SPANDIDOS PUBLICATIONS

ONCOLOGY REPORTS 43: 655-661, 2020

Association of *FOSB* exon 4 unmethylation with poor prognosis in patients with late-stage non-small cell lung cancer

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Received April 18, 2019; Accepted November 25, 2019

DOI: 10.3892/or.2019.7431

Abstract. Alterations in DNA methylation have a central role in the development and outcome of most human malignancies. Non-small cell lung cancer (NSCLC), the most common lung cancer, leads to the largest number of cancer-related deaths worldwide. FBJ murine osteosarcoma viral oncogene homolog B (FOSB) is a key component of the activator protein-1 transcription factor and regulates gene networks associated within oncogenic transformation. The role of FOSB in the development of NSCLC is still elusive. Therefore, the methylation status of the *FOSB* gene was investigated in NSCLC and its clinical significance in NSCLC progression was evaluated. The methylation status of the promoter and exon 4 regions of the *FOSB* gene were analyzed in 176 NSCLC specimens by bisulfite pyrosequencing and the association between *FOSB* methylation status and patient survival was investigated. Compared to adjacent non-malignant tissues, *FOSB* promoter exhibited exclusive unmethylation in all malignant tissues and the exon 4 region was found unmethylated in 18 (10.2% of the total) tumor samples. Exon 4 unmethylation was associated with downregulation of its mRNA and tended to occur in patients with lymph node metastasis. Univariate and multivariate analyses revealed that exon 4 unmethylation was significantly associated with unfavorable overall survival in patients with stage II-IIIA NSCLC (log-rank P=0.05, adjusted hazard ratio=2.43, 95% confidence interval=1.04-5.68, P=0.04). *FOSB* was identified as a novel gene with tumor-specific gene body unmethylation in NSCLC and a novel predictive biomarker for NSCLC prognosis. Moreover, the present results indicated that FOSB may have a tumor suppressor function in the progression of NSCLC.

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Key words: promoter, exon, unmethylation, *FOSB*, NSCLC, prognosis

Introduction

Non-small cell lung cancer (NSCLC) represents approximately 85% of all lung cancer cases and remains the leading cancer killer with globally increasing incidence (1). Despite public and medical endeavors to vanquish this disease, NSCLC has risen during the last decade, and >40% of the patients diagnosed with NSCLC have distant metastases, which are associated with unsatisfactory prognosis and high mortality (2). NSCLC is also considered as a highly heterogeneous disease that differs in epidemiological, histological, molecular and phenotypic characteristics (3), rendering it an urgent challenge for early detection and effective treatment of disease. Nowadays, it has become clear that epigenetic and genetic modifications occurring in a number of genes play an important role for the maintenance of normal cellular homeostasis and in the occurrence of numerous pathologies; on the other hand, the intervention on such modifications may improve the treatment and prevention of numerous diseases, including NSCLC.

DNA methylation is a fundamental process for eukaryotic development and leads to long term-repression of genome expression (4). DNA methylation changes are common and relatively stable in various types of cancers, providing useful tools for diagnostic, prognostic and even therapeutic intervention (5). It has been reported that a wide spectrum of aberrantly methylated genes in NSCLC regulate proliferation and maintenance of genome stability (6,7). Notably, tobacco smoking, a major etiological factor for NSCLC, may predispose to aberrant methylation of key regulatory genes (8).

FBJ murine osteosarcoma viral oncogene homolog B (FOSB), a member of the FOS family, heterodimerizes with JUN family proteins to form activator protein-1 (AP-1) transcription factor complexes, which play a central role in the transcriptional regulation of numerous genes that are associated with cell proliferation, differentiation, migration, metastasis, and apoptosis (9). Recent data indicated that FOSB

can act as a common target for anticancer drugs (10,11). In contrast to the amount of data on the function of c-FOS, far less is known about the role of FOSB (12). In addition, despite the numerous studies that have revealed its oncogenic function in tumor formation (13-17), FOSB has been revealed to be down-regulated in breast, gastric, and pancreatic cancer (18,19,20). Furthermore, there are controversial data reporting the expression and functional role of FOSB in lung cancer (11,21). Therefore, to understand the biological role of FOSB and its clinical significance in NSCLC progression, the methylation status of the promoter and exon 4 of the *FOSB* gene was evaluated in 176 specimens from patients with NSCLC using bisulfite sequencing and its association with patient outcomes was investigated.

