Aberrant methylation of dipeptidyl peptidase-like 6 as a potential prognostic biomarker for lung adenocarcinoma

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Abstract. We previously performed the genome-wide screening of aberrantly methylated CpG islands (CGIs) using the paired tumorous and non-tumorous tissues of 12 lung adenocarcinomas (LADC). In comparisons with paired normal lung tissues, dipeptidyl peptidase-like 6 (*DPP6*) has been identified as the most significantly hypermethylated CGI in LADC. *DPP6* is a protein that modulates A-type potassium channels in the somatodendritic compartments of neurons, which play a role in synaptic plasticity. Previous studies have showed that *DPP6* is downregulated in cancers, such as acute myeloid leukemia and melanoma, but upregulated in colon cancer, which is attributed to hyper- and hypomethylation, respectively. The present study investigated the methylation and expression levels of *DPP6* and its prognostic value in patients with LADC. The DNA methylation and mRNA expression levels of *DPP6* in surgically resected LADC tissues were examined by bisulfite pyrosequencing and reverse transcription-quantitative PCR, respectively. The DNA methylation and mRNA expression levels of *DPP6* were both significantly higher in LADC tissues compared with in normal lung tissues (n=25; P<0.0001). Overall and disease-free survival rates were significantly higher in LADC with high mRNA expression levels compared with those with low levels. In conclusion, epigenetic alterations in *DPP6* were significantly higher in LADC tissues compared with in normal lung tissues, which may contribute to the malignant features and worse prognosis of these patients.

Introduction

According to Global Cancer Observatory data, lung cancer is the second most common cancer worldwide (1). Although smoking is still a dominant risk factor for lung cancer, other risk factors, including environmental and occupational exposure, chronic lung disease, and lifestyle factors, contribute to the risk of developing lung cancer. Lung adenocarcinoma (LADC) is the most common type of lung cancer, accounting for approximately 50% of all cases, and its frequency is increasing. Even though the major cause of LADC is smoking, it is also the most common type of lung cancer in non-smokers (2).

In the past decade, advances have been achieved in the epidemiology and prevention of lung cancer. Nevertheless, lung cancer remains the leading cause of cancer death in both sexes and in all ages. The lack of specific symptoms in the early stages and practical biomarkers for screening and the prediction of patient outcomes partially explain the poor survival rate and mortality of these patients (3).

Oncogenesis is a highly complex, multi-step process involving genetic and epigenetic changes. Lung cancer has a higher tumor mutational burden than other cancer types, which may be related to smoking and exposure to tobacco xenobiotics (4). A previous study examined the rates of protein-altering mutations in 441 tumors. The findings obtained showed that non-small cell lung cancer (NSCLC) had one of the highest rates of protein-altering mutations, with rates in adenocarcinomas and squamous cell carcinomas of 3.5 and 3.9 per megabase, respectively (5). NSCLC treatment strategies and prognoses are markedly affected by the stage of the disease at diagnosis. Surgery is highly effective in the early stages, but ineffective in patients with metastasis, which accounts for the majority of cases at the time of diagnosis. In a previous study, up to 69% of advanced NSCLC patients were estimated to have targetable mutations in a number of

genes, including epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase, c-ROS oncogene 1, Kirsten rat sarcoma virus, V-RAF murine sarcoma oncogene homolog B1, mesenchymal-epithelial transition factor receptor, and human EGFR-related 2 (6).

Epigenetic alterations have been detected in all human cancers, and these changes, including DNA methylation, play an important role in the early development and progression of diseases, particularly cancer (7). The DNA methylation of intermittently distributed CpG sites down-regulates the expression of tumor suppressor genes. Furthermore, aberrant DNA methylation patterns have been identified in many cases of lung cancer, with most being located in promoter sequences (8,9). Moreover, a progressive CpG island (CGI) methylation pattern was detected in atypical adenomatous hyperplasia and adenocarcinoma *in situ* that was followed by further methylation and the development of LADC (10,11). These findings suggest that DNA methylation has potential in the development of biomarker candidates for the early detection of cancer and outcome predictions.

We previously performed the genome-wide screening of aberrantly methylated CGIs using the paired tumorous and non-tumorous tissues of 12 LADC samples. The top 10 significantly methylated genes were noted and the gene showing the highest methylation level was *dipeptidyl peptidase-like 6 (DPP6)*. Furthermore, the expression of *DPP6* was down-regulated in cancer cell lines (12). *DPP6* is a type II transmembrane protein and a member of the prolyl oligopeptidase family of serine proteases (13,14). *DPP6* is mostly studied as an auxiliary subunit of voltage-gated K+ channels of the Kv4 family. However, *DPP6* hypermethylation has been identified in several cancer types (15-19).

In the present study, we examined the DNA methylation and gene expression levels of *DPP6* in resected LADC tissues to assess its potential as a prognostic marker for LADC.

Materials and methods

Patients and tissue samples. Seventy-three LADC tissues, including 25 pairs of tumor-matched normal tissues, were collected from patients who underwent surgery at Tokushima University Hospital between April 1999 and November 2013. Tissue samples were snap-frozen and stored at 80°C for the later isolation of DNA and RNA. A methylation analysis and RT-PCR were performed on 73 tumor samples and 25 normal lung samples. Tumor staging was conducted based on the seventh tumor-node-metastasis classification for lung cancer (20). Tumors were classified according to the predominant histological subtype proposed by the 2015 WHO classification (21). The mean follow-up duration for 162 patients with LADC was 48 months (range, 0.6-147 months), with 45 cases of recurrence in (27.8%) and 34 of death (21.0%).

