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ORIGINAL ARTICLE

TP53 inactivation and expression of methylation-associated proteins in gastric adenocarcinoma with enteroblastic differentiation

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Abstract

Gastric adenocarcinoma with enteroblastic differentiation (GAED) is a rare variant of aggressive adenocarcinoma. We demonstrated previously that GAED is genetically characterized by frequent *TP53* mutation. In this study, we aimed to further clarify the mechanism of inactivation of *TP53* in GAED in the light of promoter methylation of *TP53*, and expression of methylation-associated proteins such as Ten-eleven translocation (TET) 1 and 5-hydroxymethylcytosine (5-hmc) in addition to *ATM* mutations. We analyzed 51 cases of GAED. The *ATM* mutation was detected in only one case. Promoter methylation of *TP53* was detected in 18% and frequency of loss of heterozygosity (LOH) at *TP53* locus was 37.2%. Reduced TET1 expression was found in 29 cases (56.9%) and was significantly associated with advanced stage ($p = 0.01$), lymph node metastasis ($p = 0.04$), and macroscopic type ($p = 0.01$). Reduced 5-hmc expression was found in 28 cases (54.9%) and was significantly associated with advanced stage ($p = 0.01$), gender ($p = 0.01$), tumor location ($p = 0.03$), tumor size ($p = 0.01$), and lymph node metastasis ($p = 0.01$). Among 9 cases with *TP53* promoter methylation, reduced expression of TET1 was observed in 6 cases, and reduced expression of 5-hmc was observed in 5 cases. Reduced expression of both TET1 and 5-hmc was significantly associated with adverse clinical outcomes. In summary, promoter methylation of *TP53* is partly involved in loss of p53 expression. Aberrant methylation by reduced TET1 and 5-hmc may be involved in the development of aggressive GAED.

Keywords Gastric adenocarcinoma · Enteroblastic differentiation · TP53 · LOH · Methylation · TET1

Introduction

Gastric adenocarcinoma with enteroblastic differentiation (GAED) is a rare variant of gastric adenocarcinoma, characterized by fetal gut-like structures with glycogen-rich clear cytoplasm [1–3]. Clinicopathologically, GAED shows aggressive behavior characterized by frequent lymphovascular invasion, lymphatic and liver metastasis even in early stages, in comparison with that seen in conventional gastric adenocarcinoma (CGA) [4, 5]. Histologically, this tumor has been characterized as being composed of cuboidal or columnar cells with clear cytoplasm and a tubulo-papillary or a solid sheet-like growth pattern, and it is almost always accompanied with conventional adenocarcinoma [3, 6, 7]. Furthermore, oncofetal proteins, such as AFP, glypican-3 (GPC3), and spalt-like transcription factor 4 (SALL4), are used as immunohistochemical diagnostic markers for GAED [8, 9]. GAED partially overlaps with alpha-fetoprotein (AFP)–producing gastric cancer (AFP-GC) or hepatoid adenocarcinoma (HAC) of the stomach; however, we concluded in our previous study that HAC may be subcategorized as solid-type GAED [10].

Recently, our group reported that GAED shows high frequency of *TP53* mutation associated with p53 overexpression, by comprehensive analysis using next generation sequencing

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(NGS) [10]. Furthermore, we also revealed that *ERBB2* amplification and *HER2* overexpression were observed in GAED as well as in CGA, and that trastuzumab could be used in a molecular target therapy for GAED [10]; however, the mechanism of inactivation of *TP53* in GAED has not been completely elucidated.

ATM was first cloned in 1995 in studies involving the ataxia telangiectasia (A-T) syndrome [11]. Interestingly, we found an *ATM* mutation in a case from among 24 GAED tissue samples examined using NGS. ATM activates the p53 tumor suppressor protein by phosphorylation of p53 in response to DNA damage [12]. Thus, the *ATM* mutation is associated with the inactivation of *TP53*, and could be the substitute for the *TP53* mutation in *TP53*-wild type in GAED.

Promoter methylation at CpG islands is an important epigenetic modification that is frequently found to be altered in cancers. Ten-eleven translocation (TET) proteins are active CpG demethylases converting 5-methylcytosine (5-mc) to 5-hydroxymethylcytosine (5-hmc), and reduced 5-hmc expression levels in cancers indicate critical roles of TET proteins in epigenetic modifications associated with tumorigenesis. Previous studies have reported frequent downregulation of TET1 in various malignancies, including nasopharyngeal, esophageal, gastric, colorectal, renal, breast and cervical carcinomas, non-Hodgkin, Hodgkin, and nasal natural killer/T cell lymphomas [13]. TET1 downregulation induces reduced 5-hmC expression levels, which are significantly associated with some clinicopathological features and poor prognosis in various cancers, including gastric cancer [14, 15]; however, the role of TET1 and 5-hmc in GAED remains unknown.

