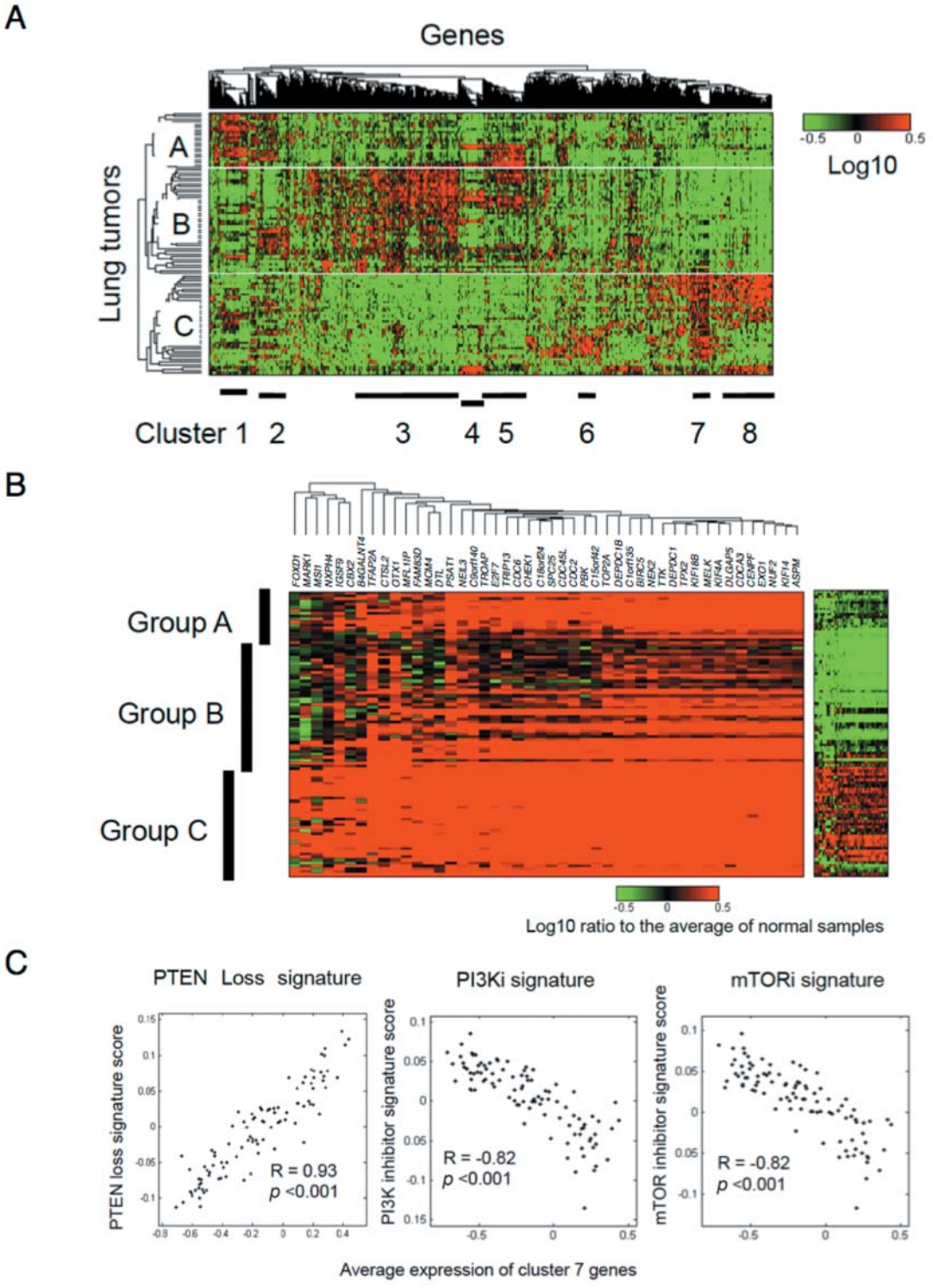

Figure 1. *(A) Hierarchical clustering of the 90 lung tumor samples (rows) by genes (columns). In the heat map, fold change is relative to the median for each gene according to the color scale shown (red, high expression; green, low expression). (B) Heat map of "Cluster 7" genes. Each column represents a sample and each row represents a gene transcript. Red and green colors indicate higher and lower gene expressions than the matched normal tissue (left panel) or the average of all tumor tissues (right panel). (C) Scatter diagrams of "Cluster 7" genes expression and each pathway signature score. "Cluster 7" gene score and each pathway score were calculated and plotted for each sample. The X-axis shows "Cluster 7" gene expression scores and the Y-axis shows each pathway signature score. The p-value was calculated using a simple linear regression model. R indicates the correlation coefficient.*

protein 1), were found to show an extremely higher expression in tumor samples than in the matched normal tissues in group C. Among these, focus was placed on *FOXD1*, since the role of *FOXD1* was unknown in lung cancer and because *FOXD1* is involved in other cancers, including cancers of the prostate and ovarian carcinomas (15, 16).

Samples were classified in two groups according to the level of *FOXD1* expression. As shown in Table II, high *FOXD1* expression was significantly associated with squamous cell carcinoma ($p<0.001$), male gender ($p=0.004$), history of heavy smoking ($p=0.03$) and absence of *EGFR* mutations ($p=0.005$) (*i.e.*, absence of exon 19 deletion, exon 21 L858R and exon18 G817S). No association was found between *FOXD1* expression and advanced age ($p=0.67$), *K-RAS* mutation status ($p=1$), tumor size ($p=0.39$) or lymph node metastasis ($p=1$).