The Ethics Committee of the University of Tokushima approved the present study (Tokushima University Hospital, approval no. 4071), and procedures were conducted according to the Declaration of Helsinki. All patients provided written informed consent.

Clinical characteristics. Clinical information on 73 LADC patients, including survival data and basic characteristics,

such as sex, age at diagnosis, tumor stage, histology, smoking history, and EGFR mutations, was obtained and shown in Table I.

Global methylation analysis. We previously screened 12 paired tumorous/non-tumorous LADC sample sets obtained from freshly frozen specimens using Illumina HumanMethylation450 K Bead Chip to identify aberrantly methylated CGIs in a genome-wide manner. Nineteen CGIs were identified as differentially hypermethylated in the *DPP6* gene, with a false discovery rate of <0.05 and β difference (tumor-non-tumorous tissue) of >0.25. CGI in *DPP6* was ranked as the most hypermethylated CGI with a high P-value. A schematic diagram of the *DPP6* mRNA structure is shown in Fig. 1A. Among the 26 exons of *DPP6* mRNA, the CGI was located around exon 1. Fig. 1B shows the results of the array-based methylation status of each CpG site within *DPP6*. We examined the DNA methylation of 4 CpG sites near cg00017489 of the *DPP6* gene.

Nucleic acid isolation. DNA and RNA were extracted from frozen tissue using the QIAamp DNA Mini Kit 50 and RNeasy Mini kit (Qiagen GmbH) according to the manufacturer's instructions.

Bisulfite pyrosequencing. Genomic DNA was converted to bisulfite using the manufacturer's specified EpiTect Bisulfite Kit 48 (Qiagen). Transformed DNA was amplified by PCR with Pyromark PCR-Kit 200 (Qiagen) before pyrosequencing. Sequencing primers were developed using PyroMark Assay Design 2.0: forward, TTTGGGTGGGTT TGTATATGAATTTG; reverse, (biotin)-TACCCCTAAACC CTATTCCAT-CAATCATC. Pyrosequencing was performed according to published protocols using PSQ 96MA (Qiagen) to obtain the methylation rate at the mean of four CpG sites. The methylation rate at each CpG was calculated using PyroQ-CpG 1.0.9.

Real-time quantitative PCR. Reverse transcription was performed with iScript Reverse transcription Supermix. SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad) and the *DPP6* Taqman Gene expression assay were used for quantitative PCR employing the following primers: *DPP6* forward 5'-ATGCAGGGGAACGTGATG-3', *DPP6* reverse 5'-GCAGTGCAATTGCTATTCCTT-3. GAPDH forward 5'-AGCCACATCGCTCAGACAC-3', *GAPDH* reverse 5'-GCC CAATACGACCAAATCC-3' *GAPDH* was used as the internal control gene.

Statistical analysis. The paired *t*-test and Wilcoxon signed-rank test were used for comparisons between paired samples when data were and were not normally distributed, respectively. The relationships between methylation and mRNA expression levels and clinical characteristics, including tumor stage, histological patterns, smoking history, blood vessel invasion, lymph vessel invasion, pleural invasion, and lymph node metastasis, were examined using the unpaired *t*-test for normally distributed data and the Mann-Whitney test for not normally distributed data. Multiple group comparisons were conducted using a one-way analysis

Table I. Patient characteristics.

Characteristics	Value, n	% or IQR
Sex		
Male	39	53%
Female	34	47%
Age	66.8±9.8	(43-84)
Non-smoker	35	48%
Smoker	38	48%
Brinkman index	783±487	
Pathology		
Lepidic	23	31.5%
Papillary	26	35.6%
Solid	3	4.1%
Acinar	5	6.8%
Mixed or others	16	21.9%
Operation		
Pneumonectomy	1	1.4%
Lobectomy	69	94.5%
Segmentectomy	1	1.4%
Lobectomy + segmentectomy	3	4.1%
Complete resection	73	100%
Preoperative chemotherapy	1	1.4%
Postoperative chemotherapy	20	27.4%
Cisplatin-based	6	
UFT	13	
Other	1	
Pathological stage		
IA	38	52.1%
IB	20	27.4%
IIA	9	12.3%
IIB	4	5.5%
III	2	2.7%
pN factor		
0	65	89.0%
1	6	8.2%
2	2	2.7%
Pl factor positive	16	21.9%
v factor positive (n=71)	12	16.9%
ly factor positive	12	16.4%
EGFR mutation (n=24)	9	37.5%

of variance followed by Tukey's multiple comparison test for normally distributed data and the Kruskal-Wallis test followed by Dunn's multiple comparison test for not normally distributed data. The relationships between the methylation and mRNA expression levels of *DPP6* and basic clinical characteristics were examined by the chi-square test. Survival data analyzed by the Kaplan-Meier analysis with the Log-rank test were used to compare overall survival (OS) and disease-free survival (DFS) rates across high/low methylation and high/low mRNA expression levels. The cut-off values were made from ROC curves.

The multivariate survival analyses were calculated using the likelihood ratio test of the stratified Cox's proportional hazard regression analysis. All statistical analyses were performed using GraphPad Prism, version 5.00; and SPSS (version 24.0; IBM Corp.).