Materials and methods

NSCLC tissue samples. Fresh tissue samples were obtained from 176 patients (aged 35 to 83 years) with primary NSCLC and corresponding normal tissues. The present study was approved by the Institutional Review Board (IRB) of Kyungpook National University Hospital (KNUH; Daegu, Korea) (No. 2014-04-210) and all patients provided signed informed consent. The clinical characteristics of the patients are summarized in Table I. NSCLC and normal tissue samples were obtained at the time of surgery, and were immediately frozen in liquid nitrogen and stored at -80˚C until DNA extraction. Only tumors with >80% of tumorous components were used for methylation analysis and the histological adequacy of tissue specimens was verified by hematoxylin-eosin (H&E) staining. Tumors were staged according to the American Joint Cancer Committee (AJCC) criteria.

Cell culture, total RNA isolation, and reverse transcription PCR (RT-PCR). The NCI-H157 and NCI-H187 cell lines were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium/F12 or RPMI-1640 medium (Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum and 1% penicillin-streptomycin. Genetic characteristics of the NCI-H157 cell line were determined by PCR-single-locus-technology at Eurofins Genomics. Cells were maintained in an incubator at 37˚C in 5% CO_2. 5-Aza-2'-deoxycytidine (5-AzadC) was added daily to the culturing medium at the indicated concentration for 72 h. Total RNA was isolated from clinical tissue samples and cell lines using TRIzol (Thermo Fisher Scientific, Inc.) and then first-strand cDNA was synthesized using the SuperScript First-Strand Synthesis Kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The relative expression of *FOSB* mRNA was measured with semi-quantitative RT-PCR using *GAPDH* as an internal control for normalization. All primer sequences are listed in Table SI. The PCR thermal cycling began with initial denaturation for 2 min at 95˚C, followed by 30 amplification cycles (1 min at 95˚C, 1 min at 54˚C, 1 min at 72˚C) and a final 5 min incubation at 72˚C. PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide (0.5 μg/ml) for 15 min at room temperature and visualized using the Syngene DigiGenius Gel Documentation system (Syngene).

DNA extraction, bisulfite treatment, and methylation analysis. DNA samples were extracted using the QIAamp DNA Mini Kit (Qiagen, Inc.) and bisulfited using the EZ DNA Methylation-Gold Kit (Zymo Research) following the manufacturer's instructions. The converted DNA was pyrosequenced using a PyroMark Q96MD system (Qiagen, Inc.) as previously described (22). PCR primer sequences and pyrosequencing primers are presented in Table SI. *FOSB* methylation for each sample was calculated from the average value of examined CpGs [mC/total C x100 (%)] and represented as a mean methylation index (MI).

Statistical analysis. Differences in the methylation level of *FOSB* promoter and exon 4 between tumor and matched normal tissues were analyzed using paired t-tests. A comparison of unmethylation proportion of *FOSB* exon 4 according to the clinical characteristics was evaluated using chi-square test. Overall survival (OS) was estimated with Kaplan-Meier method (log-rank test) and multivariate Cox regression analysis. A P-value <0.05 was considered to indicate a statistically significant difference. All analyses were performed using SAS ver. 9.4 (SAS, Inc.).

Results

Methylation status and expression of the FOSB gene in NSCLC samples. Bisulfite pyrosequencing was performed to analyze the methylation status of the human *FOSB* gene in malignant and adjacent non-malignant lung tissue of 176 NSCLC patients. Due to DNA methylation changes in the promoter and gene body regions of *FOSB* (23,24), pyrosequencing primers encompassing nine and seven CpGs within the promoter and exon 4 regions of the *FOSB* gene were designed, respectively. Representative examples of pyrosequencing are presented in Fig. 1 and revealed that non-CpG cytosine residues were converted to thymine, indicating the completeness of bisulfite treatment. The present results revealed that there were no statistically significant differences of the mean DNA methylation level of *FOSB* promoter (P=0.089) and exon 4 (P=0.175) between tumor and matched normal tissues (Fig. 2). The CGI of the *FOSB* promoter region was completely unmethylated in all nonmalignant and malignant lung tissues. However, compared to the MI of normal tissues, the DNA methylation level of the *FOSB* exon 4 region was markedly (>2-folds) decreased in 18 (10.2% of the total) tumor tissues, indicating that the unmethylation of *FOSB* exon 4 may be a tumor-associated event during NSCLC tumorigenesis.