FOXD1 regulates cell proliferation in lung cancer cells. To investigate whether *FOXD1* is related to cell proliferation *in vitro*, siRNA knockdown was performed. *FOXD1* siRNA was transfected into H358, H520, H522 cells, since *FOXD1* expression in these cells was higher than that in normal human bronchial epithelial cells. To validate the efficiency of different siRNAs, transcript levels were measured by quantitative TaqMan PCR. *FOXD1*-specific siRNAs significantly inhibited *FOXD1* mRNA by the same order of magnitude in all cell lines (Figure 2A).

Next, it was determined if inhibition of *FOXD1* decreased cell proliferation by counting viable cells using an automated cell counter. As shown in Figure 2B, *FOXD1* siRNA suppressed cell growth in all cell lines. In H522 cells, viable cells were under the limits of detection ($<5\times10^4$ cells/ml). These data suggest that *FOXD1* regulates cell proliferation in lung-cancer cell lines.

FOXD1 knockdown decreases anchorage-independent growth of lung cancer cells. To determine if *FOXD1* is involved in clonogenicity of lung cancer cells, a colony formation assay was performed. *FOXD1* knockdown cells transfected with siRNA #1 showed significantly inhibited colony formation of all three cancer cell lines in soft agar (Figure 3). This indicates that *FOXD1* is involved in clonogenicity, which is one of the hallmarks of cancer cells.

Low expression of FOXD1 is associated with poor prognosis. To assess whether *FOXD1* expression was related to prognosis, the Kaplan-Meier analysis was performed. Surprisingly, survival of patients with high *FOXD1* expression was substantially shorter than of those patients with low *FOXD1* expression (Figure 4). At a median follow-up time of 63 months, 17 patients (18.9%) had died in the *FOXD1* high-expression group, among them, 13 (76.5%) deaths were due to disease recurrence and 4 (23.5%) were due to unrelated causes. Median survival time was not reached. A statistically significant difference in survival was observed (hazard ratio (HR) 3.39; 95% CI, 1.19-9.63, $p=0.02$) among the high-expression and low-expression groups. Five-year survival rates were 90% (95% CI, 75%-96%) and 68% (95% CI, 49%-81%) in patients with low and high *FOXD1* expression, respectively ($p=0.015$).