Results

Methylation and mRNA expression levels of DPP6 in LADC samples and paired adjacent normal tissues. The methylation and mRNA expression levels of the *DPP6* gene were examined in 25 matched samples. Fig. 2A shows the methylation levels of the *DPP6* gene in LADC and normal lung tissues. DNA methylation levels were significantly higher in tumor samples than in normal samples (the Wilcoxon signed-rank test, P<0.0001). Fig. 2B shows the mRNA expression levels of *DPP6* in LADC and normal lung tissues. mRNA expression levels were significantly lower in tumor samples than in normal samples (the Wilcoxon signed-rank test, P<0.0001).

The relationship between the DNA methylation and mRNA expression levels of *DPP6* in 98 samples /73 tumor and 25 normal/ was examined with Spearman's rank correlation (Fig. 2C). The obtained results revealed a negative relationship between methylation and mRNA expression levels (P=0.0001, ϱ=-0.5231).

Relationships between methylation and mRNA expression levels of DPP6 and the malignant behavior of LADC. To examine the effects of the hypermethylation of *DPP6* on the malignancy and carcinogenesis of LADC, we measured methylation and mRNA expression levels in 73 samples and compared the results obtained with histology patterns and stage grading.

DPP6 methylation levels were lower in the IA group than in the other groups. Furthermore, slightly higher mRNA expression levels were observed in the IA group (Fig. 3A and 3B). No significant differences were observed in methylation levels in samples with the lepidic and other histological patterns (Fig. 3C). However, *DPP6* mRNA expression