It was then investigated whether the unmethylation of *FOSB* exon 4 could regulate its mRNA expression in representative lung tissue specimens. Methylation status combined with RT-PCR results revealed low or undetectable levels of *FOSB* transcripts in tumor tissues with unmethylated alleles (213T, 236T and 291T), and high *FOSB* levels in tumor and non-tumor lung tissues with methylated alleles (Fig. 3A and B). This observation was further substantiated by 5-AzadC treatment of NSCLC cell lines. *FOSB* mRNA was absent in H187 cells with unmethylated exon 4, and present in H157 cells with methylated alleles; notably, *FOSB* expression decreased upon 5-AzadC treatment (Fig. 3C). Collectively, these results

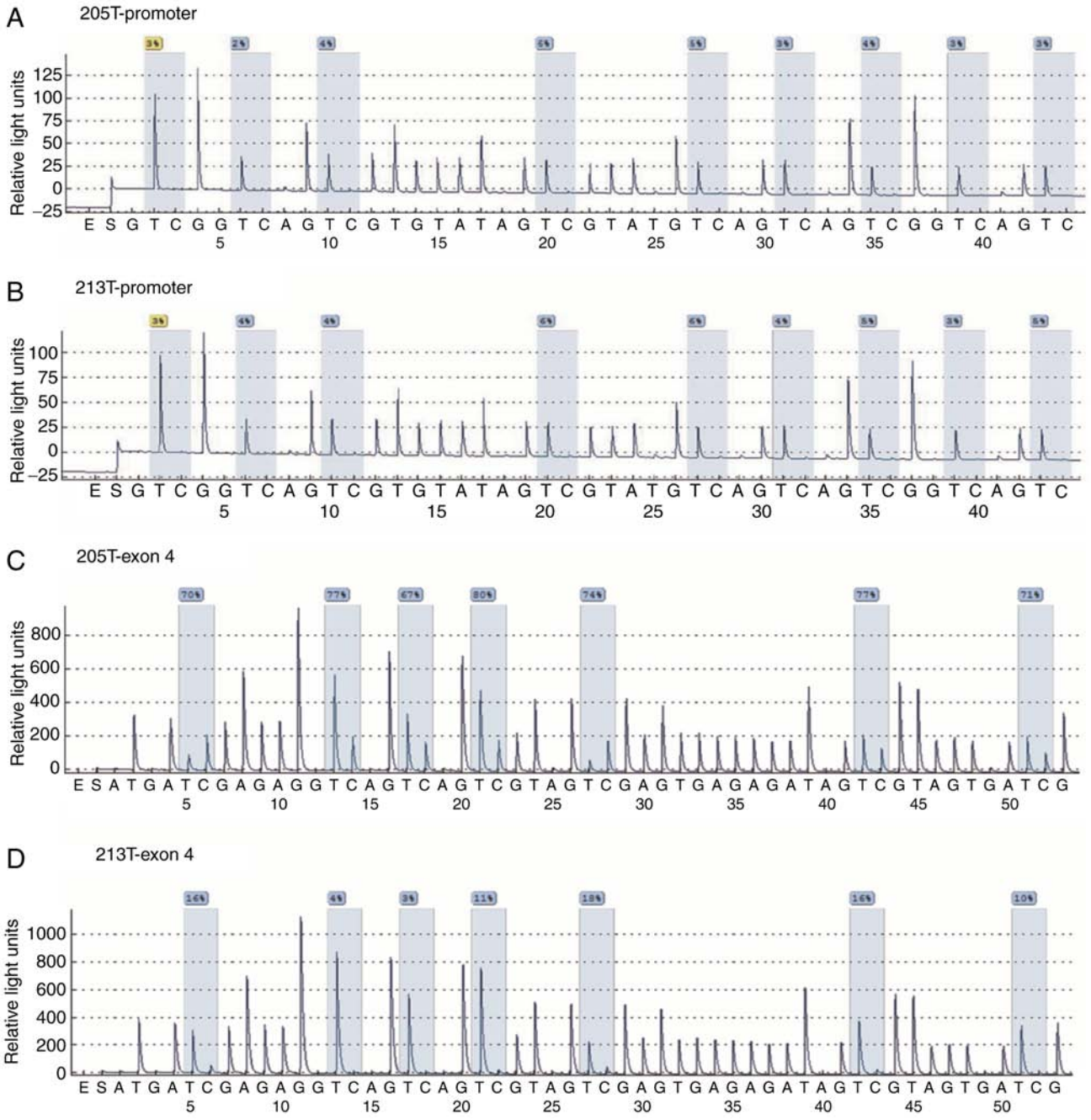


Figure 1. Representative pyrograms of *FOSB* in lung tissues of NSCLC patients. The methylation status of the (A and B) promoter and (C and D) exon 4 regions of the *FOSB* gene was determined in NSCLC primary lung tissues using pyrosequencing. The letters on the axis represent the dispensation order; E, enzyme mix; S, substrate; A, G, C, and T, nucleotides. Shaded bars encompassing T/C pairs indicate the eight investigated CpGs. The methylation of each CpG site was calculated as a percentage of C incorporation. *FOSB*, FBJ murine osteosarcoma viral oncogene homolog B; NSCLC, non-small cell lung cancer.

indicated that exon 4 hypomethylation may be indicative of *FOSB* gene silencing.

Association of FOSB methylation status with clinicopathological parameters and survival outcome. Unmethylated *FOSB* exon 4 was more frequent in patients with lymph node metastasis than in those without metastasis with a borderline significance (17.4 vs. 7.7%, P=0.062; Table