DNA methylation and mRNA expression of ZNF577 as biomarkers for the detection and prognosis of lung adenocarcinoma

BATKHISHIG MUNKHJARGAL[1], KAZUYA KONDO[1], SHIHO SOEJIMA[1], BILGUUN TEGSHEE[2], MICHIKO YAMASHITA[3], NAOYA KAWAKITA[3,4], HIROAKI TOBA[3,4] and HIROMITSU TAKIZAWA[3,4]

[1]Department of Oncological Medical Services, Graduate School of Biomedical Sciences, Tokushima University, Tokushima 770-8509, Japan; [2]Division of Cancer Biology, Department of Medicine, Case Western Reserve University at MetroHealth Medical Center, Cleveland, OH 44109, USA; [3]Department of Analytical Pathology, Graduate School of Biomedical Sciences, Tokushima University, Tokushima 770-8503, Japan; [4]Department of Thoracic, Endocrine Surgery and Oncology, Graduate School of Biomedical Sciences, Tokushima University, Tokushima 770-8503, Japan

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Abstract. Despite advances in science and technology, lung cancer remains a major public health issue. The discovery of early diagnostic and prognostic markers is still needed to reduce the mortality rate of lung cancer, which is the highest among all cancer types. Aberrations in the DNA methylation system have an important role in human cancer and are promising for the development of early diagnostic and prognostic markers. The present study focused on zinc finger protein (ZNF)577, whose encoding gene was indicated to exhibit promoter hypermethylation together with 9 other genes in lung adenocarcinoma (LADC) in a previous study by our group. ZN577 is a member of the ZNG family and its functional role has so far remained elusive. LADC tissue samples surgically resected at Tokushima University Hospital (Tokushima, Japan) between April 1999 and November 2013 were collected. A total of 73 tumors and 27 paired tumor-adjacent normal tissues were examined for DNA methylation and mRNA expression of ZNF577. A total of 149 LADC tissue samples were collected and evaluated by immunohistochemistry (IHC) for the tissue expression of ZNF577. High methylation (n=27, P<0.0001) and low mRNA expression levels (n=27, P<0.031) of ZNF577 were identified in LADC tissues, and it was demonstrated that methylation levels were inversely correlated with mRNA expression levels (P=0.0116, ρ=-0.2515). Among the LADC tissues, lepidic-patterned samples had lower methylation levels of ZNF577 than other pathological types. In addition, mRNA expression levels of ZNF577 were significantly higher in females, non-smokers and stage I samples. Overall survival [P<0.0001; area under curve (AUC)=0.8658] and disease-free survival (DFS; P<0.0004; AUC=0.7232) rates were significantly higher in the ZNF577 high mRNA expression group than in the ZNF577 low mRNA expression group. Among the 149 LADC samples examined by IHC, 105 were negative and 44 were positive for the tissue expression of ZNF577. Cox regression analysis showed poorer DFS (hazard ratio: 3.917; P=0.023) in patients with lower expression of ZNF577. In conclusion, higher methylation levels of ZNF577 were observed in LADC tissues than in normal lung tissue and low mRNA expression of ZNF577 was associated with unfavorable prognosis.

Correspondence to: Professor Kazuya Kondo, Department of Oncological Medical Services, Graduate School of Biomedical Sciences, Tokushima University, 3-18-15 Kuramotocho, Tokushima 770-8509, Japan
E-mail: kzykondo@tokushima-u.ac.jp

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Introduction

The World Health Organization (WHO) predicts that lung cancer will become the leading cause of death not only among cancers, but also all diseases by 2060 (1). The most common type of epithelial lung cancer is non-small cell lung cancer (NSCLC), including lung adenocarcinoma (LADC), which accounts for ~85% of all lung cancer diagnoses (2). Although our understanding of the epidemiology of lung cancer and the development of strategies for its prevention have advanced in the past 10 years, it is still the primary cause of cancer-related death regardless of sex and age (3). The diagnosis of lung cancer in its early stages continues to be a challenge because numerous techniques and methodologies currently in use, such as low-dose CT, X-rays, sputum examinations, bronchoscopy and lung tissue biopsies, are often only effective at detecting cancer in its advanced stage (4).

NSCLC exhibits a complex genomic landscape, with various genetic and epigenetic mechanisms being implicated in its development, and it has diverse genomic alterations (5). Tumor protein p53 and LDL receptor related protein 1B mutations are prevalent across all subtypes of NSCLC. However, LADC shows higher rates of somatic mutations in genes, such as Kirsten rat sarcoma (RAS) viral oncogene homolog, EGFR, Kelch-like ECH associated protein 1, serine/threonine

kinase 11, mesenchymal epithelial transition factor receptor, V-Raf murine sarcoma viral oncogene homolog B, anaplastic lymphoma kinase, human EGFR2, Ret proto-oncogene and Ros proto-oncogene 1, receptor tyrosine kinase. These mutations primarily affect the RAS-MAPK kinase-ERK, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha-mTOR and MAPK pathways (6-10).

Epigenetic changes, including DNA methylation, are present in all human cancers and contribute not only to the initiation, but also the progression of diseases, particularly cancer (11). Hypermethylation silences critical tumor suppressor genes or regulatory regions within the genome. This silencing may lead to the dysregulation of cell proliferation or modify responses to cancer therapy (12).

The hypermethylation of numerous genes has been reported in numerous cases of lung cancer, with the majority being detected in promoter sequences (13,14). Genes such as cyclin-dependent kinase-2, adenomatous polyposis coli, cadherin-13, p16 (also known as cyclin-dependent kinase inhibitor 2A) and Ras-association domain family protein1 isoform A are hypermethylated, and this methylation has been linked to a higher likelihood of relapse after the surgical resection of stage I NSCLC (15,16). Previous studies by our group revealed that the hypermethylation of the glutamate decarboxylase 1 and dipeptidyl protease-like 6 genes was associated with poor outcomes in patients with LADC (17,18). Therefore, DNA methylation may contribute to the development of biomarkers to detect cancer in the early stages and predict patient outcomes.

In a previous study by our group, the paired tumorous and non-tumorous tissues of 12 LADC samples were subjected to genome-wide screening for aberrantly methylated CpG islands (CGIs), and the top 10 significantly methylated genes were listed, including zinc finger protein (ZNF)577 (19). Individual gene studies were then performed to examine the methylation and expression of ZNF577 and their relationships with the clinical characteristics of patients. However, only a small number of studies have examined ZNF577, particularly in lung cancer (13), and therefore, further research is needed.