levels were higher in samples with the lepidic pattern than in those with other histological patterns (Fig. 3D) (the Mann-Whitney test P=0.0393). The relationships between the methylation and mRNA expression levels of *DPP6* and basic clinical characteristics are shown in Table II. Data were analyzed by cross-tabulation with the chi-square test. Methylation levels were significantly higher in LADC with than in that without pleural invasion (Table II). mRNA levels were significantly lower in LADC with than in that without pleural invasion (Table II). LADC with vascular invasion had higher methylation and lower mRNA expression levels of *DPP6*. The relationships between the mRNA expression levels of DPP6 and blood and lymph vessel invasions are shown in Fig S1.

Prognostic value of DPP6 mRNA expression and methylation levels in LADC. We performed DFS and OS analyses of the mRNA expression and methylation levels of *DPP6* (Fig. 4). Samples were divided into high/low expression and high/low methylation levels using cut-off values
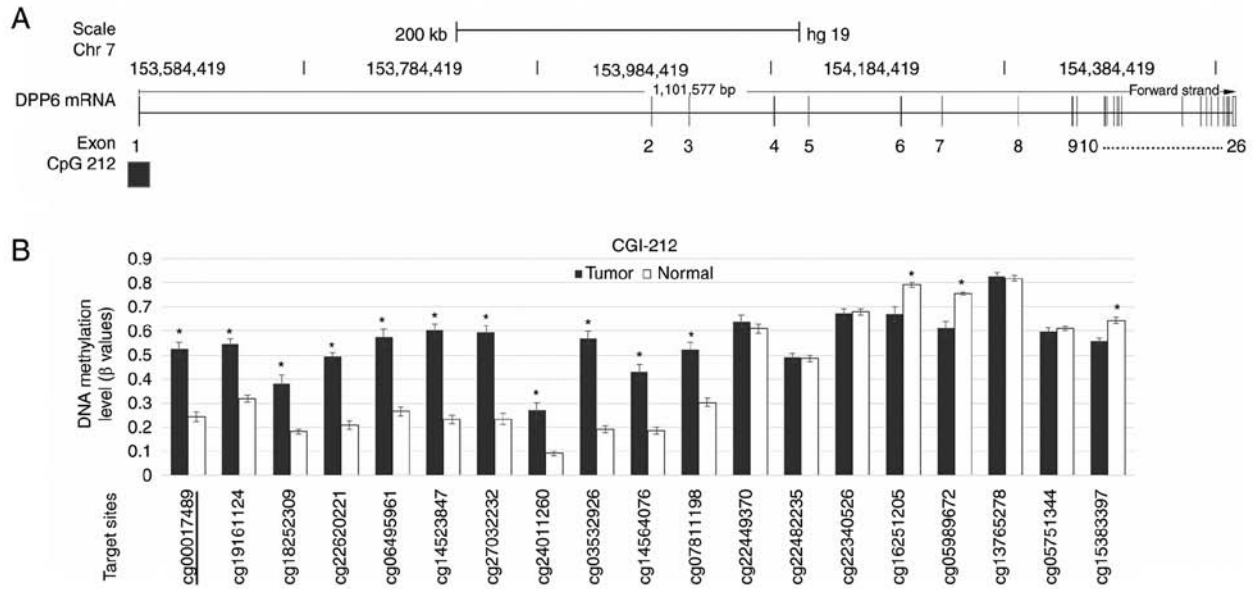
Figure 1. Methylation levels of CpG sites in the DPP6 gene. (A) Schematic structure of human DPP6 mRNA. The mRNA of DPP6 has 26 exons. CpG island 212 (black box) is located around exon 1. (B) DNA methylation levels. The vertical scale indicates the average β-value for the methylation level of each CpG site between LADC tissue (black bars) and normal tissues (white bars). All target sites are located in CpG island 212. The methylation value of underlined CpG site was evaluated in the present study. *P<0.05.
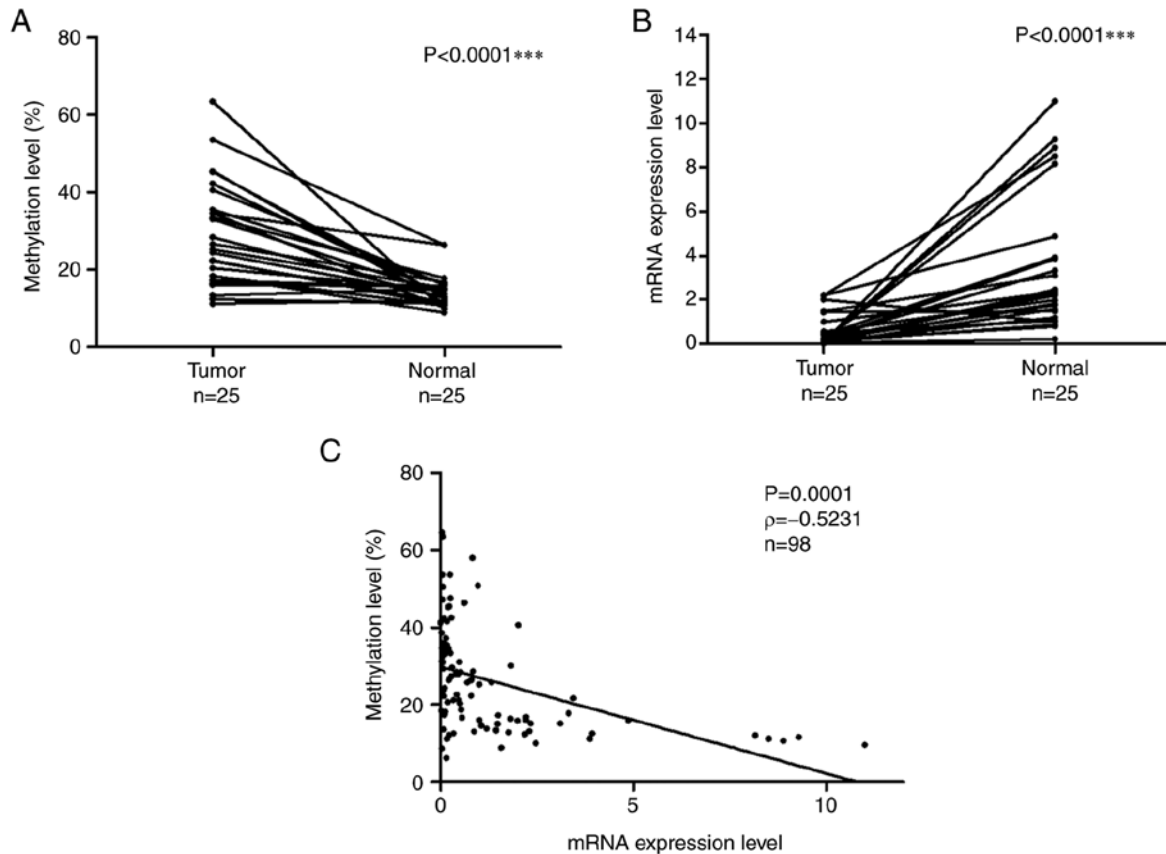