I). However, no significant association was revealed in other clinicopathological factors, such as age, sex, smoking status, histology, and pathologic stage (Table I). The methylation status of the *FOSB* exon 4 in adenocarcinoma and squamous cell carcinoma, respectively, was specifically analyzed (Table SII).

Next, Kaplan-Meier survival analysis was performed to determine the prognostic potential of *FOSB* methylation status. The patients with unmethylated *FOSB* had significantly shorter OS than those with *FOSB* methylation (P_{L-R}=0.045, Fig. 4A). After stratification, according to the clinicopathological features, *FOSB* unmethylation was markedly associated with an unfavorable OS in patients with stage II-IIIA (P_{L-R}=0.056; Fig. 4B). To evaluate whether *FOSB* unmethylation is an independent prog-

Table I. Comparison of methylation status of the *FOSB* exon 4 according to characteristics of NSCLC patients.

Variables	Unmethylation, n (%)	Methylation, n (%)	P-value[a]
All subjects	18 (10.2)	158 (89.8)	
Age (years)			
≤64	7 (8.9)	72 (91.1)	0.589
>64	11 (11.3)	86 (88.7)	
Sex			
Men	15 (12.2)	108 (87.8)	0.189
Women	3 (5.7)	50 (94.3)	
Smoking status			
Ever	15 (12.6)	104 (87.4)	0.132
Never	3 (5.3)	54 (94.7)	
Histological types			
SQC	11 (13.1)	73 (86.9)	0.230
ADC	7 (7.6)	85 (92.4)	
Lymph node metastasis			
N0	10 (7.7)	120 (92.3)	0.062
N1 and N2	8 (17.4)	38 (82.6)	
Pathologic stage			
Stage I	6 (6.6)	85 (93.4)	0.100
Stage II-IIIA	12 (14.1)	73 (85.9)	

[a]Comparison between unmethylation and methylation frequency was analyzed using Chi-square test. *FOSB*, FBJ murine osteosarcoma viral oncogene homolog B; NSCLC, non-small cell lung cancer; SQC, squamous cell carcinoma; ADC, adenocarcinoma.