ZNF577 belongs to the Krüppel-associated box (KRAB) C_2H_2-type ZNF family (20). ZNFs have a crucial role in the regulation of gene transcription (21,22). While the specific function of ZNF577 remains unclear and requires further investigation, other individual ZNFs are frequently subjected to hypermethylation and subsequent silencing in various types of tumors. These findings indicate that the disrupted epigenetic pathway involving ZNFs is a common occurrence in cancer progression (22).

Numerous studies have recognized specific ZNFs as potential tumor suppressors that are responsible for controlling cellular proliferation through the inhibition of MAPK signaling and the repression of various oncogenes (23). Oxidative stress is a potential mechanism contributing to the hypermethylation of ZNFs during malignant transformation because it disrupts the interaction between CCCTC-binding factor and poly(ADP-ribose) polymerase 1, consequently leading to increases in DNA methylation (24).

To assess the potential of *ZNF577* as a prognostic marker for LADC, the present study set out to investigate its DNA methylation, gene expression and tissue expression in resected LADC tissues.

Materials and methods

Patients and tissue samples. The present study was conducted using a retrospective, observational design. A total of 149 LADC tumor samples and 27 paired tumor-matched normal lung tissue samples were acquired from patients surgically treated at Tokushima University Hospital (Tokushima, Japan) between April 1999 and November 2013. Tissue samples were snap-frozen in liquid nitrogen and then stored at -80˚C for the later extraction of DNA and RNA. All samples were subjected to an IHC analysis to detect tissue expression of ZNF577. Of the 149 samples, 73 tumor and 27 paired tumor-matched samples were examined by methylation analysis and reverse transcription-quantitative (RT-q)PCR. The 7th edition of the tumor-nodes-metastasis classification for lung cancer was used to grade the stages of tumors in samples (25). Tumors were also categorized according to the predominant histological subtype proposed by the 2015 WHO classification (26). A total of 162 patients with LADC were followed up for a mean duration of 48 months (range, 0.6-147 months). Recurrence was detected in 45 patients (27.8%) and there were 34 deaths (21.0%). The patients' age ranged from 43 to 84 years, with a median age of 66 years.

The present study was approved by The Ethics Committee of the University of Tokushima (Tokushima University Hospital, Tokushima, Japan; approval no. 4071-1) and procedures were performed in accordance with the tenets of the Declaration of Helsinki. Written informed consent, which included the key elements of a research study and what their participation will involve, was obtained from all patients.

Global methylation analysis. In a previous study by our group to detect aberrantly methylated CGIs in a genome-wide manner (18), 12 paired tumorous/non-tumorous LADC sample sets from freshly frozen specimens were screened using the Illumina HumanMethylation450 K Bead Chip (Illumina, Inc.). The findings obtained revealed 17 differentially hypermethylated CGI sites in the *ZNF577* gene. The false discovery rate was <0.05 and the β difference (tumor vs. non-tumorous tissue) was >0.22. CGI sites in *ZNF577* ranked 3rd amongst differentially methylated CGIs with a low P-value. Fig. 1A shows a schematic of the mRNA structure of *ZNF577*. Among the 6 (4 coding and 2 non-coding) exons of *ZNF577* mRNA, CGIs were located around exon 1. The array-based methylation status of each CpG site within *ZNF577* is shown in Fig. 1B. Two DNA methylation sites in CpG47 of the *ZNF577* gene, cg09547119 and cg22331349, which were underlined, were subsequently analyzed.

Nucleic acid isolation. The QIAamp DNA Mini Kitand RNeasy Mini kit (Qiagen GmbH) were used to isolate DNA and RNA from frozen tissue, respectively, using the protocols described by the manufacturer.

Bisulfite conversion and pyrosequencing. The EpiTect Bisulfite Kit 48 (Qiagen GmbH) was used to convert genomic DNA to bisulfite according to the manufacturer's protocols. Prior to pyrosequencing, the PyroMark PCR-Kit 200 (Qiagen GmbH) was used according to the manufacturer's protocols to amplify the transformed DNA by PCR using the following primers:

