GAD1 expression and its methylation as indicators of malignant behavior in thymic epithelial tumors

SHIHO SOEJIMA[1], KAZUYA KONDO[1], MITSUHIRO TSUBOI[2], KYOKA MUGURUMA[1], BILGUUN TEGSHEE[1], YUKIKIYO KAWAKAMI[2], KOICHIRO KAJIURA[2], NAOYA KAWAKITA[2], HIROAKI TOBA[2], MITSUTERU YOSHIDA[2], HIROMITSU TAKIZAWA[2] and AKIRA TANGOKU[2]

[1]Department of Oncological Medical Services, Graduate School of Biomedical Sciences, Tokushima University, Tokushima 770-8509; [2]Department of Thoracic, Endocrine Surgery and Oncology, Graduate School of Biomedical Sciences, Tokushima University, Tokushima 770-8503, Japan

Abstract. Thymic epithelial tumors (TETs) comprise thymomas and thymic carcinoma (TC). TC has more aggressive features and a poorer prognosis than thymomas. Genetic and epigenetic alterations in thymomas and TC have been investigated in an attempt to identify novel target molecules for TC. In the present study, genome-wide screening was performed on aberrantly methylated CpG islands in thymomas and TC, and the glutamate decarboxylase 1 gene (*GAD1*) was identified as the 4th significantly hypermethylated CpG island in TC compared with thymomas. *GAD1* catalyzes the production of γ-aminobutyric acid from L-glutamic acid. *GAD1* expression is abundant in the brain but rare in other tissues, including the thymus. A total of 73 thymomas and 17 TC tissues were obtained from 90 patients who underwent surgery or biopsy at Tokushima University Hospital between 1990 and 2017. DNA methylation was examined by bisulfite pyrosequencing, and the mRNA and protein expression levels of *GAD1* were analyzed using reverse transcription-quantitative PCR and immunohistochemistry, respectively. The DNA methylation levels of *GAD1* were significantly higher in TC tissues than in the normal thymus and thymoma tissues, and *GAD1* methylation exhibited high sensitivity and specificity for discriminating between TC and thymoma. The mRNA and protein expression levels of *GAD1* were significantly higher in TC tissues than in thymomas. Patients with TET with high *GAD1* DNA hypermethylation and high mRNA and protein expression levels had significantly shorter relapse-free survival rates than those with low levels. In conclusion, significantly more epigenetic alterations were observed in TC tissues compared with in thymomas, which may contribute to the clinical features and prognosis of patients.

Introduction

Thymomas and thymic carcinoma (TC) are rare primary tumors of the anterior mediastinum. Although they are derived from the thymic epithelium, their biological characteristics markedly differ (1). Thymomas are low-grade malignant tumors with a variable population of immature, but non-neoplastic T cells associated with myasthenia gravis and other autoimmune diseases. In most cases, thymomas are successfully treated with surgery and/or adjuvant radiotherapy. Outcomes depend on the tumor subtype, and patients sometimes require supplementary chemotherapy. When recurrence and/or residual tumors are present, the clinical course is indolent. In contrast, TC is a malignant tumor without immature T-cell infiltration and autoimmune disease (2). It has more aggressive features and a poorer prognosis (5-year overall survival rate: Approximately 50%) (3,4). The outcomes of metastatic or inoperable refractory/recurrent TC are extremely poor due to the lack of a standard chemotherapy regimen (5,6). The rarity of this tumor makes clinical trials difficult, and, thus, the development of new drugs is slow. Previous studies examined the effects of targeted therapies and immunomodulatory agents for thymic epithelial tumors (TET) (7). The findings obtained revealed that only sunitinib (an oral tyrosine kinase inhibitor of VEGFR, KIT, and PDGFR) and pembrolizumab (an immune checkpoint inhibitor) were active against TC (response rates of 26 and 22.5%, respectively) (8,9). To identify novel target molecules for TC therapy, a more detailed understanding of the aberrant pathways involved in TC using new molecular methods is needed.

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

Abbreviations: TET, thymic epithelial tumor; TC, thymic carcinoma; CGI, CpG islands; GAD1, glutamate decarboxylase 1; GABA, γ-aminobutyric acid; CTCF, CCCTC-binding factor; IHC, immunohistochemistry; IQR, interquartile range; ROC, receiver operating characteristic; HR, hazard ratio; RFS, relapse-free survival; PRC2, polycomb repressive complex 2; AUC, area under the ROC curve; H3K27me3, histone H3 lysine 27 trimethylation; MMP-7, matrix metalloproteinase-7

Key words: TC, thymoma, GAD1, DNA methylation, paradoxical expression, poor prognosis

Genetic alterations in thymomas and TC have been comprehensively examined using next-generation sequencing, and the findings obtained showed that the frequency of genetic alterations was significantly higher in TC than in thymomas (10,11). Genetic mutations have been detected in TP53, TET2, CYLD, BRD7, SETD2, and CDKN2A in TC (10-13). Petrini *et al* (14) demonstrated that GTF2IL424H mutations were the most common in type A and AB thymomas. Limited information is currently available on epigenetic alterations in TET (15-18). We previously reported that the aberrant methylation of 4 cancer-related genes (DAPK, p16, MGMT, and HPP1) was significantly more frequent in TC than in thymomas (15,16). We also performed genome-wide screening on aberrantly methylated CpG islands (CGI) in TET and identified 92 that were significantly hypermethylated in TC (17). We selected 4 genes (GNG4, GHSR, HOXD9, and SALL3) and demonstrated that the promoter methylation of cancer-related genes was significantly higher in TC than in thymomas (18). These epigenetic alterations were of high diagnostic value for TC and may be used in targeted therapy for TC.

Genome-wide screening on CGI identified the glutamate decarboxylase 1 gene (GAD1) as the 4th significantly hypermethylated CGI in TC in relation to B3 thymomas. GAD1 catalyzes the production of γ-aminobutyric acid (GABA) from L-glutamic acid, the principal inhibitory neurotransmitter (19,20). The overexpression of GAD1 has been reported in various neoplastic tissues, such as oral, nasopharyngeal, colorectal, liver, gastric, and lung cancers (21-26). Yan *et al* showed that the GAD1 promoter was hypermethylated in both colorectal and liver cancers, leading to the production of high levels of GAD1 (21). GAD1 is a target gene that is silenced by histone H3 lysine 27 trimethylation (H3K27me3). The key locus responsible for the reactivation of GAD1 was mapped to a DNA methylation-sensitive CCCTC-binding factor (CTCF)-binding site (CTCF-3) within the third intron of GAD1 (Fig. 1A).