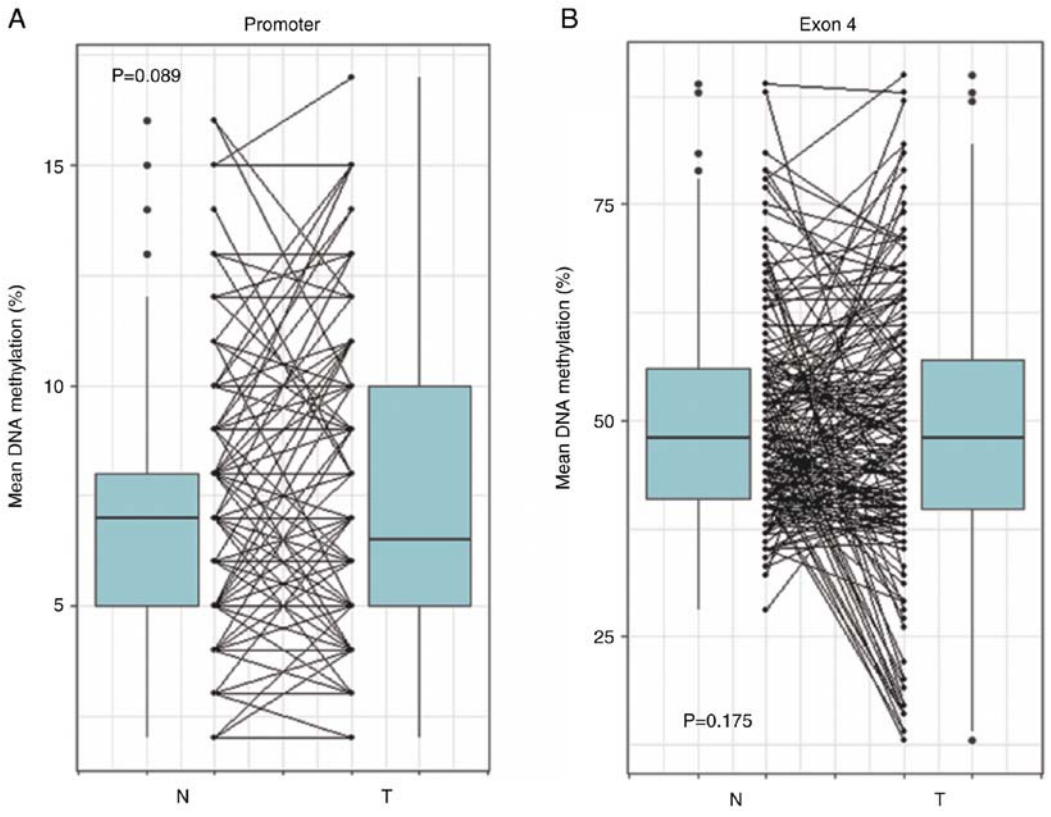
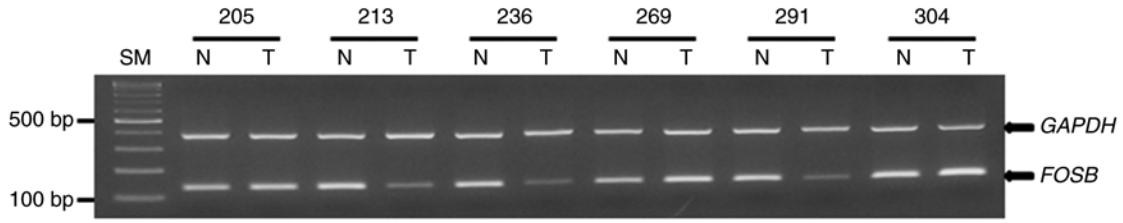


Figure 2. Comparison of the mean methylation level of *FOSB* between malignant and non-malignant tissues. The mean DNA methylation levels of the tested CpG sites in the *FOSB* (A) promoter and (B) exon 4 regions were used to compare their differences between NSCLC tumors and the adjacent normal tissues. P-values were obtained using paired t-tests. *FOSB*, FBJ murine osteosarcoma viral oncogene homolog B; N, adjacent normal tissue; T, tumor tissue; NSCLC, non-small cell lung cancer.

A Pyrosequencing analysis

Tissues	205N	205T	213N	213T	236N	236T	269N	269T	291N	291T	304N	304T
Promoter-MI (%)	6.7	3.7	8.1	4.4	11.2	10.0	6.6	8.9	14.6	15.4	9.3	5.9
Exon 4-MI (%)	62.9	73.7	50.9	11.1	69.4	17.4	71.5	56.7	68.5	27.6	51.4	64.9

B RT-PCR analysis



C 5-AzadC treatment

Cell lines	H187	H187	H157	H157
5-AzadC (μM)	0	20	0	20
Promoter-MI (%)	8.1	8.8	12.4	9.0
Exon 4-MI (%)	21.4	17.0	86.4	28.1