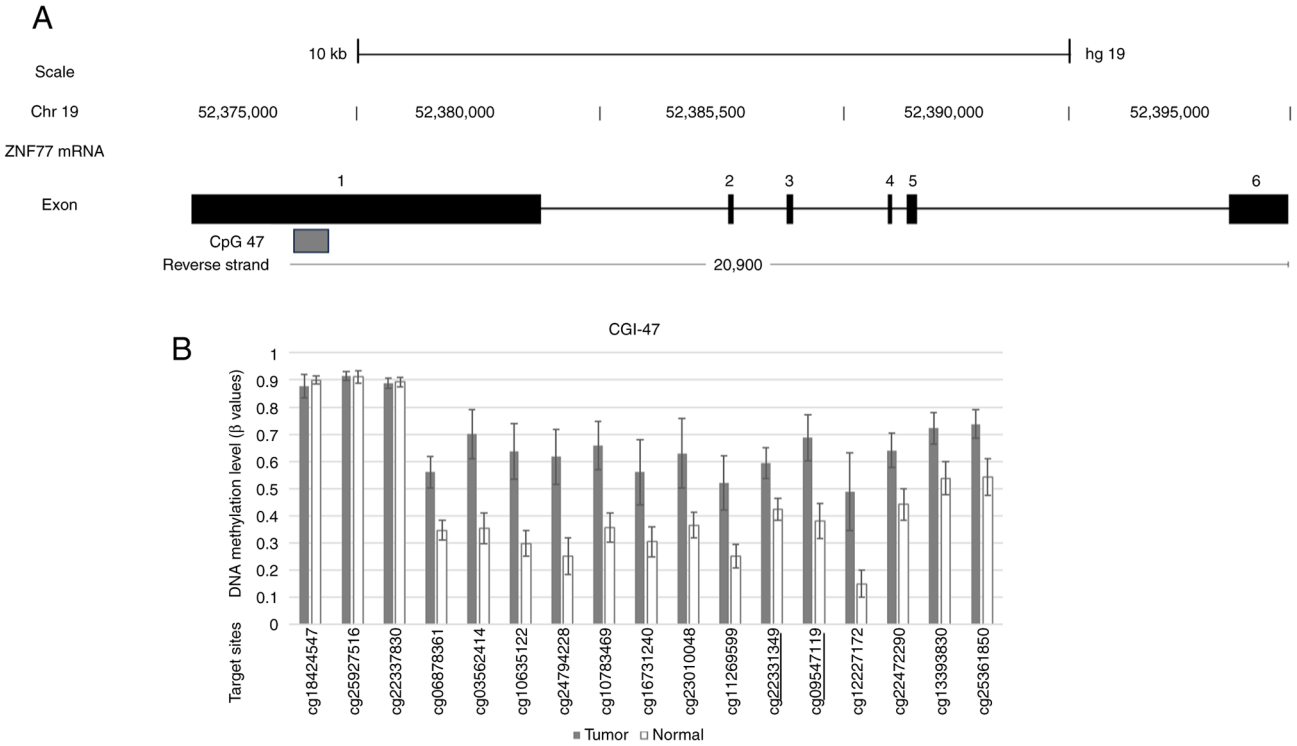

Figure 1. Methylation levels at CpG sites in the ZNF577 gene. (A) Schematic of human ZNF577 mRNA. There are 6 exons in ZNF577 mRNA. CpG island 47 (black box) is located around exon 1. (B) DNA methylation levels. The average β-value difference (0.2190) for the methylation level of each CpG site between lung adenocarcinoma tissue (black bars) and normal tissues (white bars) is shown on the vertical scale. The target sites are all located in CpG island 47. The present study assessed the methylation level at the two underlined CpG sites. ZNF, zinc finger protein; Chr, chromosome.

Forward, 5'-GGGAAGTTTGTTGGGAGTAGTTAT-3' and reverse, (Biotin)-5'-ATATTACAAAACCAAAATCTAACA ATTCAC-3' (target sequence before bisulfite treatment: Forward, 5'-CCTACTGCCGTAGAGCAGGCGGAGTCC CTCTTTTCGCGCCTTAGACAGGTTCTGA-3' and reverse, 3'-CCTACTACTGCCGTAGAGCAGGCGGAGTCCCTCTTT TCGCGCCTTAAGACAGGTAGGTTCTGA-5').

The ZNF577 PyroMark Custom Assay Design 2.0 (Qiagen GmbH) was employed to develop the following sequencing primer according to the manufacturer's intstructuions: 5'-GTT GGGAGTAGTTATTTTTAAT-3'.

In order to assess the mean methylation rate of CpG sites, pyrosequencing was conducted using PSQ 96MA (Qiagen GmbH) according to the manufacturer's protocols. PyroQ-CpG 1.0.9 was used to calculate the methylation rate at each CpG.

RT-qPCR. iScript™ Reverse Transcription Supermix (Bio-Rad Laboratories, Inc.) was used for RT. Real-time PCR was conducted with SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad Laboratories, Inc.) and the PrimePCR™ SYBR® Green Assay (cat. no. 10025716; Bio-Rad Laboratories, Inc.) for ZNF577 and *GAPDH* (cat. no. 10025637; Bio-Rad Laboratories, Inc.). The internal control gene was *GAPDH*. All the reagents were used and reactions were performed according to the manufacturer's protocols. The quantification of qPCR data was performed according the $2^{-\Delta\Delta Cq}$ method (27).

Immunohistochemistry (IHC). IHC staining was performed on paraffin-embedded sections using anti-ZNF577 rabbit polyclonal antibodies (cat. no. HPA046761; 1:50 dilution; Atlas Antibodies) overnight at 4˚C. The tissue samples were incubated at room temperature for 1 h with EnVision + Dual Link System-HRP secondary antibody (cat. no. K4063; Dako; Agilent Technologies, Inc.). Antigen retrieval was conducted by heating dewaxed and dehydrated sections in Dako Real Target Retrieval Solution (Dako; Agilent Technologies, Inc.), pH 9 at 98˚C for 30 min. A total of 149 tumor samples were analyzed, excluding positive control and normal tissues. IHC images were evaluated and grouped as follows: Negative (intensity: Not stained or weakly stained in a small area) or positive (intensity: Moderate to strong with ≥30% area).

Statistical analysis. Comparisons between paired samples, when data were and were not normally distributed, were performed using the paired t-test and the Wilcoxon signed-rank test, 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 investigated using the unpaired t-test for normally distributed data and the Mann-Whitney U-test for data that were not normally distributed. One-way analysis of variance was employed for multiple-group comparisons and was followed by Tukey's multiple-comparisons test for normally distributed data and the Kruskal-Wallis test followed by Dunn's multiple-comparisons test for non-normally distributed data. Overall survival (OS) and disease-free survival (DFS) rates were compared between high/low methylation, high/low mRNA expression and ZNF577 negative/positive expression using Kaplan-Meier analysis with the log-rank

(Mantel-Cox) test. Cut-off values were selected from receiver operating characteristic (ROC) curves. Multivariate survival analyses were performed using the likelihood ratio test of the stratified Cox proportional hazard regression analysis. GraphPad Prism version 5.00 (GraphPad; Dotmatics) and SPSS (version 24.0; IBM Corp.) were used for all statistical analyses.

Results

Patient characteristics. Table I shows the clinical characteristics of the 73 patients with LADC examined by PCR and methylation analysis, including survival data, and their basic characteristics, such as sex (male, 52%; female, 47.9%), age at diagnosis (66.3±9.7 years; range, 43-84 years). The clinical characteristics of the 149 patients with LADC evaluated by IHC, including survival data, and their basic characteristics, such as sex (male, 45%; female, 55%), age at diagnosis (66.9±9.1 years; range, 43-84 years) are provided in Table SI.

ZNF577 methylation and mRNA expression levels in LADC tissues and paired normal tissues. *ZNF577* methylation and mRNA expression levels in LADC tissues were assessed in comparison with 27 matched samples. *ZNF577* methylation levels in LADC and normal lung tissues are presented in Fig. 2A. Tumor samples had significantly higher DNA methylation levels than normal samples (Wilcoxon signed-rank test, P<0.0001). ROC curves were used to evaluate the ability of ZNF577 methylation levels and mRNA expression levels to discriminate tumor tissue from non-tumor tissue. Fig. 2B presents the ROC for methylation levels between tumor and normal samples with an area under the curve (AUC) of 0.8402. *ZNF577* mRNA expression levels in LADC and normal lung tissues are shown in Fig. 2C. Tumor samples had significantly lower mRNA expression levels than normal samples (Wilcoxon signed-rank test, P<0.031). Fig. 2D shows the ROC for *ZNF577* mRNA expression levels in tumor and normal samples with an AUC of 0.8658.