The primary role of CTCF is in the regulation of the 3D structure of chromatin and also in the regulation of genes (27). CTCF binds to the consensus sequence CCGCGNGGNGGCAG (28). The binding of CTCF was shown to be disrupted by CpG methylation of the DNA to which it binds (4).

In the present study, we investigated the DNA methylation and mRNA and protein expression levels of GAD1 in TET. We also examined the prognostic significance of GAD1 expression in TET.

Materials and methods

Patients and tissue samples. TET samples were obtained from 90 patients with histologically proven TET who underwent surgery or biopsy at Tokushima University Hospital (Tokushima, Japan) between 1990 and 2017. Fifty-four tumors and 21 paired thymic tissues were snap-frozen and stored at -80°C. Thymic tissues located away from tumors were obtained during surgery. DNA was extracted from 47 tumor samples and 21 thymic samples and used in a DNA methylation analysis. RNA was extracted from 37 tumor samples and used for RT-PCR. DNA and RNA were extracted from 30 tumor samples. The formalin-fixed and paraffin-embedded materials of 81 samples were used for immunohistochemistry (IHC) (Table SI).

All TET were classified according to the World Health Organization (WHO) histological classification system (29). The clinical stage of each TET was evaluated according to the criteria of the Masaoka-Koga staging system (30). The clinical and pathological characteristics (sex, age, WHO histological classification, Masaoka-Koga stage classification, and myasthenia gravis) of patients subjected to pyrosequencing, RT-PCR, and IHC analyses are shown in Tables I and SI. This study included a case which was diagnosed with thymic carcinoma and type B2 thymoma. Several papers reported tumors combined thymic carcinoma and thymoma (31,32). As main area of this tumor showed thymic carcinoma histologically and the area extracted DNA showed thymic carcinoma histologically, we included it as 'thymic carcinoma' group.

The present study was performed in accordance with the principles outlined in the Declaration of Helsinki. Following the approval of all aspects of these studies by the local Ethics Committee (Tokushima University Hospital, approval no. 2205-4), formal written consent was obtained from all patients.

DNA and RNA preparation and bisulfite conversion of genomic DNA. Tumors were snap-frozen and stored at -80°C until DNA and RNA analyses. DNA and RNA were extracted using standard methods. The bisulfite conversion of DNA was conducted using the EpiTect Bisulfite Kit (Qiagen GmbH) according to the manufacturer's instructions.

Global methylation analysis. We previously screened 7 TC and 8 B3 thymoma samples obtained from freshly frozen specimens with Illumina HumanMethylation450 K BeadChip to identify differentially methylated CGI in a genome-wide manner (17,18). The hypermethylation of 92 CGI in TC samples differed from that in B3 thymoma samples [FDR <0.05 and β-difference (TC-B3 thymoma) >0.3] (18). CGI within the GAD1 gene were the 4th significantly hypermethylated CGI in TC in relation to B3 thymomas (18).

A schematic diagram of the GAD1 structure is shown in Fig. 1A. GAD1 mRNA had 16 exons and four CGI were located in the promoter and first three exons of GAD1. Four CTCF-binding sites of GAD1 and the sequence of the four putative CTCF-binding sites at the human GAD1 locus were shown in Fig. 1A. The CpG dinucleotide in the CTCF-binding site is underlined. Yan *et al* reported that since the CTCF-3 site had four CpG sites, the methylation of these sites in CTCF-3 inhibited CTCF binding and promoted the GAD1 reaction (21). The CTCF-3 site was sensitive to DNA methylation, which may have contributed to the overexpression of GAD1. Fig. 1B shows the results of the array-based methylation status of each CpG site within the four CGI of GAD1. The CGI-3 (from cg07420274 to cg14005211) and CGI-4 (from cg14486905 to cg08863440) regions had significantly higher methylation levels in TC samples (P<0.05). The CpG site cg15126544 in the CTCF-3 site of GAD1 is shown as a dotted box. We also assessed the methylation status of the 4 CpG sites within CTCF-3, including cg15126544 of GAD1.

Bisulfite pyrosequencing. Bisulfite-treated genomic DNA was amplified using a set of primers designed with PyroMark Assay Design Software (version 2.0.01.15; Qiagen; Table SII). The target region of the sequence was 10 nucleotides (nt)

before and 26 nt after cg15126544 (CpG site). There were four CpG sites in this region of the CTCF-3 site. PCR product pyrosequencing and methylation quantification were performed using the PyroMark 24 Pyrosequencing System (version 2.0.6; Qiagen) with sequencing primers according to the manufacturer's instructions.

Reverse transcription-quantitative PCR (RT-qPCR). Complementary DNA was generated from isolated total RNA using the iScript Reverse Transcription Supermix for RT-PCR (Bio-Rad). qPCR was conducted using the KAPA PROBE FAST qPCR Kit (Kapa Biosystems) and GAD1 TaqMan Gene Expression Assay (Thermo Fisher Scientific, Inc.) according to the manufacturers' instructions. Primers used for qPCR on *GAD1* and *GAPDH* are listed in Table SII. *GAPDH* mRNA levels were employed as internal controls for normalization. The relative expression of *GAD1* mRNA was calculated using Human Thymus Total RNA (Takara) as a normal thymus control.

IHC staining. Three-micrometer-thick paraffin-embedded sections were subjected to IHC staining using the Envision system (ChemMate Envision kit; Dako) according to the manufacturer's instructions. A mouse monoclonal anti-GAD67 antibody (Sigma-Aldrich; Merck KGaA; G5419), diluted 1:500 with antibody diluents (Dako), was used as the primary antibody. Antigen retrieval was performed by heating dewaxed and dehydrated sections in Dako Real Target Retrieval Solution, pH 9 (Dako) using a 2100 retriever (Aptum Biologics, Ltd.). The proportion and intensity of the GAD1 stain in TET samples were scored. We defined the stain score as the sum of the proportion and intensity scores. A stain score of ≥4 indicated the overexpression of the GAD1 protein (Table SIII). IHC data scoring was performed independently by two different researchers (SS and KKo).