Lastly, the possibility that *FOXD1* expression was an independent risk factor for poor prognosis was investigated. *FOXD1* expression and clinicopathological factors were analyzed by Cox's univariate and multivariate hazard regression models. In univariate analysis, only the *FOXD1* expression status significantly correlated with overall survival of NSCLC patients. Multivariate analysis further indicated that high *FOXD1* expression was an independent risk factor for overall survival (Table III).

Table II. *Association between FOXD1 expression and clinicopathological characteristics*.

	FOXD1 expression		
	Low	High	*p*-Value
Age			
<70	29	23	0.67
>=70	18	20	
Brinkman Index			
<600	29	16	0.03
>=600	18	27	
Gender			
Male	23	34	0.004
Female	24	9	
EGFR mutation			
Negative	27	37	0.005
Positive	20	6	
Histology			
Squamous	3	21	<0.001
Non squamous	44	22	
KRAS mutation			
Negative	44	41	1
Positive	3	2	
Tumor Size (mm)			
<30	23	16	0.39
>=30	24	27	
LN Metastasis			
Negative	39	35	1
Positive	8	8	

LN, Lymph node.

Discussion

It is known that the FOX family of genes, which encode for transcription factors with forkhead motifs, regulate a wide spectrum of biological processes, including differentiation, development and tumorigenesis (14). *FOXD1* with the