Spearman's rank correlation analysis was used to investigate the relationship between *ZNF577* DNA methylation and mRNA expression levels in 100 samples (73 tumors and 27 normal tissues) (Fig. 2E). The results obtained showed that methylation levels were inversely correlated with mRNA expression levels (P=0.0116, ρ=-0.2515).

Relationship between ZNF577 methylation and mRNA expression levels and clinical characteristics of patients with LADC. To establish whether the malignancy and progression of LADC were affected by the hypermethylation of *ZNF577*, the methylation and mRNA expression levels were measured in 73 samples and compared with histology patterns and stage grading.

The IA group had slightly lower *ZNF577* methylation levels than the other, advanced-stage groups but it was not statistically significant. Furthermore, the IA group had significantly higher *ZNF577* mRNA expression levels (Mann-Whitney U-test, P=0.0184; Fig. 3A and B). Samples with the lepidic pattern had significantly lower *ZNF577* methylation levels than those with the other patterns (unpaired t-test, P=0.0217; Fig. 3C). However, *ZNF577* mRNA expression levels did not significantly differ between samples with the lepidic and other histological patterns (Mann-Whitney U-test; Fig. 3D).

Table I. Patient characteristics (n=73).

Item	Value
Sex	
Male	38 (52.0)
Female	35 (47.9)
Age, years	66.3±9.7 (43-84)
Smoking status	
Non-smoker	36 (49.3)
Smoker	37 (50.6)
Brinkman index[a]	381.3±501.8
Pathology	
Lepidic	26 (35.6)
Papillary	26 (35.6)
Solid	3 (4.1)
Acinar	5 (6.8)
Mixed or others	13 (17.8)
Surgery	
Pneumonectomy	1 (1.36)
Lobectomy	68 (93.1)
Segmentectomy	1 (1.36)
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[b]	
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	14 (19.1)
V factor-positive (n=72)	12 (16.6)
Ly factor-positive	12 (16.4)
EGFR mutation (n=25)	11 (44)

Values are expressed as n (%) or the mean ± standard deviation and range. [a]The Brinkman index is the number of cigarettes smoked per day multiplied by the number of years of smoking [b]Stage according to the 7th edition of the tumor-nodes-metastasis classification (25). UFT, tegafur/uracil; Pl, pleural; V, vascular; Ly, lymph node; EGFR, epidermal growth factor receptor.

To examine differences in the hypermethylation and mRNA expression levels of *ZNF577* between the sexes and individuals with different smoking statuses, methylation and mRNA expression levels were assessed in 73 samples.

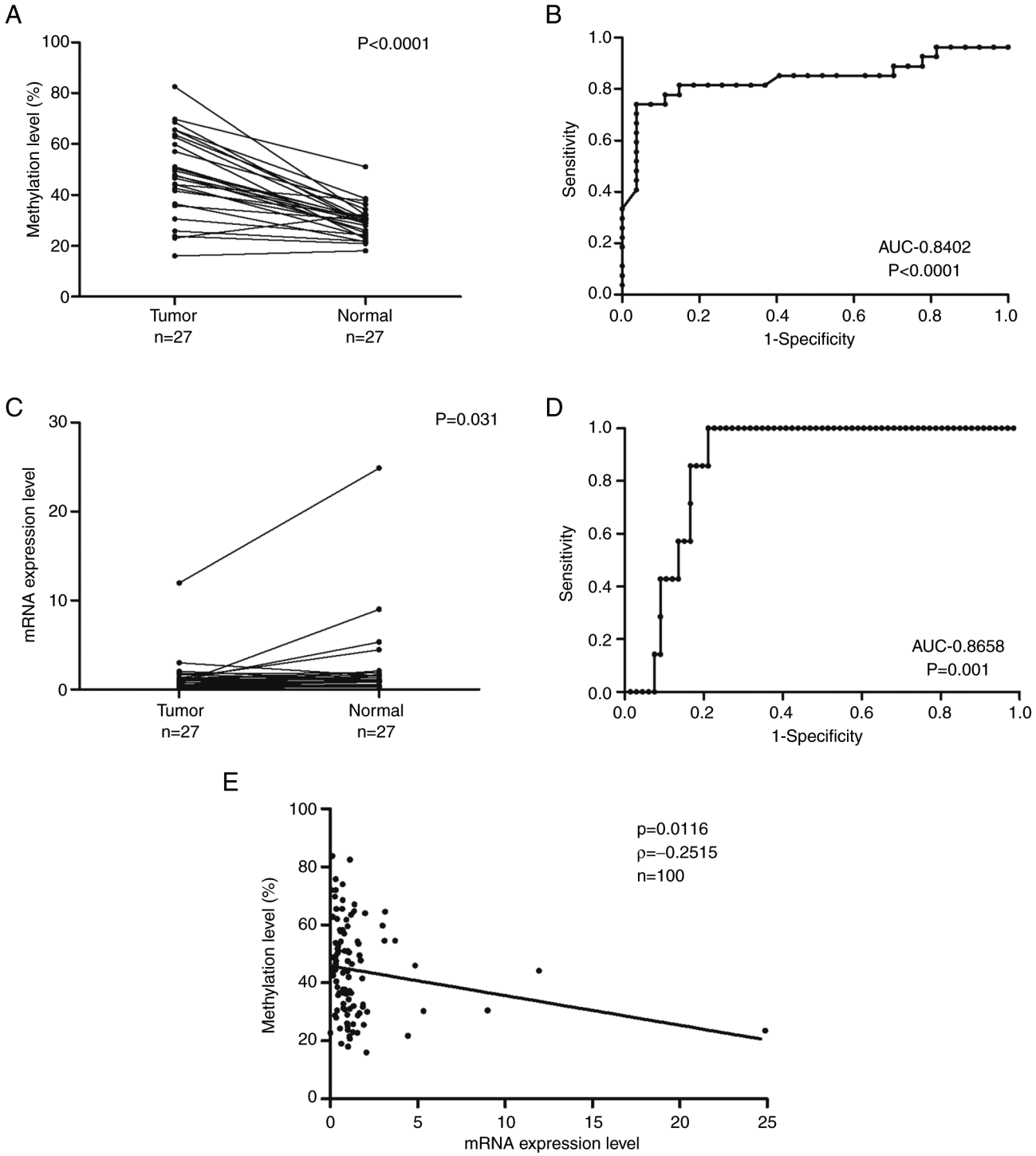

Figure 2. ZNF577 DNA methylation and mRNA expression levels. (A) Pyrosequencing was performed to measure the difference between ZNF577 methylation levels in 27 LADC tumors and paired normal tissues. (B) ROC curve for ZNF577 methylation levels to distinguish between in tumor and normal samples. (C) ZNF577 mRNA expression levels in 27 LADC tumors and paired normal tissues. (D) ROC curve for ZNF577 mRNA expression to distinguish between in tumor and normal samples. (E) Relationship between ZNF577 DNA methylation and mRNA expression levels in 100 samples. Spearman's rank correlation was used to analyze data. ZNF, zinc finger protein; AUC, area under the ROC curve; ROC, receiver operating characteristic; LADC, lung adenocarcinoma.