Statistical analysis. The Shapiro-Wilk test was used to evaluate whether numerical datasets were normally distributed. Parametric tests (a paired t-test, the Student's t-test, or Welch's t-test) were used when numerical datasets were normally distributed. On the other hand, non-parametric tests (the Wilcoxon signed-rank test or Mann-Whitney test) were used when numerical datasets were not normally distributed. Continuous data were expressed as medians and ranges or interquartile ranges (IQR, 25th to 75th percentile). Data were analyzed using Kruskal Wallis test and Steel-Dwass test for multiple comparisons in histology and stage. The unpaired t-test was used for age distribution, Fisher's exact test for sex, histology, and stage distributions, and the chi-squared test for the distribution of myasthenia gravis. The area under the receiver operating characteristic (ROC) curve [AUC; ranging between 0.5 (chance) and 1.0 (perfect discrimination or accuracy)] was measured to characterize the accuracy of the DNA methylation signature to discriminate TC from thymomas. Correlation analysis was performed using Spearman's rank correlation. Relapse-free survival (RFS) was defined as 'the time from surgical resection to relapse of disease'. Univariate and multivariate survival analyses were performed using the likelihood ratio test of the stratified Cox's proportional hazard regression analysis. Survival curves were estimated using the Kaplan-Meier method and compared with the Log-rank test

and the Gehan-Breslow-Wilcoxon test. All statistical analyses were performed using a commercial software program (GraphPad Prism, version 5.00; GraphPad Software and SPSS, version 24.0; IBM Corp.). A P-value <0.05 was considered to be significant.

Results

CGI methylation status of GAD1 genes in TET and a paired normal thymus in pyrosequencing. Fig. 1C (left side) shows the relationship of the DNA methylation rate of the four CpG sites of CTCF-3 in the *GAD1* gene between thymomas and the normal thymus. No significant difference was observed in the DNA methylation rate between thymomas and the normal thymus (the paired t-test, P=0.917). Fig. 1C (right side) shows the relationship of the DNA methylation rate of the *GAD1* gene between TC and the normal thymus. The DNA methylation rate was significantly higher in TC than in the normal thymus (the Wilcoxon signed-rank test, P=0.003).

CGI methylation status of the GAD1 gene in the pyrosequencing of TET according to the WHO histological classification. Fig. 1D shows the median DNA methylation rate of the *GAD1* gene in TET according to the WHO histological classification. Median DNA methylation rates in A+AB+B1, B2, B3, and TC were 4.0 (range, 2.8-17.5, IQR, 3.0-5.9), 3.5 (range, 2.5-5.5, IQR, 3.1-4.7), 4.5 (range, 3.0-28.0, IQR, 3.4-16.4), and 32.9 (range, 12.3-75.3, IQR, 19.5-51.7), respectively. Fig. 1E shows that the median DNA methylation rate was significantly higher in TC than in thymomas [32.9 (range, 12.3-75.3, IQR, 19.5-51.7] versus (vs.) 4.0 (range, 2.5-28.0, IQR 3.3-5.3)) (the Mann-Whitney U test, P<0.001). No significant differences were observed in the median DNA methylation rate according to the WHO histological classification in thymomas (A+AB+B1 vs. B2, B2 vs. B3, and A+AB+B1 vs. B3). The median DNA methylation rate of TC was significantly higher than in each thymoma group (A+AB+B1, B2 and B3) (P<0.01). We examined the accuracy of the methylation signature of *GAD1* for the detection of TC using the ROC analysis. We used the area under the ROC curve (AUC) as the criterion of accuracy. AUC may range, in value from 0.5 (chance) to 1.0 (perfect discrimination or accuracy). Fig. 1F shows ROC curves for the accuracy of the *GAD1* methylation signature to differentiate TC from all tumors. The results obtained revealed high sensitivity and specificity for discriminating between TC and thymomas (AUC=0.9758).