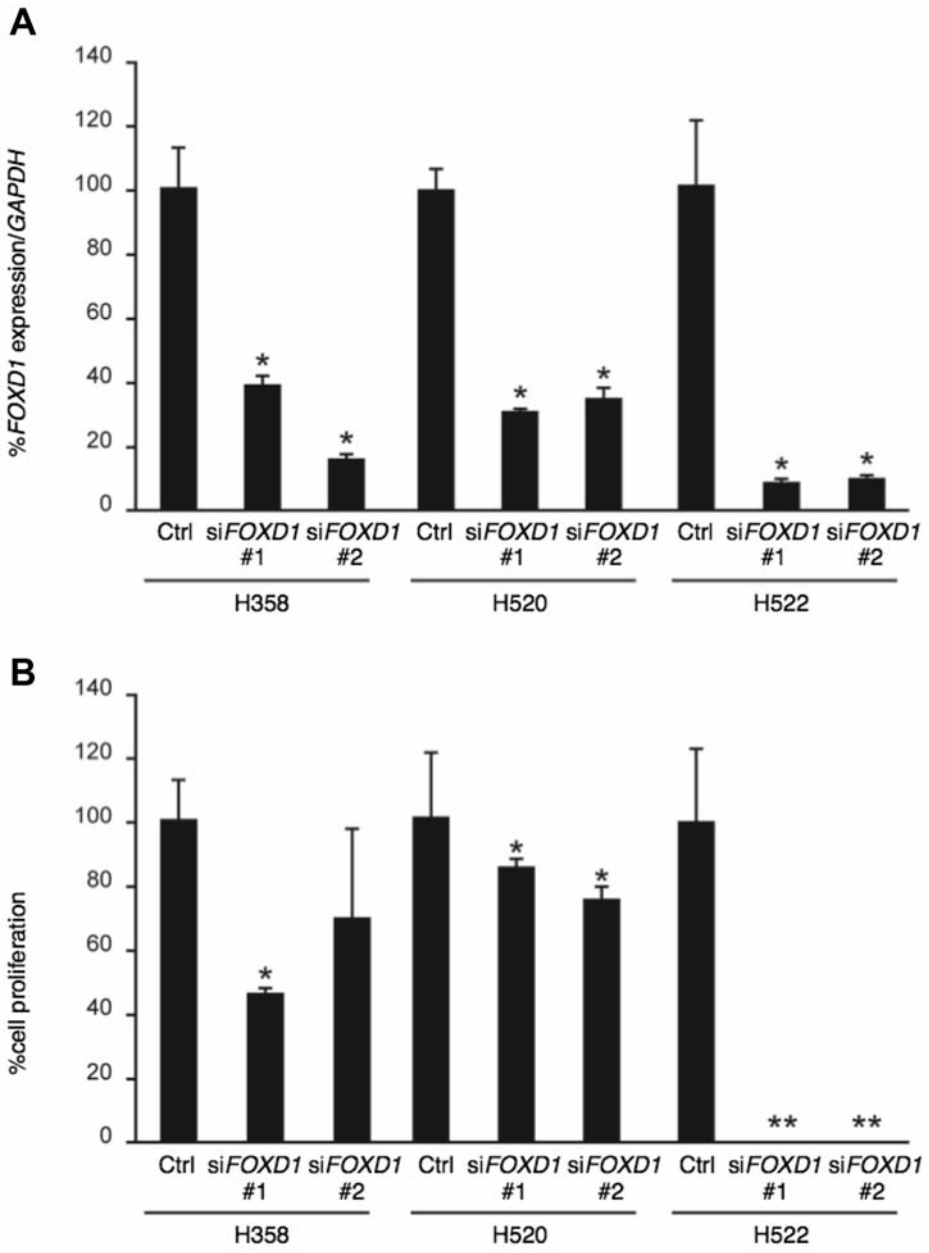

Figure 2. *The H520, H522 and H358 cell lines were transiently transfected with either control siRNA or FOXD1 siRNA. (A) TaqMan quantitative PCR for FOXD1 at 48 hours. The average of TaqMan quantitative PCR experiments for each sample in triplicate is shown. Error bars represent standard error (n=3). *p<0.05. (B) Cells were counted using an automated cell counter 72 h after siRNA treatment. Error bars indicate standard error (n=3). *p<0.05, **under the limits of detection.*

forkhead motif (*FKHL8, FREAC4*) was originally identified as an important transcription factor from human DNA libraries (21). *FOXD1* is essential for development of the kidney and forebrain, and specification of the temporal retina in mammals (11-13). In addition to their normal function, members of the FOX family have also been shown to be related to the pathogenesis of various malignancies, including prostate and ovarian cancers (15, 16).

In the present study, *FOXD1* expression was related to loss of the PTEN signature as determined by microarray analysis of NSCLC. Interestingly, high levels of *FOXD1* expression significantly associated with several clinicopathological findings, including presence of squamous cell carcinoma, male gender, history of heavy smoking and the absence of *EGFR* mutations. The characteristics of patients with high *FOXD1* expression are different from those of patients with

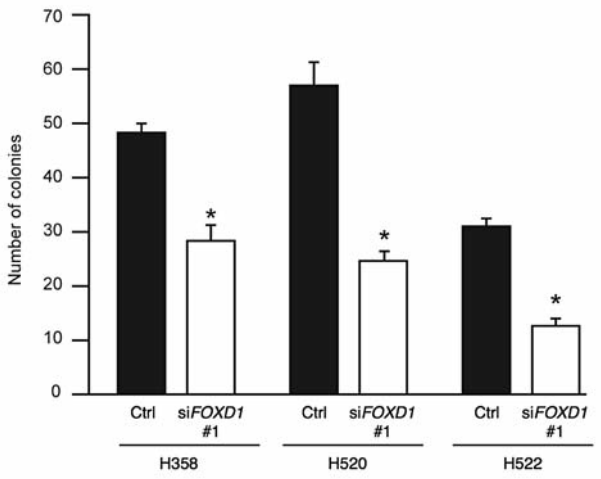

Figure 3. *Colony formation assay using H358, H522 and H520 cells transfected with siFOXD1#1 or control siRNA. The average number of foci for each sample in triplicate is shown. Error bars represent standard error (n=3). *p<0.05.*

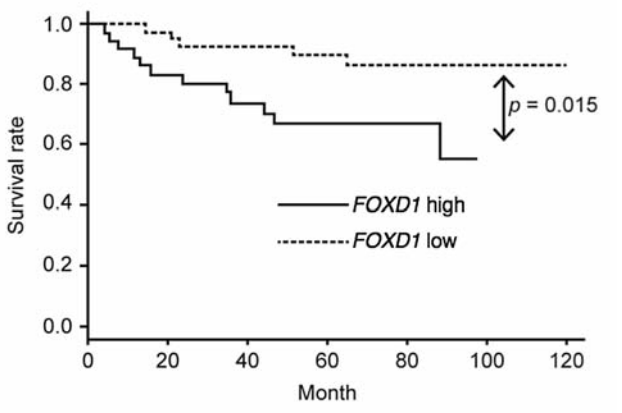

Figure 4. *Survival curves for patients with NSCLC according to FOXD1 expression status (p=0.015). The p-value was calculated with the log rank test.*