ZNF577 methylation levels were slightly lower in females (unpaired t-test, P=0.1621) and non-smokers (unpaired t-test, P=0.1548; Fig. 3E and F) but the differences were not statistically significant. However, mRNA expression levels of *ZNF577* were significantly higher in females (Mann-Whitney U-test, P=0.0276) and non-smokers (Mann-Whitney U-test, P=0.0277; Fig. 3G and H).

Prognostic value of mRNA expression and methylation levels of the ZNF577 gene in LADC. DFS and OS analyses of *ZNF577* mRNA expression and methylation levels were conducted (Fig. 4). Cut-off values obtained from ROC curves were used to divide samples into those with high/low expression and high/low methylation levels (Fig. 4A, C, E and G). Samples with high *ZNF577* mRNA expression levels had

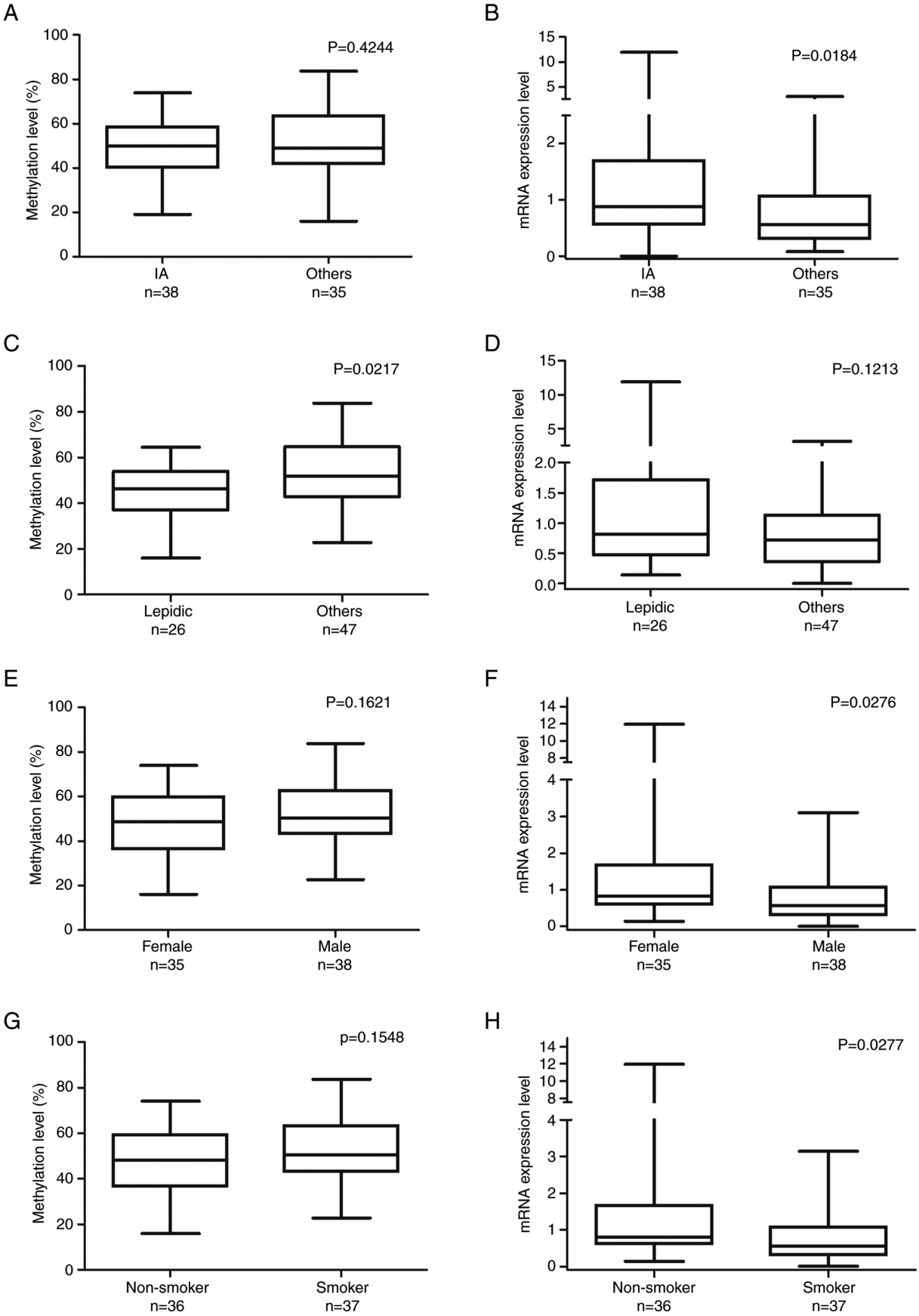

Figure 3. Comparison of ZNF577 DNA methylation and mRNA expression levels with patient characteristics. ZNF577 methylation and mRNA expression levels were analyzed in lung adenocarcinoma tumor samples (n=73) using pyrosequencing and reverse transcription-quantitative PCR, respectively. (A) ZNF577 methylation (%) and (B) mRNA expression levels in lung cancer stages categorized by the World Health Organization histological classification (IA, n=38; others: IB, n=21; IIA, n=9; IIB, n=4; IIIA, n=1). (C) ZNF577 methylation (%) and (D) mRNA expression levels in lung cancer with different histological patterns (lepidic, n=26; others: Papillary, n=26; acinar, n=5; solid, n=3; mixed, n=13). (E) ZNF577 methylation (%) and (F) mRNA expression levels according to sex. (G) ZNF577 methylation (%) and (H) mRNA expression levels according to smoking status. ZNF, zinc finger protein.

Table II. Cox proportional hazard regression analysis of DFS and OS in patients with lung adenocarcinoma (n=73).