Figure 2. DNA methylation and mRNA expression levels of DPP6. (A) Methylation levels of DPP6 in 25 paired LADC tumors and adjacent normal tissues evaluated by pyrosequencing. (B) mRNA expression levels of DPP6 in 25 paired LADC tumors and adjacent normal tissues. (C) Relationship between DNA methylation and mRNA expression levels of DPP6 in 98 samples. Data were analyzed by Spearman's rank correlation. ***P<0.0001 DPP6, dipeptidyl peptidase-like 6.

generated from the ROC/Receiver-Operating character-istic/curves (Fig. 5). DFS and OS rates were significantly higher in samples with high mRNA expression levels of

DPP6 than in those with low levels (the Kaplan-Meier analysis, DFS P=0.0016 cut-off value 0.4 and OS P=0.0184 cut-off value 0.3).
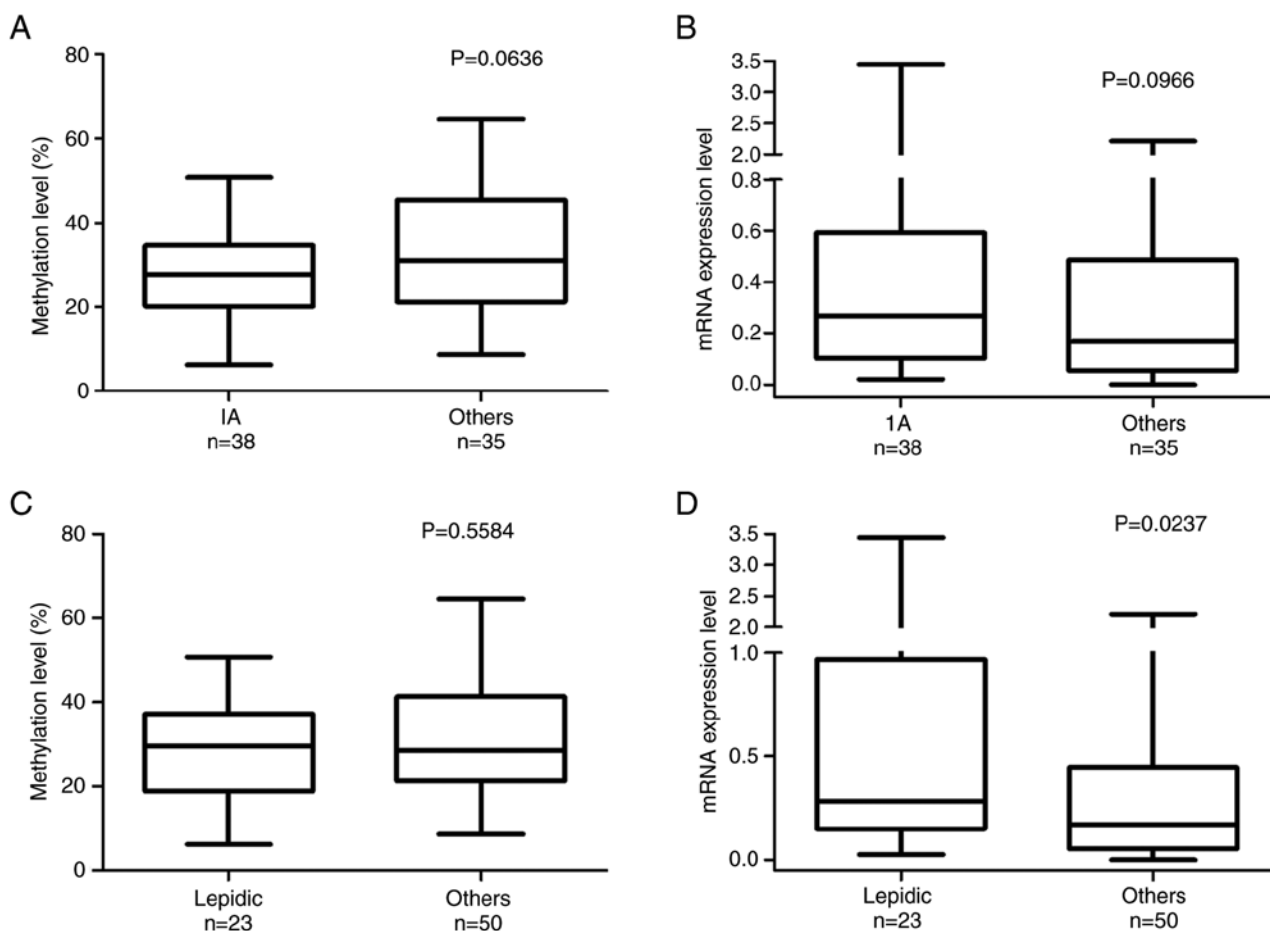
Figure 3. DNA methylation and mRNA expression levels of DPP6 in lung cancer stages and histological patterns. The methylation and mRNA expression levels of DPP6 in LADC tumor samples (n=73) were analyzed using pyrosequencing and reverse transcription-quantitative PCR, respectively. According to the World Health Organisation histological classification, the (A) methylation (%) and (B) mRNA expression levels of DPP6 are shown in lung cancer stages (IA, n=38; others: IB, n=20; IIA, n=9; IIb, n=4; IIIa, n=1). The (C) methylation (%) and (D) mRNA expression levels of DPP6 are shown in lung cancer histological patterns (lepidic, n=23; others: papillary, n=26; acinar, n=5; solid, n=3; and mixed n=16). DPP6, dipeptidyl peptidase-like 6.

However, no significant differences were observed in DFS or OS between samples with high and low *DPP6* methylation levels (the Kaplan-Meier analysis, DFS P=0.1589 cut-off value 27 and OS P=0.233 cut-off value 34.5). The ROC curves of DFS and OS are shown in (Fig. 5).

The multivariate Cox's regression analysis revealed that the tumor staging (HR; 0.289, 95% CI; 0.094-0.885 P=0.030) was an independent prognostic factor for DFS (Table III) and the patients' age (HR; 5.377, 95% CI; 1.086-26.623 P=0.039) for OS (Table III). According to the multivariate analysis the DNA methylation and mRNA expression level of DPP6 were not significant to predict DFS and OS.

Discussion

In addition to its role in the modulation of A-type potassium channels in neurons and also in neuronal development and synaptic plasticity (13), *DPP6* is involved in tumorigenesis (22,23). Furthermore, the hypermethylation of *DPP6* has been identified in several cancer types and is associated with survival rates, which will be discussed in the following paragraphs.

We examined the methylation and gene expression levels of the *DPP6* gene. Low methylation and high mRNA expression levels of *DPP6* were observed in normal tissues. On the other hand, high methylation and low mRNA expression levels of *DPP6* were detected in tumor tissues. These results indicate an inverse relationship between the methylation and mRNA expression levels of *DPP6* in LADC. The relationship between the DNA methylation and mRNA expression levels of *DPP6* was analyzed by Spearman's rank correlation (P=0.001).

DPP6 is primarily a tumor suppressor gene. A previous study on pancreatic ductal adenocarcinoma reported that the β-value of the *DPP6* promoter was significantly higher in tumor samples than in normal samples, while the methylation levels of the *DPP6* promoter negatively correlated with mRNA expression levels in 72 paired tissues (15). Another study detected the hypermethylation of *DPP6* in esophageal adenocarcinoma, which showed the down-regulated mRNA expression of *DPP6* in 78 paired samples (16). Furthermore, *DPP6* was shown to be significantly methylated in acute myeloid leukemia, and analyses of its gene expression revealed its down-regulation (17). Collectively, these findings indicate that *DPP6* is tightly regulated during oncogenic development. Moreover, the hypermethylation of *DPP6* was reported in clear cell renal cell carcinoma (ccRCC) and was associated with advanced and high-grade tumors (18). *DPP6* was also identified as one of the differentially methylated genes in LADC.