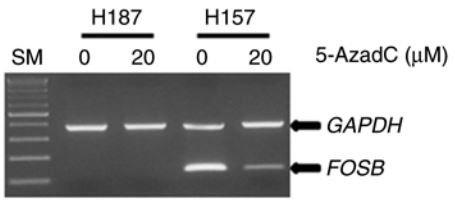


Figure 3. Pyrosequencing and RT-PCR analysis of the *FOSB* gene in patients with NSCLC and cell lines. *FOSB* methylation status and mRNA expression were determined by (A) pyrosequencing and (B) semi-quantitative RT-PCR in NSCLC specimens and (C) cell lines. H157 and H187 cell lines were incubated with 20 μM 5-AzadC for 3 days. *GAPDH* was loaded as an internal control for normalization. SM, 100-bp DNA ladder. *FOSB*, FBJ murine osteosarcoma viral oncogene homolog B; NSCLC, non-small cell lung cancer; MI, mean methylation index; N, adjacent normal tissue; T, tumor tissue; 5-AzadC, 5-aza-2'-deoxycytidine.

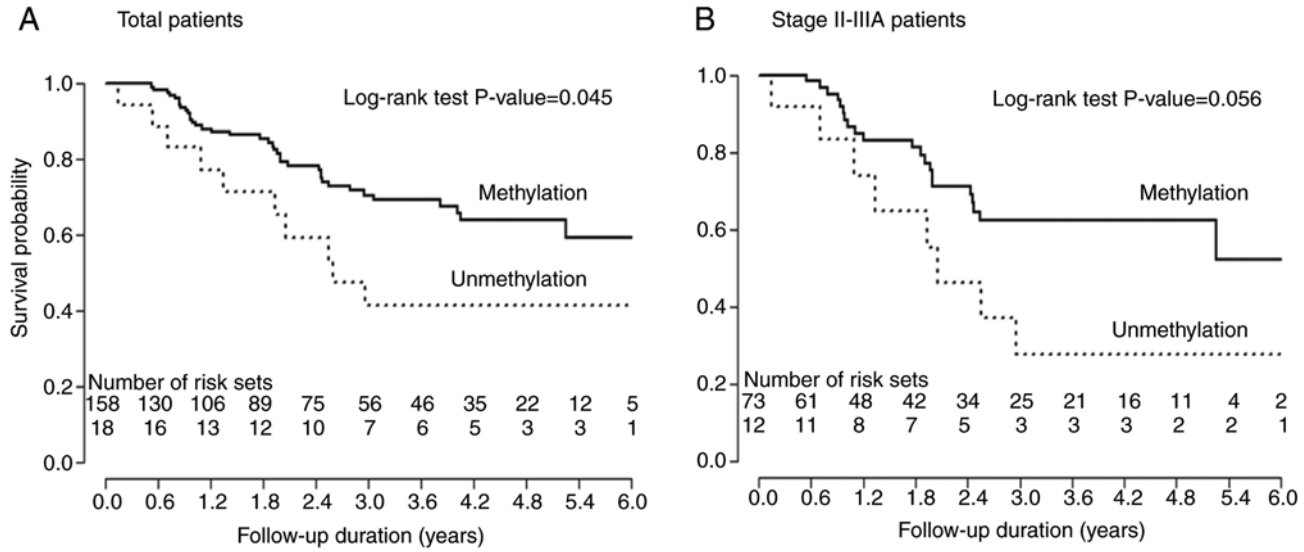


Figure 4. Association of *FOSB* exon 4 unmethylation with worse survival outcome of NSCLC patients. Kaplan-Meier survival curves for (A) all patients and (B) patients with stage II-IIIA NSCLC according to *FOSB* methylation status. P-values are based on the log-rank test. *FOSB*, FBJ murine osteosarcoma viral oncogene homolog B; NSCLC, non-small cell lung cancer.

nostic predictor in NSCLC, the data was further analyzed using the Cox proportional hazards regression model after adjusting for possible cofounding variables of survival. Multivariate analysis revealed that *FOSB* unmethylation was associated with poor survival in patients with stage II-IIIA [adjusted hazard ratio (HR)=2.43, 95% confidence interval (CI)=1.04-5.68, P=0.040], but not in all subjects (Table II).

Table II. Overall survival according to methylation status of *FOSB* genes in NSCLC patients.