A, Disease-free survival			
	Multivariate analysis		
Factor	Hazard ratio	95% CI	P-value
Sex, female (n=35) vs. male (n=38)	0.264	0.043-1.605	0.148
Age <67 (n=43) vs. ≥67 (n=30) years	0.364	0.106-1.253	0.109
Smoking, smoker (n=37) vs. non-smoker (n=36)	13.740	1.778-106.1	0.012
Stage[a], IA (n=38) vs. others (n=35)	0.159	0.041-0.612	0.007
Metastasis, No (n=65) vs. Yes (n=8)	0.270	0.066-1.103	0.068
LVI, No (n=61) vs. Yes (n=12)	0.180	0.037-0.888	0.035
Pathology, lepidic (n=26) vs. others (n=47)	0.894	0.227-3.517	0.873
ZNF577 mRNA expression, Low (n=37) vs. High (n=36)	3.917	1.212-12.660	0.023
ZNF577 DNA methylation, Low (n=36) vs. High (n=37)	0.932	0.336-2.580	0.892
B, OS			
Sex, female (n=35) vs. male (n=38)	0.014	0.000-2.048	0.093
Age,<67 (n=43) vs. ≥67 (n=30) years	0.022	0.001-0.667	0.028
Smoking, smoker (n=37) vs. smoker (n=36)	356.2	0.802-158303	0.059
Stage[a], IA (n=38) vs. others (n=35)	0.021	0.000-0.960	0.048
Metastasis, No (n=65) vs. Yes (n=8)	11.90	0.248-570.8	0.210
LVI, No (n=61) vs. Yes (n=12)	0.005	0.000-0.729	0.037
Pathology, lepidic (n=26) vs. others (n=47)	0.270	0.016-4.532	0.363
ZNF577 mRNA expression, Low (n=37) vs. High (n=36)	3173475	0.000-2.658E+	0.949
ZNF577 DNA methylation, Low (n=36) vs. High (n=37)	5.198	0.490-55.194	0.171

[a]Stage according to the 7th edition of the tumor-nodes-metastasis classification (25). CI, confidence interval; DFS, disease-free survival; OS, overall survival; ZNF, zinc finger protein; LVI, lymphovascular invasion.

significantly higher DFS and OS rates than those with low levels [DFS: Area under the ROC curve (AUC)=0.7232; log-rank P=0.0004; cut-off value, 0.5737; and OS: AUC=0.8658; log-rank P=0.0001; cut-off value, 0.4227; Fig. 4A-D]. However, no significant differences were observed in DFS or OS between samples with high and low *ZNF577* methylation levels (DFS: Log-rank P=0.2593; cut-off value, 55.75; and OS: Log-rank P=0.5752; cut-off value, 54.38) (Fig. 4E-H).

In the multivariate Cox regression analysis, ZNF577 mRNA expression levels [hazard ratio (HR): 3.917; 95% CI: 1.212-12.66; P=0.023], tumor stage (IA vs. others; HR: 0.159; 95% CI: 0.041-0.612; P=0.007), smoking status (HR: 13.740: 95% CI: 1.778-106.1; P=0.012) and lymphovascular invasion (No vs. Yes; HR: 0.180; 95% CI: 0.037-0.888; P=0.035) were identified as independent prognostic factors for DFS (Table IIA). Patient age (HR: 0.022; 95% CI: 0.001-0.667; P=0.028), tumor stage IA vs. others; HR: 0.021; 95% CI: 0.000-0.960; P=0.048) and lymphovascular invasion (No vs. Yes; HR: 0.005; 95% CI: 0.000-0.729; P=0.037) were identified as independent prognostic factors for OS (Table IIB).

Tissue expression of ZNF577 in LADC. The tissue expression of ZNF577 was examined in 149 samples by IHC. In a total of 105 samples (70.5%), ZNF577 was not expressed, while the remaining 44 (29.5%) showed positive IHC staining. The staining indicated that the protein was located in the cytoplasmic/membranous area. Representative images are shown in Fig. 5A-C. In addition, the impact of the tissue expression of ZNF577 on patient survival rates was analyzed. Survival rates did not significantly differ (DFS: Log-rank P=0.4683; OS: Log-rank P=0.8103) between the groups (Fig. 5D and E).

Discussion

Methylation and gene expression analyses of the ZNF577 gene in 73 patients with LADC were performed in the present study. Tissue microarray was performed on 149 tumor samples to detect ZNF577 expression in the tissue. To the best of our knowledge, the present study was the first to report the methylation and gene expression levels of ZNF577 and their correlation with survival rates in patients with LADC.

In pyrosequencing and gene expression analyses, significantly higher methylation levels and lower expression levels of ZNF577 in tumor samples than in their matched normal tissues were identified. The correlation analysis between the methylation and expression of ZNF577 showed an inverse relationship, with higher methylation levels being associated with lower expression levels. These results confirm the property