Table II. Characteristics of patients grouped by the median value of DNA methylation or mRNA expression.

A, Characteristics of patients grouped by the median value of DNA methylation

Factors	Group	DPP6 methylation level		P-value
		High	Low	
Sex	Female/male	20/17	14/22	0.194[a]
Age±SD		67.56±10.29	66.25±9.506	0.778[b]
Smoking	Non-smoker/smoker	15/21	20/17	0.290[a]
Stage (ver.7)	IA/others	15/21	23/14	0.080[a]
IP	-/+	35/2	32/4	1.000[a]
Emphysema	-/+	35/2	34/2	1.000[a]
Tumor size (mm±SD)		28.72±13.17	25.44±14.31	0.202[b]
Metastasis	-/+	33/3	32/5	0.711[a]
Pleural	-/+	23/13	34/3	0.004[a]
Vascular	-/+	26/9	33/3	0.050[a]
Lymph vessel	-/+	30/6	31/6	0.959[a]
Pathology	*Lep/others*	12/24	11/26	0.740[a]

B, Characteristics of patients grouped by the median value of mRNA expression.

Factors	Group	DPP6 methylation level		P-value
		High	Low	
Sex	Female/male	15/21	19/18	0.407[a]
Age±SD		67.38±8.597	66.25±11.02	0.620[b]
Smoking	Non-smoker/smoker	20/17	15/21	0.290[a]
Stage (ver.7)	IA/others	21/16	17/19	0.415[a]
IP	-/+	34/3	33/3	1.000[a]
Emphysema	-/+	36/1	33/3	0.358[a]
Tumor size (mm±SD)		25.35±10.44	29±16.29	0.690[c]
Metastasis	-/+	33/3	32/5	0.711[a]
Pleural	-/+	21/6	26/10	0.233[a]
Vascular	-/+	34/2	25/10	0.010[a]
Lymph vessel	-/+	34/3	27/9	0.050[a]
Pathology	Lep/others	14/23	9/27	0.238[a]

[a]Chi-square test, [b]Unpaired t-test, [c]Mann-Whitney test. IP, Interstitial pneumonia

This study used data obtained from UCSC Xena, which included 29 paired samples. The methylation levels of *DPP6* were higher in tumor samples than in normal samples (19). These findings indicate that the hypermethylation of *DPP6* occurs in several cancer types and contributes to the low mRNA expression levels of *DPP6*.

In the present study, the methylation and expression levels of *DPP6* were associated with lung cancer stages and histological patterns. Low methylation and high expression levels of *DPP6* were detected in the early stage of LADC (IA). In contrast, high methylation and low expression levels of *DPP6* were observed in the advanced stages of LADC (including IB, II, and III). The methylation levels of *DPP6* were lower in samples with the lepidic pattern than in those with other histological patterns. Furthermore, the mRNA expression levels of *DPP6* were significantly higher in the former than in the latter. LADC with a lepidic histology pattern is less malignant than that with other histological patterns.

DPP6 was identified as one of the genetically altered genes that regulate vascular invasion in human pancreatic cancer (24). In the present study, positive blood and lymph vessel invasion was associated with low mRNA expression levels of *DPP6* (Fig. S1). Collectively, these findings and the present results suggest that high methylation and low expression levels of *DPP6* have a negative impact on the prognosis of patients with several types of cancers, including LADC.