mRNA expression of GAD1 in TET. Relative mRNA expression levels were normalized to *GAPDH* mRNA levels and expressed as the mean of experiments performed in triplicate. Median mRNA expression levels in A+AB+B1, B2, B3, and TC were 0.20 (range, 0.02-4.40, IQR, 0.08-1.26), 0.40 (range, 0.02-3.85, IQR, 0.07-0.87), 0.40 (range, 0.01-1.70, IQR, 0.08-0.78), and 1.85 (range, 0.25-29.76, IQR, 1.21-6.65), respectively (Fig. 2A). The RT-qPCR analysis showed that median *GAD1* mRNA expression levels were 1.85 (range, 0.25-29.76, IQR, 1.21-6.65) in TC samples and 0.38 (range, 0.01-4.40, IQR, 0.08-0.86) in thymoma samples. *GAD1* mRNA expression levels were significantly higher in TC than in thymomas (the Mann-Whitney U test, P=0.0004) (Fig. 2B). A positive association was observed between mRNA expression levels
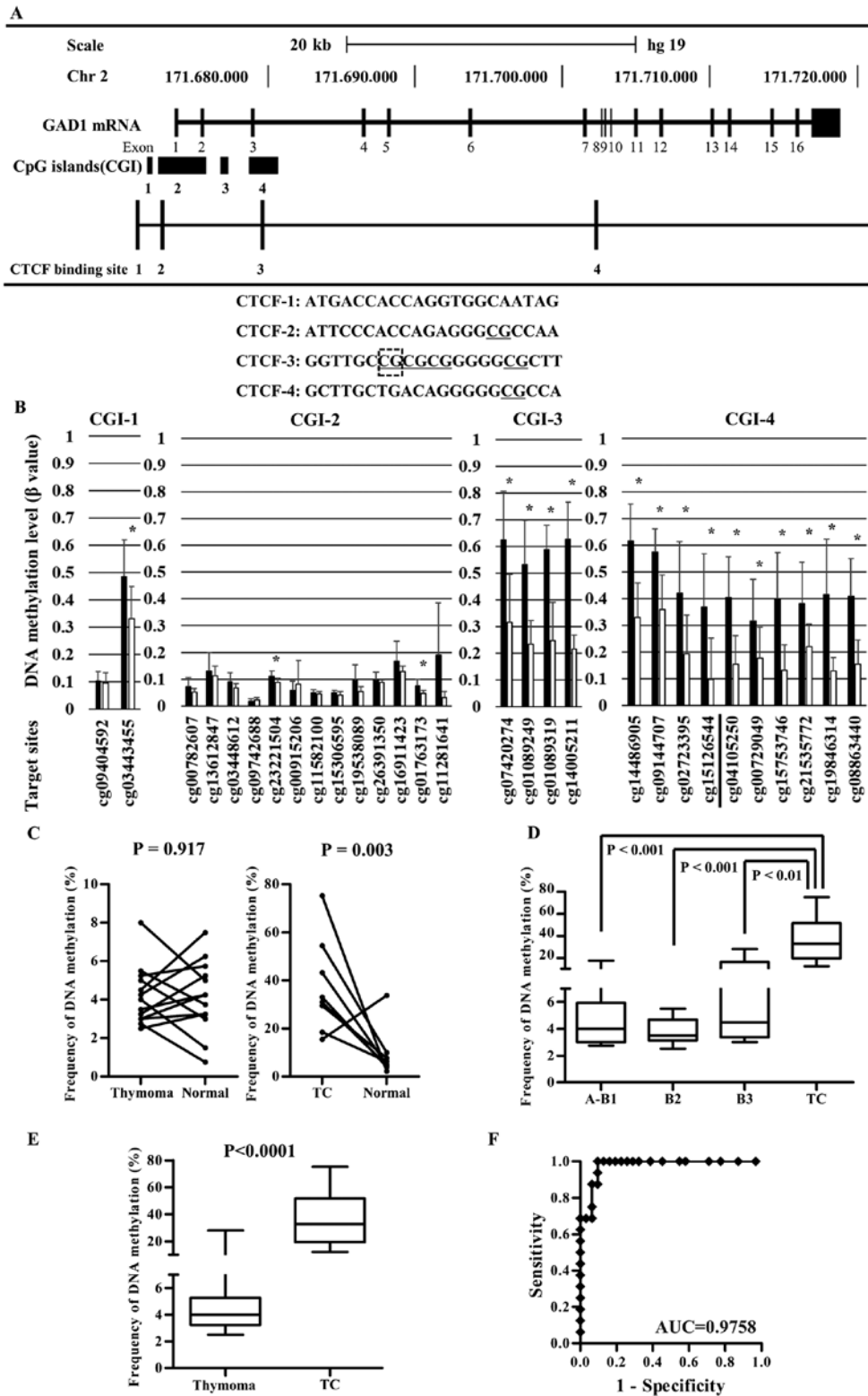
Figure 1. (A) Schematic diagram of the *GAD1* structure. The mRNA of *GAD1* has 16 exons. Four CGI (black boxes) are located in the promoter and the first three exons of *GAD1*. Four CTCF-binding sites of *GAD1* are shown as vertical lines. The sequences of the four putative CTCF-binding sites at the human GAD1 locus are shown. CpG dinucleotides in the CTCF-binding site are underlined. The CpG site cg15126544 in the CTCF-3 site of *GAD1* is shown as a dotted box. (B) DNA methylation levels. The vertical scale indicates average β-values of the DNA methylation level of each CpG site between TC (black bar) and B3 thymomas (white bar). The target region for the pyrosequencing analysis began 10 nucleotides before and 26 nucleotides after cg15126544 (underlined). *P<0.05 TC vs. thymoma. (C) DNA methylation rate of the *GAD1* gene in thymomas and paired normal thymic tissues, and in TC and paired normal thymic tissues. Average DNA methylation values are shown as percentages from the quantitative pyrosequencing of 13 thymomas and paired thymic tissues, and 8 TC and paired thymic tissues. (D) DNA methylation rate of the *GAD1* gene in thymic epithelial tumors according to the World Health Organization histological classification (A+AB+B1, B2, B3 and TC). The upper and lower ends of the whiskers, the upper and lower edges of the boxes, and the horizontal lines across each box represent the upper and lower extremes, the upper (75th) and lower (25th) quartiles, and the medians, respectively. Data were analyzed using Kruskal Wallis test (P<0.0001) and Steel-Dwass test. (E) Median DNA methylation rate between TC and thymomas (A+AB+B1+B2+B3) was 32.88 (range, 12.25-75.25; IQR, 19.50-51.69) vs. 4.0 (range, 2.5-28.0; IQR, 3.25-5.25), respectively. (F) Receiver operating characteristic curve of the accuracy of the methylation signature for detecting TC among all tumors using *GAD1* methylation. CTCF, CCCTC-binding factor; TC, thymic carcinoma; GAD1, glutamate decarboxylase 1; IQR, interquartile range; AUC, area under the curve.
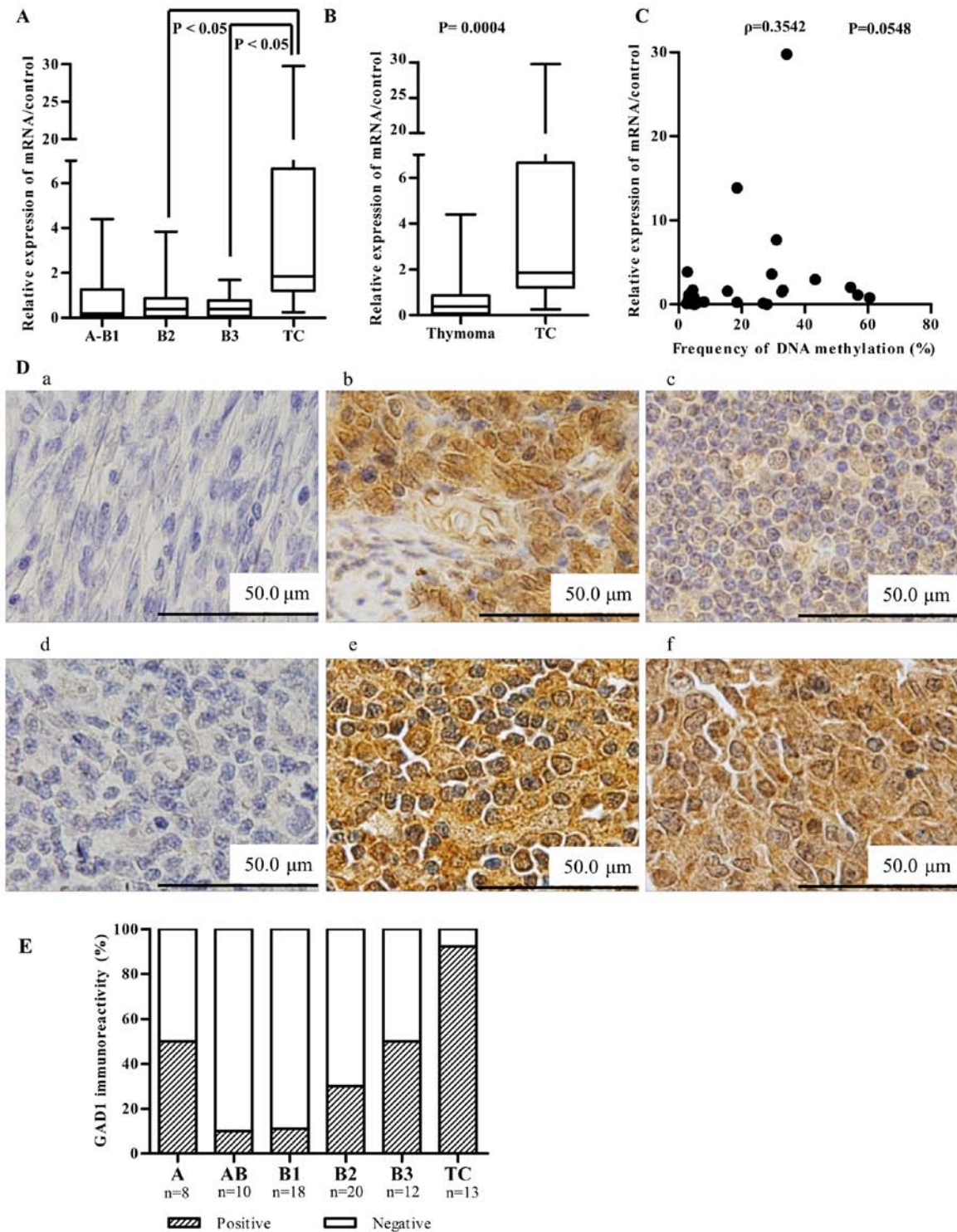
Figure 2. (A) GAD1 mRNA expression in TET according to the WHO histological classification (A+AB+B1, B2, B3 and TC). The upper and lower ends of the whiskers, the upper and lower edges of the boxes, and the horizontal lines across each box represent the upper and lower extremes, the upper (75th) and lower (25th) quartiles, and the medians, respectively. Data were analyzed via Kruskal Wallis test (P=0.0058) and the Steel-Dwass test. (B) GAD1 mRNA expression between TC and thymomas (A+AB+B1+B2+B3) was 1.85 (range, 0.25-29.76, IQR, 1.21-6.65) vs. 0.38 (range 0.01-4.40, IQR, 0.08-0.86), respectively. (C) Correlation between DNA methylation levels of four CpG sites within the CTCF-3 of *GAD1* and *GAD1* mRNA expression in 18 thymomas and 12 TC tissues. Data were analyzed via Spearman's rank correlation. (D) Representative images of the immunohistochemically detected GAD1 protein in tumors. (a) Type A thymoma: the cytoplasm of tumor cells was not stained; (b) type AB thymoma: the cytoplasm of tumor cells was strongly stained; (c) type B1 thymoma: the cytoplasm of tumor cells was weakly stained and lymphocytes were not stained; (d) type B2 thymoma: the cytoplasm of tumor cells was not stained; (e) type B3 thymoma: the cytoplasm of tumor cells was strongly stained and lymphocytes were not stained; and (f) TC: the cytoplasm of tumor cells of thymic carcinoma was strongly stained. Scale bar, 50 μm. (E) GAD1 protein expression in TET according to the WHO histological classification. TC, thymic carcinoma; GAD1, glutamate decarboxylase 1; IQR, interquartile range; TET, thymic epithelial tumor; WHO, World Health Organization.