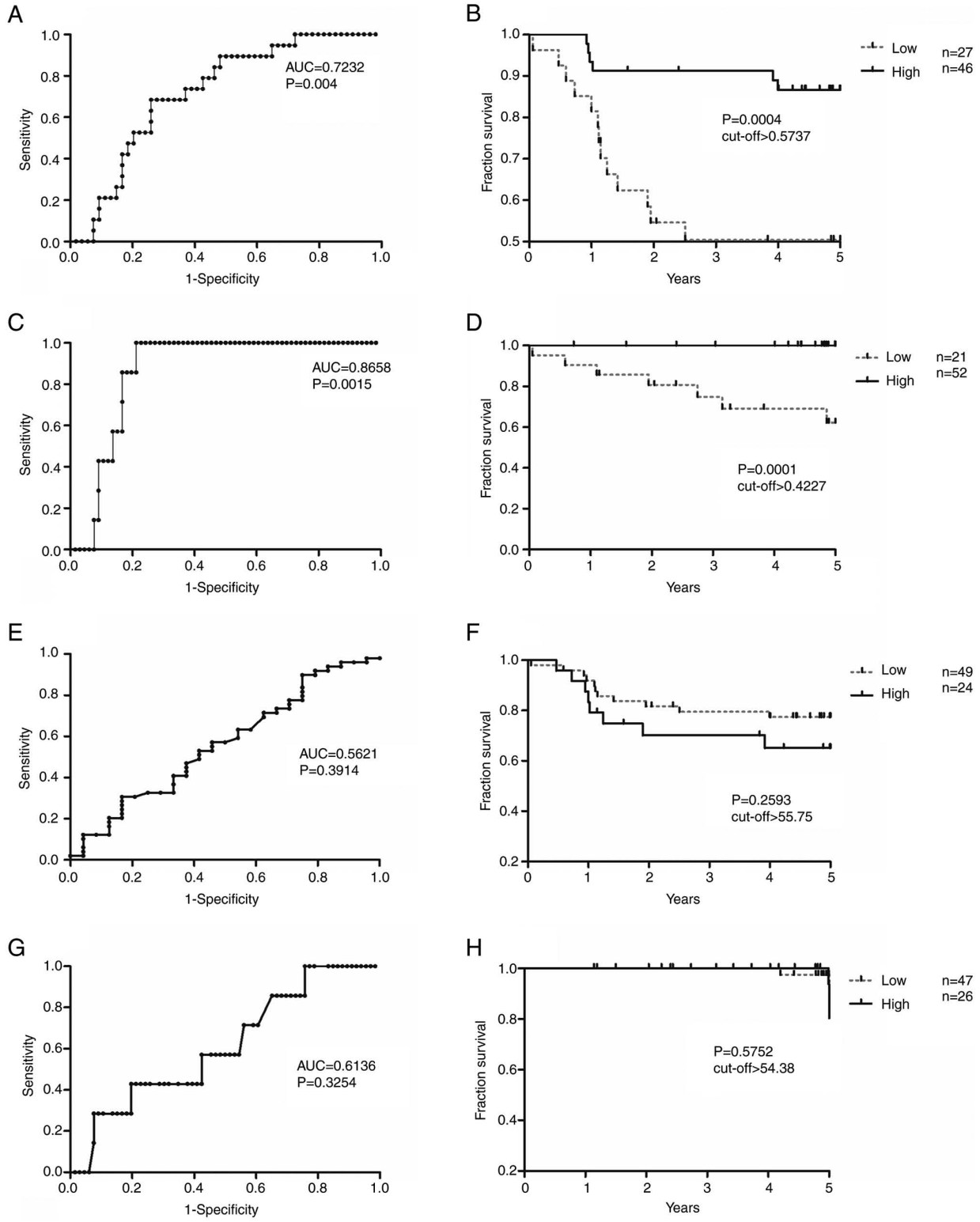

Figure 4. Relationship between ZNF577 mRNA expression and methylation levels and survival rates. (A) ROC curve for disease-free survival according to ZNF577 mRNA expression and (B) Kaplan-Meier curve for disease-free survival according to ZNF577 mRNA expression levels (high or low). (C) ROC curve for overall survival according to ZNF577 mRNA expression and (D) Kaplan-Meier curve for overall survival according to ZNF577 mRNA expression levels (high or low). (E) ROC curve for disease-free survival according to ZNF577 DNA methylation levels and (F) Kaplan-Meier curve for disease-free survival according to ZNF577 DNA methylation levels (high or low). (G) ROC curve for overall survival according to ZNF577 DNA methylation levels and (H) Kaplan-Meier curve for overall survival according to ZNF577 DNA methylation levels (high or low). ZNF, zinc finger protein; AUC, area under the ROC curve; ROC, receiver operating characteristic.

of DNA methylation that silences or inhibits gene expression. Based on ROC curve data, the present study revealed excellent efficiency for discriminating between tumor and normal samples using the methylation and mRNA levels of ZNF577. In addition, a previous study by our group indicated that the mRNA expression level of ZNF577 was low in 10 out

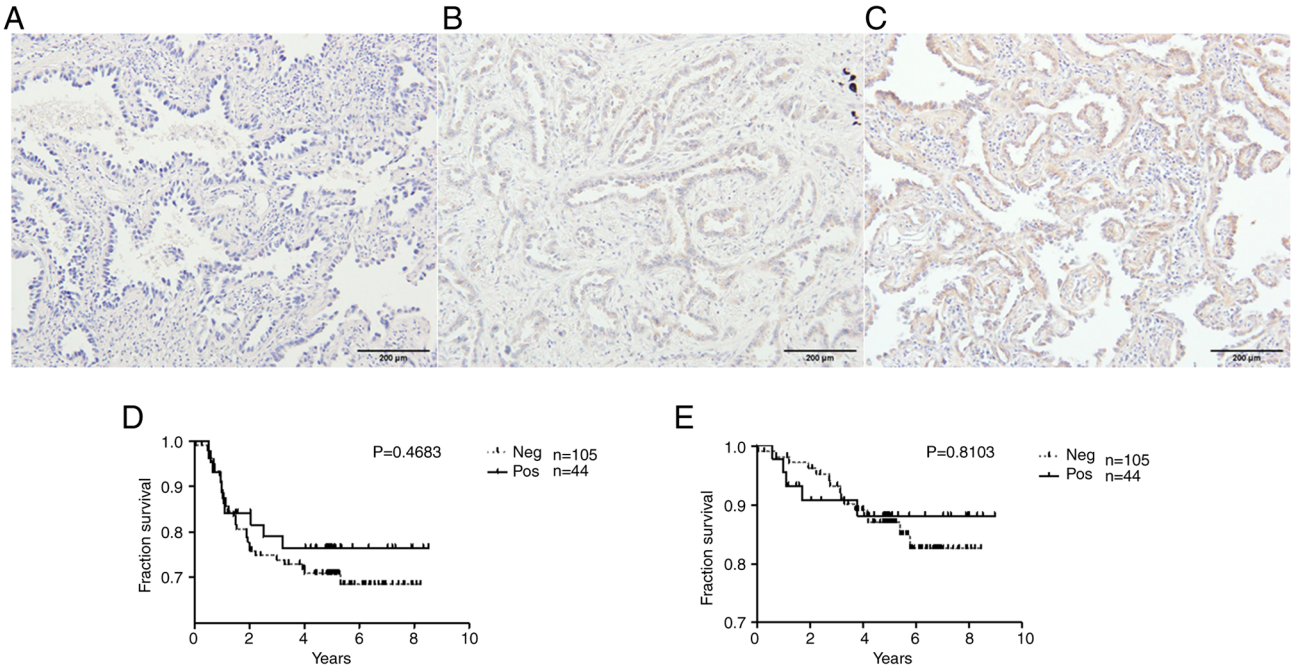

Figure 5. Expression of ZNF577 in lung adenocarcinoma tissue samples and its relationship with patient survival rates. (A) A representative IHC image of the ZNF577-negative group. (B) A representative IHC image of the group that moderately stained for ZNF577. (C) A representative IHC image of the group that strongly stained for ZNF577 (scale bar, 200 μm). (D) Kaplan-Meier curve for disease-free survival based on ZNF577 tissue expression. (E) Kaplan-Meier curve for overall survival based on ZNF577 tissue expression. Neg, negative; Pos, positive; ZNF, zinc finger protein; IHC, immunohistochemistry.

of 14 lung cancer cell lines. Furthermore, the demethylating agents 5-aza-2-deoxycytidine and trichostatin effectively increased its mRNA levels in lung cancer cell lines (19).