Variables	No. of cases	No. of deaths (%)[a]	5-year survival rate (%)[b]	P_{LR}[c]	Adjusted HR (95% CI)	P-value
All subjects	176	47 (26.7)	61.2			
Methylation	158	37 (23.4)	64.2	0.045	1	0.250
Unmethylation	18	10 (55.6)	41.7		1.53 (0.74-3.16)[d]	
Stage I						
Methylation	85	16 (18.8)	65.8	0.812	1	0.905
Unmethylation	6	2 (33.3)	66.7		0.91 (0.20-4.12)[e]	
Stage II-IIIA						
Methylation	73	21 (28.8)	62.5	0.056	1	0.040
Unmethylation	12	8 (66.7)	27.8		2.43 (1.04-5.68)[e]	

[a]Row percentage. [b]Five-year survival rate, proportion of patients who survived, according to Kaplan-Meier analysis. [c]Log-rank P-value. [d]HR, 95% CI and their corresponding P-values were calculated using multivariate Cox proportional hazards model, adjusted variables included age, sex, smoking status, histological type, and pathologic stage. [e]Adjusted variables included age, sex, smoking status, and histological type. *FOSB*, FBJ murine osteosarcoma viral oncogene homolog B; NSCLC, non-small cell lung cancer; HR, hazard ratio; CI, confidence interval.

Discussion

The present study revealed that unmethylation of *FOSB* exon 4 was detected in 18 (10.2%) out of the 176 NSCLC cases and its unmethylation was related to loss of *FOSB* mRNA expression. The genomes of cancer cells tend to show widespread gene body hypomethylation alongside hypermethylation of gene promoter (25). Numerous studies have investigated the diagnostic and prognostic values of promoter methylation (5). However, data on the role and nature of gene body methylation in specific genes are currently limited. Thus, the present results represent the first demonstration that the gene body (exon 4) region of the *FOSB* gene can undergo a DNA methylation alteration in NSCLC patients. Similarly, Suzuki *et al* revealed that increased DNA methylation in the gene body led to the upregulation of *FOSB* in liver tumors of arsenic-exposed mice (24). DNA methylation in promoter sequences is well known to silence genes and is the presumed therapeutic target of methylation inhibition (26). Notably, DNA methylation is more prevalent within gene bodies than appears for promoters (27,28), and gene body methylation appears to be actively involved in multiple gene regulation processes including alternative promoter usage, regulation of short and long non-coding RNAs, alternative splicing and enhancer activity (29,30), indicating a contrasting association between DNA methylation and expression across the promoter (negative association) vs. gene body (positive association).

One notable finding of the present study is the association of *FOSB* methylation changes with survival outcomes in a subset of patients with NSCLC, indicating that *FOSB* may be a new biomarker for the prognosis of NSCLC. Moreover, the present results provide evidence that FOSB may possess tumor-suppressive properties in NSCLC. These observations are in line with a study revealing that FOSB is an anti-metastatic protein in lung cancer because it negatively regulates MMP9 expression, which induces cell migration and invasion (21). Notably, the present data revealed that the unmethylation of *FOSB* exon 4 tends to occur in patients with lymph node metastases. *FOSB* expression was significantly downregulated in gastric and pancreatic cancer tissues and its downregulation was associated with reduced survival (18,19). *FOSB* overexpression also triggered cell death by regulating the expression of MMP9 in MCF breast cancer cells (31). Collectively, the present investigation indicated that FOSB exerts a tumor-suppressive function in NSCLC. The mechanism of its action requires future investigations. However, FOSB may be used as a novel biomarker or a specific therapeutic target for lung cancer.

Some of the limitations of this study include its retrospective design and the relatively small sample size, indicating that the results may be influenced by selection bias. Thus, further large-scale studies and longer follow-up periods are required to verify the clinical significance of *FOSB* expression. Collectively, it was revealed that the decrease in DNA methylation levels of the *FOSB* exon 4 was associated with decreased expression of *FOSB* mRNA in tumor tissues and with unfavorable outcomes of patients with later stages of lung cancer. It is anticipated that accumulating knowledge on gene body methylation will provide valuable information in the future, especially to develop effective tools for DNA methylation-targeted therapy.

Acknowledgements

We would like to thank the National Biobank of Korea, KNUH for providing patient material and data.

Funding

The present study was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) (No. NRF-2016R1D1A1B03931462).

Availability of data and materials

The accompanying data underlying our findings are available upon request to corresponding author.

Authors' contributions

DSK and JYP drafted the manuscript, conceived and coordinated the study, and were responsible for interpretation of the data. WKL performed the statistical analyses. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was conducted with the approval of IRB, KNUH (approval no. 2014-04-210). Participants provided their written informed consent to participate in this study and for the publication of all associated data.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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