and methylation levels at the 4 CpG sites (Spearman's rank correlation coefficient, ϱ=0.3542, P=0.0548) (Fig. 2C).

Protein expression of GAD1 using IHC. To assess GAD1 protein expression levels in TET, IHC was performed on 81
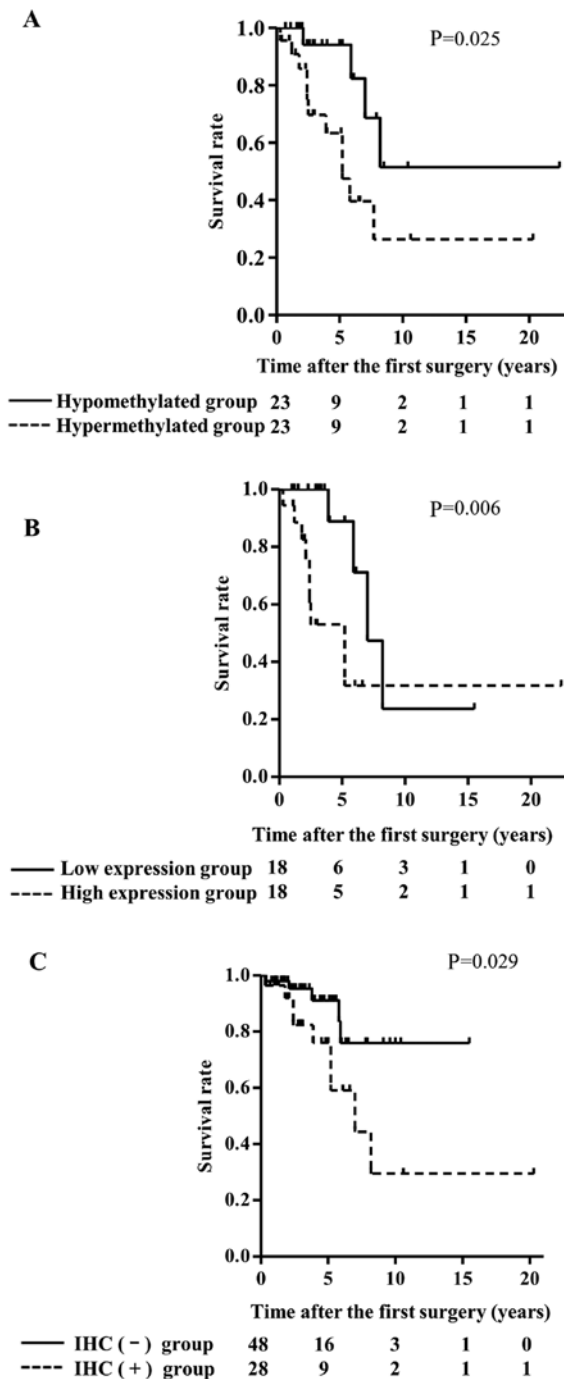
Figure 3. (A) Relapse-free survival curves of patients with TET with higher and lower levels of DNA methylation in *GAD1*. The median value (5.375) of the frequency of the DNA methylation of *GAD1* was used to divide patients into hypermethylated and hypomethylated groups. P-value was calculated using the log-rank test. (B) Relapse-free survival curves of patients with TET with higher and lower levels of *GAD1* mRNA expression. The median value (0.69) of *GAD1* mRNA expression was used to divide patients into high and low expression groups. P-value was calculated using the Gehan-Breslow-Wilcoxon test. (C) Relapse-free survival curves of patients with TET with higher and lower levels of *GAD1* protein expression. The immunoreactivity (staining score) of *GAD1* was used to divide patients into IHC(+) (staining score ≥4) and IHC(-) (staining score <4) groups. P-value was calculated using the log-rank test. GAD1, glutamate decarboxylase 1; IHC, immunohistochemistry; TET, thymic epithelial tumor.