Table III. *Multivariate analysis for variables considered for survival.*

	Univariate analysis			Multivariate analysis		
	HR	95%CI	*p*-Value	HR	95%CI	*p*-Value
FOXD1 Expression	3.39	1.19-9.63	0.02	3.91	1.23-12.35	0.02
EGFR mutation	0.69	0.23-2.12	0.52	0.98	0.29-3.34	0.98
Squamous cell carcinoma	1.40	0.49-4.00	0.50	0.70	0.21-2.27	0.55
Female	0.47	0.15-1.45	0.19	-	-	-
Brinkman Index>=600	1.38	0.53-3.64	0.51	-	-	-
Age>=70	1.13	0.43-2.99	0.81	-	-	

HR, Hazard ratio. CI, confidence interval.

EGFR mutations, who are predominantly females, have never smoked and have adenocarcinoma (22). Furthermore, it has been reported that simultaneous homozygous deletion of PTEN and *EGFR* mutation has been found in only one of 24 cases with *EGFR* mutations (23).

In breast cancer, loss of PTEN expression has been observed in nearly 40% of cases (24). In addition, breast tumors expressing the loss of PTEN signature have a significantly shorter disease-free survival and overall survival (19). However, classification by loss of PTEN signature in lung carcinoma by gene profiling could not separate samples into groups with significant differences in survival (19). On the other hand, loss of PTEN protein expression has been associated with shorter progression-free survival and overall survival (25, 26). This suggests that multiple mechanisms regulate PTEN expression, including transcription, mRNA stability, microRNA targeting and translation (27, 28). Additionally, phosphorylation, ubiquitylation and oxidation can also post-translationally regulate PTEN expression (29). Such intricate regulation of PTEN suggests that mRNA levels of *PTEN* are not reflective of the expression of PTEN protein. Indeed, mRNA expression levels of *PTEN* do not correlate with survival, while those of *FOXD1* did show a clear relationship to survival herein. Moreover, high levels of *FOXD1* mRNA expression were closely related to loss of the PTEN signature in addition to several clinicopathological characteristics in NSCLC, although the functional relationship between FOXD1 and PTEN needs to be elucidated further. To the best of our knowledge, this is the first study to investigate the expression and role of *FOXD1* in NSCLC.

Prognosis of patients was also investigated using univariate analysis and multivariate Cox analysis, and it was found that only high *FOXD1* mRNA expression significantly correlated with prognosis. Although the role of the other forkhead factors including FOXA2 (30, 31) and FOXC1(32) have been reported in lung cancer, there is no information

about *FOXD1*. Since a favorable survival trend in patients with low expression of FOXA2 and FOXC1 has been previously noted, together with the results of *FOXD1* in this study, it is possible to believe that other forkhead factors may have prognostic relevance in lung cancer.

In summary, FOXD1 was demonstrated to regulate cell proliferation in NSCLC cell lines. A high level of *FOXD1* mRNA expression correlated with poor prognosis; hence, *FOXD1* expression was identified as an independent prognostic factor. Although the mechanisms by which the regulation of *FOXD1* expression occurs remain elusive, greater understanding of these may provide innovative treatment strategies for patients with NSCLC.

Conflicts of Interest

The Authors declare that they have no conflicts of interest to disclose.

Acknowledgements

The Authors thank Ms. Mikiko Shibuya for her technical assistance. This work was supported in part by Grants-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan to K.S. (Grant #22590870).

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Received September 7, 2014
Revised September 23, 2014
Accepted September 26, 2014