The survival rate of patients with lung cancer is low due to the lack of specific symptoms in the early stages and practical
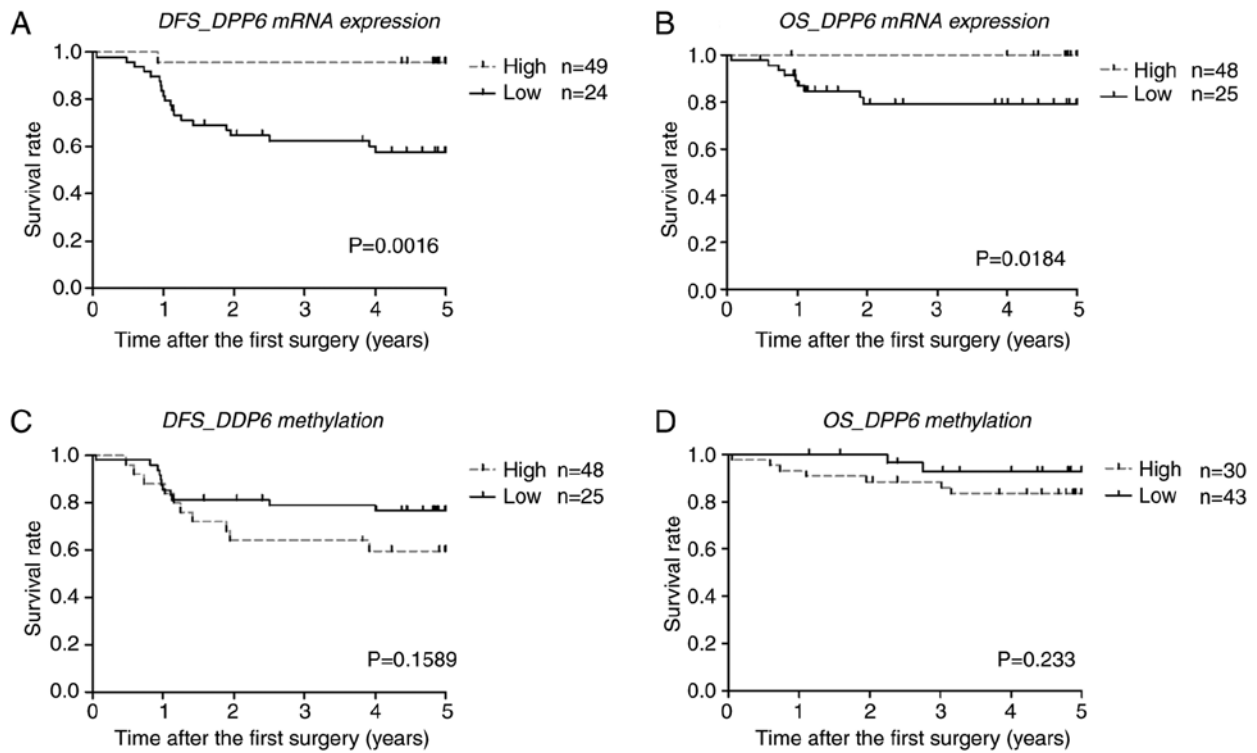
Figure 4. Relationships between DNA methylation and mRNA expression levels of DPP6 and survival rates. (A) Disease-free survival rates according to DPP6 mRNA expression levels. (B) Overall survival rates according to DPP6 mRNA expression levels. (C) Disease-free and (D) overall survival rates according to DPP6 methylation levels. DPP6, dipeptidyl peptidase-like 6.
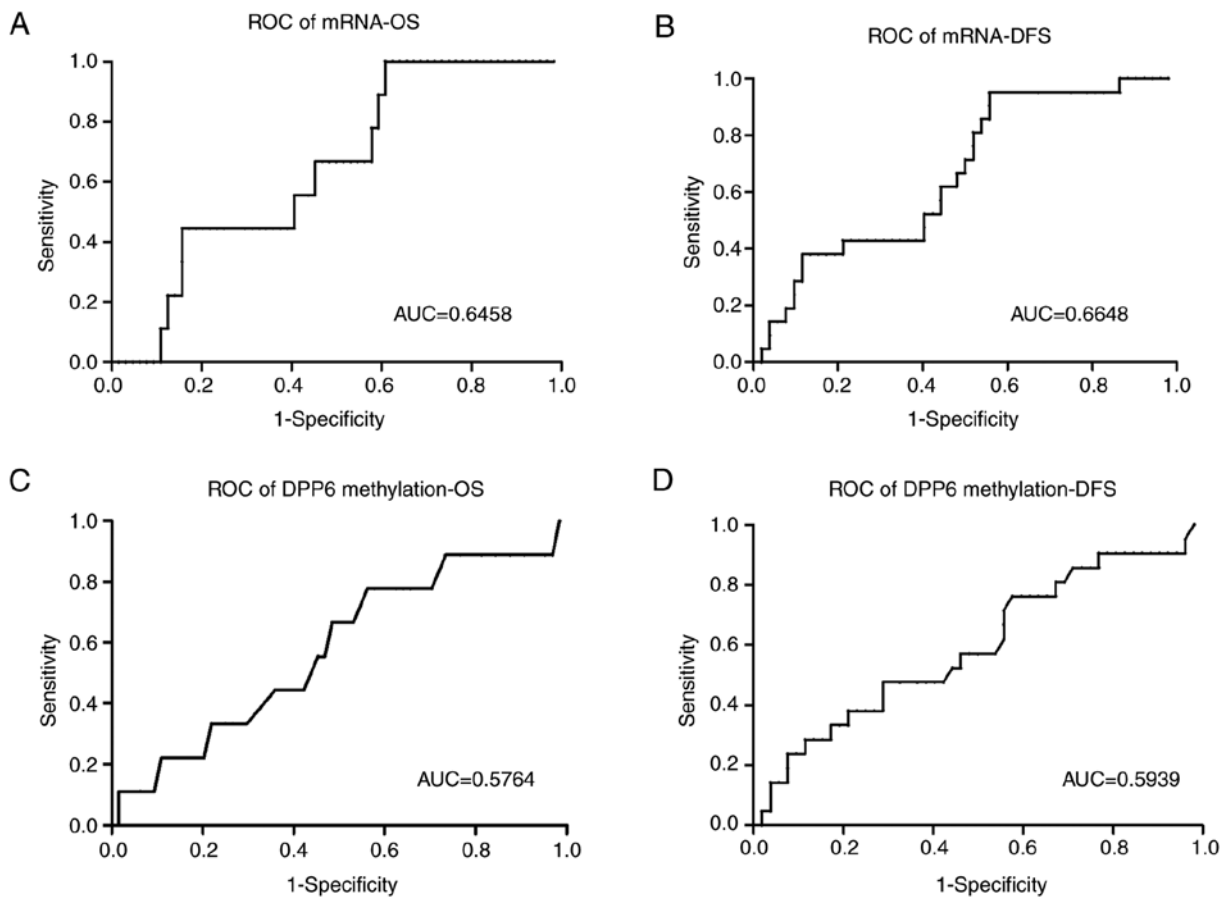

Figure 5. ROC curve of disease-free survival and overall survival. (A) ROC curve of disease-free survival using DPP6 mRNA expression. (B) ROC curve of overall survival using DPP6 mRNA expression. (C) ROC curve of disease-free survival using DPP6 DNA methylation level. (D) ROC curve of overall survival using DPP6 DNA methylation level. AUC, area under the curve; ROC, receiver operating curve; DPP6, dipeptidyl peptidase-like 6.