TET, including 68 thymomas and 13 TC. Patient characteristics are shown in Table I. Cytoplasmic staining of GAD1 was observed in the tumor cells of TC (Fig. 2D-f), whereas almost

no staining was noted in thymoma cells (Fig. 2D-a and d). Some cells of type A and B3 thymoma were strongly stained (Fig. 2D-b and c). Fig. 2D-b showed weakly stained tumor cells in type B1 thymomas. Fig. 2E showed the immunoreactivity of GAD1 in the WHO histological classification. Reactivity increased in order from type AB thymomas to TC. A significant difference was observed in the expression of GAD1 between TC and thymomas (92.31 vs. 27.94%, P<0.001). Among the various clinicopathological factors examined, the Masaoka-Koga staging system, WHO histological classification, mRNA expression, and DNA methylation correlated with GAD1 immunoreactivity (Table II).

Univariate and multivariate survival analyses using the stratified Cox's proportional hazard regression analysis. The univariate Cox's regression analysis confirmed that the WHO histological classification (Hazard ratio (HR); 3.974, 95% confidence interval (95% CI); 1.990-7.938), Masaoka-Koga staging system (HR; 5.532, 95% CI; 1.941-15.771), and GAD1 DNA methylation (HR; 3.424, 95% CI; 1.082-10.835) correlated with a worse prognosis for RFS (Table III). The multivariate Cox's regression analysis revealed that the WHO histological classification (HR; 7.517, 95% CI; 2.156-26.202) and Masaoka-Koga staging system (HR; 5.727, 95% CI; 1.689-19.423) were independent prognostic factors for RFS (Table III). *GAD1* immunoreactivity was associated with RFS in the univariate Cox's regression analysis; (HR; 2.800, 95% CI; 0.996-7.870, P=0.051) and multivariate Cox's regression analysis; (HR; 0.213, 95% CI; 0.034-1.321, P=0.097).

RFS curve of TET according to DNA methylation, mRNA, and protein overexpression levels. In all TET cases (n=90), the median follow-up time was 3.84 years (0.36-22.41 years). Two patients died from their tumors, five from another disease, and two had no known cause of death. Seventeen patients had recurrence: pleural dissemination in 10, lung metastasis in 4, lymph node metastasis in 2, and multiple organ metastasis in 1. In thymoma cases (n=73), the median follow-up time was 3.86 years (0.75-22.41 years). One patient died due to their tumor, five from another disease, and two had no known cause of death. Nine patients had recurrence: Pleural dissemination in 7 and lung metastasis in 2.

Patients with TET were divided into 2 groups according to the median value of the frequency of DNA methylation. Twenty-three patients had a median frequency of DNA methylation of the *GAD1* gene >5.375 (higher DNA methylation level), while that for the remaining 23 patients was <5.375 (lower DNA methylation level). Patients with TET were divided into 2 groups according to the median value of the relative expression of mRNA. Eighteen patients had a median value for the relative expression of mRNA >0.69 (higher mRNA expression), while that for the remaining 18 patients was <0.69 (lower mRNA expression).

Kaplan-Meier curves of estimated RFS were generated according to the GAD1 DNA methylation, mRNA, and protein expression status of TET. Patients with high GAD1 DNA methylation levels had significantly shorter RFS (P=0.029, the Log-rank test) than those with low GAD1 DNA methylation levels (Fig. 3A). Patients with high GAD1

Table I. Clinicopathological characteristics of 90 patients with thymic epithelial tumors used for IHC analysis (n=81), pyrosequencing analysis (n=47) and RT-qPCR analysis (n=37).

Characteristics	Total	IHC analysis	Pyrosequencing analysis	RT-qPCR analysis
Sex, n (%)				
Male	31 (34.4)	27 (33.3)	19 (40.4)	14 (37.8)
Female	59 (65.6)	54 (66.7)	28 (59.6)	23 (62.2)
Age, mean ± SD (years)	59.7±13.0	59.2±13.0	59.5±12.3	60.9±12.3
Myasthenia gravis, n (%)				
With	19 (21.1)	17 (21.0)	10 (21.3)	9 (24.3)
Without	71 (7.6)	64 (79.0)	37 (78.7)	28 (75.7)
World Health Organization histological classification, n (%)				
Thymoma	73 (81.1)	68 (84.0)	31 (66.0)	25 (67.6)
A	9 (10.0)	8 (9.9)	5 (10.6)	2 (5.4)
AB	11 (12.2)	10 (12.3)	2 (4.3)	3 (8.1)
B1	19 (21.1)	18 (22.2)	5 (10.6)	3 (8.1)
B2	20 (22.2)	20 (24.7)	10 (21.3)	9 (24.3)
B3	14 (15.6)	12 (14.8)	9 (19.1)	8 (21.6)
Thymic carcinoma	17[a] (18.9)	13 (16.0)	16[a] (34.0)	12 (32.4)
Masaoka-Koga staging system, n (%)				
Thymoma				
I	34 (46.6)	30 (44.1)	10 (32.3)	10 (40.0)
II	21 (28.8)	21 (30.9)	11 (35.5)	6 (24.0)
III	7 (9.6)	6 (8.8)	4 (12.9)	3 (12.0)
IVA	8 (11.0)	8 (11.8)	4 (12.9)	4 (16.0)
IVB	3 (4.1)	3 (4.4)	2 (6.5)	2 (8.0)
Thymic carcinoma				
I	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
II	8 (47.1)	7 (53.8)	8 (50.0)	6 (50.0)
III	6 (35.3)	4 (30.8)	5 (31.3)	3 (25.0)
IVA	1 (5.9)	1 (7.7)	1 (6.3)	1 (8.3)
IVB	2 (11.8)	1 (7.7)	2 (12.5)	2 (16.7)
Treatment before and after operation, n (%)				
Thymoma				
Chemotherapy	7 (9.6)			
Radiotherapy	0 (0.0)			
Chemoradiotherapy	3 (4.1)			
Thymic carcinoma				
Chemotherapy	3 (17.6)			
Radiotherapy	1 (5.9)			
Chemoradiotherapy	2 (11.8)			

[a]One case was thymic squamous cell carcinoma combined with type B2 thymoma. IHC, immunohistochemical; RT-qPCR, reverse transcription-quantitative PCR.

mRNA expression levels had significantly shorter RFS (P=0.006, the Gehan-Breslow-Wilcoxon test) than those with low GAD1 mRNA expression levels (Fig. 3B). Patients with GAD1 protein-overexpressing tumors had significantly shorter RFS (P=0.029, the Log-rank test) than those without GAD1 protein-overexpressing tumors (Fig. 3C).