Rauch *et al* (13) identified 12 CpG islands that were confirmed to be methylated in 85-100% of stage I lung squamous cell carcinoma cases (n=5), including ZNF577. The group also tested stage I LADC samples (n=8) to identify hypermethylated genes. However, ZNF577 was not among the hypermethylated genes detected, which may be attributed to the stage of the samples as well as the small number of samples examined. Furthermore, certain studies reported the hypermethylation of ZNF577 in aggressive prostate cancer (28), breast cancer (29,30), *de-novo* skin cancer (31) and polycythemia vera cases (32). On the other hand, hypomethylation and overexpression of ZNF577 were observed in fetal alcohol spectrum disorder (33). Based on these studies, it may be predicted that ZNF577 is hypermethylated in several types of tumors.

According to the present results, the mRNA expression levels of ZNF577 were significantly higher in stage I samples and non-smokers than in later stages and smokers. In addition, lepidic samples had lower methylation levels of ZNF577 than advanced pathological patterns. These results clearly showed that ZNF577 methylation and gene expression levels were affected by sex, smoking status, cancer stage and the pathological pattern of LADC. However, the expected (i.e., 'high methylation-low expression' and 'low methylation-high mRNA expression') inverse relationship between methylation and gene expression levels (i.e., 'high methylation-low expression' and 'low methylation-high mRNA expression'), as seen in tumor vs. normal samples, was not observed for these clinical characteristics.

The hypermethylation of ZNF577 has been associated with obesity and post-menopause-related breast cancer (29,30). Furthermore, higher methylation levels of ZNF577 were detected in the leukocytes of women with breast cancer and greater adherence to the Mediterranean diet or specific foods, such as vegetables and fish. The present results showed that females had higher mRNA expression levels of ZNF577 than males. However, the methylation levels did not significantly differ between females and males. In the present study, female patients were mostly menopausal and post-menopausal, with ages ranging between 43 and 84 (median, 66) years. Therefore, it was impossible to compare methylation and gene expression levels between premenopausal and postmenopausal women in this study. A relationship between obesity and aggressive prostate cancer was previously reported (34). ZNF577 was hypermethylated in the leukocytes of these patients, 49.1% of whom were obese and 36.8% were overweight (28). Therefore, a relationship may exist between the metabolic status and the ZNF577 methylation rate.

DNA methylation patterns are linked to a greater likelihood of developing cancer, a worse prognosis and a lower chance of DFS (35). A relationship between patient survival rates and ZNF577 expression levels has not been reported. Significantly higher OS and DFS rates in the high ZNF577 mRNA expression group than in the low expression group were observed. Furthermore, the prediction of both OS and DFS rates by the high and low ZNF577 mRNA expression groups was excellent (DFS: AUC=0.7232; log-rank P=0.0004 and OS: AUC=0.8658; log-rank P=0.0001).

The tissue expression of ZNF577 was also assessed and slightly higher OS and DFS rates were found in the ZNF577-positive group than in the ZNF577-negative group. There was no relationship between the mRNA expression level and

the IHC H-score of ZNF577 (data not shown). Numerous factors, including post-transcriptional and post-translational modifications and microRNAs, can influence the abundance and stability of both mRNA and proteins. Therefore, the abundance of mRNA and the protein level may not always correlate. This may have affected the tissue expression of ZNF577. And another reason could be the availability of the ZNF577 antibody. Since this gene is less studied, particularly by IHC, immunofluorescence and western blotting, there are only a small number of antibody options. A couple of commercially available antibodies were tried by our group, which generally gave relatively weak signals. Furthermore, the tissue expression of ZNF577 was not reported in previous studies.

ZNF577 is a KRAB C_2H_2-type zinc-finger protein (20). The presence of the KRAB domain, which has a potent transcriptional repressor property, indicates that the majority of KRAB-ZNF family members have roles in regulating various biological processes, such as embryonic development, cell differentiation, cell proliferation, apoptosis, neoplastic transformation and cell cycle regulation (36). For instance, ZNF471, ZNF382 and ZNF545 have been reported as tumor suppressor genes and are hypermethylated in several cancer types (37-43).

ZNF577 may be regarded as a relatively new gene that has not been reported in many studies. The hypermethylation of ZNF577 may interfere with immune responses under conditions such as obesity, the post-menopausal stage and all of the aforementioned cancer cases. This may be because ZNFs have significant roles in regulating immune responses at both the transcriptional and post-transcriptional levels and are involved in the onset and progression of cancer (22,44).

The present study had certain limitations that need to be addressed. First, the sample size examined was small due to the limited source of collected samples. Furthermore, since ZNF577 is relatively less studied, there are only a couple of commercially available antibodies to detect ZNF577. This limits our choice to select a suitable antibody for IHC analysis. In addition, liquid biopsy samples of patients were not available for analysis. This prevented us from obtaining more profound data to compare and support the present results.

In conclusion, ZNF577 may be a candidate biomarker for the prognosis and screening of LADC as well as a therapeutic target. Therefore, additional studies are required to obtain a more detailed understanding of the functional role of ZNF577, particularly its methylation status and expression levels, in cancer biology. ZNF577 has potential in the diagnosis and outcome predictions of lung cancer.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

SS, KK, HTa and BM analyzed DNA methylation using pyrosequencing and interpreted the data. SS, BM and KK analyzed mRNA expression using RT-qPCR and interpreted the data. KK, BT, HTo and MY performed the IHC staining and interpreted the association between the clinical data and the immunoreactivity. KK, HTa and BM designed and conducted the present study. KK, NK and MY performed the histological examination of LADC tumor samples. BM, KK and BT were major contributors in writing the manuscript. BM and KK checked and confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was performed in accordance with the principles outlined in the Declaration of Helsinki. Following the approval of all aspects of the present study by the local Ethics Committee (Tokushima University Hospital; Tokushima, Japan; approval no. 4071-1), formal written informed consent for the use of their tissues and the publication of any associated data was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

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