Table III. Cox proportional hazard regression analysis of disease-free and overall survival in 73 lung adenocarcinoma samples.

A, Cox proportional hazard regression analysis of disease-free survival in 73 lung adenocarcinoma samples.

	Multivariate analysis		
Factors	Hazard ratio	95% CI	P-value
Sex, male (n=39) vs. female (n=34)	1.075	0.149-7.734	0.943
Age, ≥67 (n=36) vs. <67 (n=37)	2.475	0.967-6.334	0.059
Smoking, non-smoker (n=35) vs. smoker (n=38)	0.344	0.049-2.432	0.285
Stage, (ver.7) IA (n=38) vs. others (n=35)	0.289	0.094-0.885	0.030
Metastasis, negative (n=65) vs. positive (n=8)	2.994	0.880-10.183	0.079
Lymph vessel, negative (n=61) vs. positive (n=12)	2.048	0.591-7.101	0.259
Pathology, lepidic (n=23) vs. others (n=50)	0.611	0.165-2.262	0.461
mRNA expression, low (n=37) vs. high (n=36)	1.991	0.731-5.425	0.178
DNA methylation, low (n=36) vs. high (n=37)	0.695	0.271-1.782	0.449

B, Cox proportional hazard regression analysis of overall survival in 73 lung adenocarcinoma samples.

	Multivariate analysis		
Factors	Hazard ratio	95% CI	P-value
Sex, male (n=39) vs. female (n=34)	0.900	0.012-68.553	0.962
Age, ≥67 (n=36) vs. <67 (n=37)	5.377	1.086-26.623	0.039
Smoking, non-smoker (n=35) vs. smoker (n=38)	0.505	0.006-39.686	0.759
Stage, (ver.7) IA (n=38) vs. others (n=35)	0.389	0.076-1.983	0.256
Metastasis, negative (n=65) vs. positive (n=8)	0.351	0.025-4.898	0.436
Lymph vessel, negative (n=61) vs. positive (n=12)	1.673	0.220-12.719	0.619
Pathology, lepidic (n=23) vs. others (n=50)	0.293	0.029-2.915	0.295
mRNA expression, low (n=37) vs. high (n=36)	1.748	0.398-7.682	0.460
DNA methylation, low (n=36) vs. high (n=37)	0.562	0.127-2.480	0.447

biomarkers for screening and outcome predictions. Therefore, we analyzed OS and DFS based on a receiver operating characteristic curve. The methylation level of *DPP6* did not significantly differ between the groups examined; however, the survival rate was higher in the group with low methylation levels than in that with high methylation levels. On the other hand, the survival rate was significantly higher in the group with high mRNA expression levels than in that with low expression levels.

A previous study examined the survival rates of patients with breast cancer based on the expression of *DPP6*. According to Kaplan-Meier plots, high expression levels of *DPP6* were associated with higher DFS rates (n=2898) (25). Furthermore, a Kaplan-Meier survival analysis of candidate biomarkers and 12 genes, including *DPP6*, revealed their significant effects on the prognosis of pancreatic cancer patients. The OS rate was higher in the group with high mRNA expression levels of *DPP6* (n=150) (26). Therefore, high methylation and low mRNA expression levels of *DPP6* may have a negative impact on the prognosis of patients with cancer.

Limited information is currently available on the oncogenic properties of the *DPP6* gene. In patients with esophageal carcinoma, high expression levels of *DPP6* were associated with shorter survival, and *DPP6* was negatively regulated by activating enhancer-binding protein 2 (AP-2α) and positively by AP-2γ (27). Furthermore, in patients with esophageal squamous cell carcinoma, DPP6 was up-regulated by ARID3A and down-regulated by ZNF354C (28). *DPP6* is also involved in the regulation of cell-specific phenotypes and its deregulation may result in carcinogenesis. Low methylation and high expression levels of the *DPP6* gene have been observed in colon cancer (29). Based on these findings, *DPP6* may act as a tumor suppressor gene in LADC; however, the methylation and expression levels of *DPP6* may depend on the cancer type.

We performed immunohistochemistry to detect the expression of *DPP6*. However, we were unable to evaluate the results obtained because the protein expression of *DPP6* in healthy lungs was generally low. Therefore, we need a more sensitive assay to assess tissue protein expression. Furthermore, *DPP6* was stained in the interstitial tissues, but not tumor cells of tumor tissues, indicating non-specific staining. The function of *DPP6* in the regulation of tumor progression currently remains unknown. Therefore, further studies are needed to obtain more detailed insights into this novel biomarker.

In conclusion, the methylation levels of DDP6 differed between lung cancer tissues and normal tissues. The low

mRNA expression level of DPP6 was associated with the malignant features of lung cancer samples and poor survival rates. The present results indicate the potential of DPP6 as a biomarker for lung cancer screening and outcome predictions.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

BM, KK, SS, and BT analyzed and interpreted patient data on DNA methylation using pyrosequencing and mRNA expression by RT-qPCR. KK, HTo, and HTa designed and conducted the present study. KK, CT and NK performed histological examinations of lung cancer and combined the clinical data of patients. BM and KK were major contributors to the writing of the manuscript. BM and KK confirm the authenticity of raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The Ethics Committee of the University of Tokushima approved the present study (Tokushima University Hospital; approval no. 4071), and all procedures were conducted according to the Declaration of Helsinki. All patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

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