Discussion

We previously performed genome-wide screening on aberrantly methylated CGI in TET and identified 92 CGI that were significantly hypermethylated in TC relative to B3 thymomas. CGI within the *GAD1* gene were the 4th

Table II. Association between GAD1 immunoreactivity and clinicopathological factors in 81 patients with thymic epithelial tumors.

| | GAD1 immunoreactivity | | |
Factor	Negative (n=50)	Positive (n=31)	P-value[a]
Sex, male/female	13/37	14/17	0.075
Mean age ± SD	60.4±12.8	57.2±13.2	0.20
Masaoka-Koga staging system, stages I and II vs. III and IV	40/10	18/13	0.033
WHO histological classification, A, AB and B1 vs. B2 and B3 vs. carcinoma	29/20/1	7/12/12	<0.001
Mean GAD1 DNA methylation ± SD	5.51±5.49	22.63±20.86	0.011
Mean GAD1 mRNA expression ± SD	0.37±0.47	2.16±3.47	0.018
Myasthenia gravis, +/-	11/39	6/25	0.78

[a]P-values were calculated using the Mann-Whitney U test for age, mRNA expression and DNA methylation, while the χ^2 test was used for sex, Masaoka-Koga staging system, WHO classification and myasthenia gravis. GAD1, glutamate decarboxylase 1; WHO, World Health Organization.

Table III. Cox proportional hazard regression analysis of relapse-free survival in 90 patients with thymic epithelial tumors.

| | Univariate analysis | | | Multivariate analysis | | |
Factor	Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
Sex [male (n=31) vs. female (n=59)]	1.083	0.423-2.776	0.868	0.344	0.084-1.416	0.139
Age [≥62 (n=45) vs. <62 (n=45) years]	0.548	0.205-1.466	0.231	0.586	0.154-2.238	0.435
Masaoka-Koga staging system (stages I and II vs. III and IV)	5.532	1.941-15.771	0.001	5.727	1.689-19.423	0.005
World Health Organization histological classification (A, AB and B1 vs. B2 and B3 vs. carcinoma)	3.974	1.990-7.938	<0.0001	7.517	2.156-26.202	0.002
Myasthenia gravis [with (n=19) vs. without (n=71)]	0.811	0.265-2.477	0.713	1.407	0.332-5.960	0.643
GAD1 mRNA expression [≥0.69 (n=18) vs. <0.69 (n=19)]	3.216	0.977-10.588	0.055	NA	NA	NA
GAD1 DNA methylation [≥5.4 (n=23) vs. <5.4 (n=24)]	3.424	1.082-10.835	0.036	NA	NA	NA
GAD1 immunoreactivity [negative (n=50) vs. positive (n=31)]	2.800	0.996-7.870	0.051	0.213	0.034-1.321	0.097

GAD1, glutamate decarboxylase 1; NA, not applicable.

significantly hypermethylated CGI in TC. In the present study, we focused on the DNA methylation of *GAD1* in TET. *GAD* is crucially involved in the synthesis of GABA, which is the main inhibitory neurotransmitter system. The mammalian nervous system contains two major isoforms of *GAD*, named by their protein sizes of 67 and 65 kDa. GAD67 and GAD65 transcripts are encoded by two separate genes, GAD1 and GAD2, located on chromosomes 2q31.1 and 10p11.23, respectively, in humans (19,20). Previous studies reported that reductions in mRNA expression and DNA methylation at the promoter region of GAD1 were related to the development of several neuronal diseases, such as schizophrenia, bipolar disorder, and cerebellar disorders (33,34). GAD1 expression is abundant in the brain but rarely in other tissues, including the thymus. GAD1 has not been studied in the thymus.

We confirmed that the results obtained from genome-wide screening on aberrantly methylated CGI in TET were accurate. These results revealed that the DNA methylation rate of *GAD1* was significantly higher in TC than in the normal thymus (Fig. 1C); however, no relationship was observed for the DNA methylation rate between thymomas and the normal thymus (Fig. 1C). The DNA methylation rate was significantly higher in TC than in thymomas (Fig. 1E). Furthermore, *GAD1* methylation showed high sensitivity and specificity for discriminating between TC and thymomas (AUC=0.976) (Fig. 1F). We confirmed that GAD1 was hypermethylated in TC relative to thymomas and thymic normal tissues. In our previous study,

we examined the DNA methylation of 4 cancer-related genes (*GHSR*, *GNG4*, *HOXD9* and *SALL3*) selected from 92 CGI and revealed that the DNA methylation of 4 genes was significantly higher in TC and showed high AUC for discriminating between TC and thymomas (18). These findings demonstrated that the DNA methylation rate was significantly higher in TC than in thymomas. Recent comprehensive genetic analyses using next-generation sequencing showed that the incidence of somatic mutations was significantly higher in TC than in thymomas (10-14). Furthermore, not only genetic alterations, but also epigenetic alterations in TC significantly differed from those in thymomas. Clinically, TC differs from thymomas. Thymomas are low-grade malignant and indolent tumors with a good prognosis (5-year overall survival rate: approximately 90%) and a variable population of immature T cells associated with autoimmune diseases. In contrast, TC is an aggressive malignant tumor with a poorer prognosis (5-year overall survival rate: approximately 50%) without immature T-cell infiltration and autoimmune disease (1-6). Genetic and epigenetic differences between TC and thymomas may contribute to their clinical differences.

We also revealed that GAD1 mRNA expression levels were significantly higher in TC than in thymomas (Fig. 2A and B). To confirm mRNA results, we examined GAD1 protein expression levels in the FFPE materials of thymomas (n=68) and TC (n=13) using IHC. The immunoreactivity of GAD1 was significantly stronger in TC than in thymomas (92.31 vs. 27.94%) (Table II). Moreover, GAD1 protein expression correlated with mRNA expression (Table 2). The microarray data published in the Oncoming database showed that with the exception of some brain and kidney cancers, GAD1 was up-regulated in the majority of solid tumors, including those in colorectal, lung, and head and neck cancer patients (21). Previous studies reported the overexpression of *GAD1* in oral, nasopharyngeal, colorectal, liver, gastric, and lung cancers (21-26). In an IHC study of nasopharyngeal carcinoma (n=146), GAD1 expression correlated with the primary tumor status and advanced American Joint Committee on Cancer (AJCC) stages (23). In primary oral cancer (n=80), GAD1 expression correlated with lymph node metastasis (22). In our previous study, we revealed that GAD1 expression correlated with pleural invasion, vessel invasion, and advanced stages in non-small cell lung cancer (NSCLC) (n=162) (26). In these cancers, GAD1 expression was identified as an indicator of a poor prognosis. In the present study, we demonstrated that patients with high GAD1 mRNA expression levels had significantly shorter RFS than those with low GAD1 mRNA expression levels (Fig. 3B), and also that patients with *GAD1* protein-overexpressing tumors had significantly shorter RFS than those without GAD1 protein-overexpressing tumors (Fig. 3C). Furthermore, *GAD1* mRNA and protein expression was associated with a poor prognosis in TET as well as in other cancers.

Previous studies suggested that the GAD1/β-catenin/matrix metalloproteinase-7 (MMP-7) interaction affects malignant behaviors (22,35). GAD1 expression controls β-catenin localization to nuclei and activates MMP7. Tumors with high GAD1 expression levels have a greater potential for invasion and metastasis. Previous studies investigated β-catenin protein expression in thymomas, but not in TC (36-38). In type A, B1, and non-invasive type B2 thymomas, β-catenin

was located in the cytoplasm, in contrast to invasive type B2 and B3 thymomas, in which membranous immunopositivity was observed (36). Takahashi *et al* reported that the positive rate of MMP-7 was significantly higher in type C thymomas than in other types of thymomas, and that all TC (n=9) showed positive immunoreactivity for MMP-7 (39). We speculate that the GAD1/β-catenin/MMP7 interaction affects malignant behaviors in TC.

The present results also revealed that the DNA methylation rate of *GAD1* was significantly higher in TC than in thymomas and the normal thymus (Fig. 1C and E), and that its mRNA and protein expression levels was significantly higher in TC than in thymomas (Fig. 2B, Table II). Furthermore, a positive association was observed between mRNA expression and DNA methylation levels (Spearman's rank correlation coefficient, ϱ=0.3542, P=0.0548) (Fig. 2C). These results showed that the DNA methylation of GAD1 positively correlated with its mRNA and protein expression levels, and that a paradoxical relationship exists between promoter DNA methylation and GAD1 expression. This result is applicable to other cancers, such as colon and liver cancers (21). DNA methylation negatively regulates gene expression in the mammalian genome and inversely correlates with transcription. Recent studies reported that the DNA methylation of several genes positively correlated with mRNA expression levels (40-42). Polycomb repressive complex 2 (PRC2) is the only known complex capable of catalyzing H3K27me3 (43,44). CTCF has been shown to block the transcriptional activity of enhancer elements in a methylation-sensitive manner (45,46). In the present study, four CTCF-binding sites were detected in the GAD1 gene (Fig. 1A). The CTCF-3 site contained four CpG sites, whereas CTCF-1 had no CpG site and CTCF-2 and CTCF-4 only had one (Fig. 1A). Using electrophoretic mobility shift assays, Yan *et al* (21) confirmed that the methylation of CTCF-3 inhibited CTCF binding, whereas that of CTCF-1 and CTCF-2 had no effect. When the CpG sites of CTCF-3 are unmethylated, significant DNA-looping interactions between CTCF-3 and CTCF-1, CTCF-2, and CTCF-4 are generated, a PRC2-repressive complex is formed, and GAD1 expression is repressed. However, when the CpG sites of CTCF-3 are methylated, the DNA-looping interactions of the four CTCF are not generated and a PRC2-repressive complex cannot be formed. H3K27me3 levels are markedly reduced at the GAD1 promoter and GAD1 expression is ultimately reactivated. We speculate that the paradoxical relationship (positive correlation) between promoter DNA methylation and GAD1 expression in TET is due to the inhibition of a PRC2-repressive complex by the methylation of CTCF-3.

The present study had some limitations that need to be addressed. We examined the DNA methylation and mRNA of GAD1 using bisulfite pyrosequencing and RT-PCR in 31 and 25 thymomas and 16 and 12 TC, respectively. Thymomas are stratified into 5 entities (types A, AB, B1, B2 and B3) based on the morphology of epithelial cells and the lymphocyte-to-epithelial cell ratio. The numbers of thymoma type A, AB and B1 cases were lower (5 for type A, 3 for type AB, and 5 for type B1 thymoma) because these tumors are very rare. Although the ratio of lymphocytes to tumor cells was high in AB, B1 and B2 thymomas, we were unable to separate tumor cells from lymphocytes prior to DNA extraction. Therefore,

the presence of lymphocytes in resected AB, B1 and B2 thymomas may have influenced the promoter methylation rate. We examined GAD1 protein expression in 68 thymoma and 13 TC samples using IHC. We detected GAD1 expression in the tumor cells of each thymoma (8 type A, 10 type AB, and 18 type B1 thymomas). We also investigated the immunoreactivity of tumor cells and lymphocytes separately. The number of TC is small comparing with the number of thymomas in this study. However, TC is very rare malignant tumor. Thymomas is also a rare tumor. Incidence rates have ranged from 2.2 to 2.6 per million per year. The incidence rates of TC have ranged from 0.3 to 0.6 per million per year. Other report revealed that thymic carcinoma account for approximately 22% of all TET. We examined GAD1 DNA methylation and expression in 73 thymomas (81%) and 17 thymic carcinomas (19%). It is very difficult to make the numbers of thymomas and TC equal (47-50). Since some patients died (9 out of 90 patients), we were unable to analyze the overall survival curve.

In conclusion, we herein demonstrated that GAD1 was hypermethylated in TC relative to thymomas and normal thymic tissues. Genetic and epigenetic alterations in TC significantly differed from those in thymomas, which may contribute to their clinical differences. We revealed that the mRNA and protein expression levels of GAD1 were significantly higher in TC than in thymomas. Higher expression levels indicated malignant behavior and shorter RFS as well as other cancers. We speculate that the paradoxical relationship between promoter DNA methylation and GAD1 expression in TET is involved in the inhibition of a PRC2-repressive complex by the methylation of CTCF-3.

Availability of data and materials

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

Authors' contributions

SS, KKo, MT, KM and BT analyzed and interpreted patient data on DNA methylation using pyrosequencing. SS, YK and KKa analyzed and interpreted patient data on mRNA expression using RT-PCR. KKo, NK, HTo and MY performed the immunohistochemical staining and interpreted the association between the clinical data and the immunoreactivity. KKo, HTa and AT designed and conducted the present study. KKo and HTa performed the histological examination of thymic epithelial tumors. SS, KKo and AT were major contributors to the writing of the manuscript. SS and KKo confirm 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. 2205-4), 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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