In this study, we aimed to further clarify the mechanism of inactivation of *TP53* in GAED in the light of promoter methylation of *TP53* and expression of methylation-associated proteins such as TET1 and 5-hmc in addition to *ATM* mutations.

Materials and methods

Case selection and DNA extraction

We selected a series of 51 GAED cases from our recent study [10]. These cases were selected on the basis of their pathological records (between April 2008 and February 2017) from the Juntendo University Hospital. GAED was defined as a tumor with a predominant adenocarcinoma component with a clear cytoplasm similar to a fetal gut, growing as a tubulo-papillary or solid sheet-like structure, and with more than 10% immunohistochemical positivity for AFP, GPC3, or SALL4. The 51 GAED cases consisted of 17 cases with early gastric cancer and 34 with advanced gastric cancer. All patients followed-up every 3 months after surgery. Survival periods were determined as survival time after diagnosis. The mean follow-up time for patients with GAED was 39.2 months (range, 2 to 108 months). All specimens from patients with resection were treated using a uniform preparation protocol for formalin-fixed paraffin-embedded (FFPE) specimens. Tumoral and corresponding non-tumoral FFPE samples were collected from each patient, and genomic DNA was extracted using the QIAamp FFPE tissue kit (Qiagen, Antwerp, Belgium). Quality and integrity of the DNA samples were determined spectrophotometrically. Clinicopathological data and p53 immunohistochemical data were obtained from our previous study [10]. This study was reviewed and approved by the Juntendo University School of Medicine Institutional Review Board (#2016107).

Immunohistochemistry

Immunohistochemical analyses were performed on 4-mm FFPEs from tumors. The following antibodies were used: the mouse monoclonal TET1 antibody (1:150, OriGene), and the mouse monoclonal 5-hmc antibody (1:100, GeneTex). TET1 and 5-hmc expression in tumor cells were evaluated only in GAED area. Expression levels were compared to those of normal epithelial cells within the same slide as internal control [16]. Only nuclear staining was evaluated, and nuclear and diffuse (> 50% of GAED area) staining as strong as that of normal epithelium were considered to be positive, whereas those with weaker or less than 50% staining were considered to be negative. Immunohistochemical staining results were evaluated by two pathologists (T.S. and N.Y.). When discrepancies arose, the specimens were reviewed using a multiheaded microscope to achieve a consensus.

Sanger sequencing

Mutations in the *ATM* sequence were evaluated for specimens from the remaining 27 cases that were not examined in our previous NGS study, using genomic DNA for polymerase chain reaction (PCR), followed by direct sequencing. Genomic DNA was extracted as described as previously [10]. Seventeen primer pairs listed from the Cancer Hotspot Panel were used (Supplementary Table 1). PCR products were cut from the gel and purified using a PCR clean-up gel extraction kit (MACHEREY-NAGEL). Purified PCR products were sequenced using dideoxynucleotides (BigDye Terminator v3.1; Applied Biosystems, Foster City, CA, USA) and specific primers, purified using a BigDye XTerminator Purification Kit (Applied Biosystems), and analyzed using a capillary sequencing machine (3730xl Genetic Analyzer; Applied Biosystems). Sequences were then examined using the Sequencing Analysis version 3.5.1 software (Applied

Biosystems). Bases were considered to be mutated if the height of the peak for the base reached 20% of the height of the peak for the base in the physiological scenario. All mutations were verified using sequencing the sense and antisense strands. Mutations were evaluated by two independent researchers (N.Y. and T.S.).

Methylation-specific PCR

Methylation-specific PCR was used to detect promoter methylation of *TP53*. Bisulfite modification was performed using an EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA). Then, the bisulfate-treated DNA was amplified using specifically designed primers for methylated and unmethylated alleles [17, 18]. The sequences of the primers, annealing temperature, and product size are listed in Supplementary Table 2. After amplification, the products were electrophoresed using 2% agarose gels, stained with ethidium bromide, and visualized under ultra-violet illumination. Epitect PCR control DNA set (QUIAGEN NO. 59695) was used as the control DNA.

Loss of heterozygosity analysis

Loss of heterozygosity (LOH) analysis of the *TP53* locus was performed for all specimens. Two polymorphic microsatellite markers at the *TP53* locus were used (Supplementary Table 1). Amplified PCR products were run in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). LOH was determined as previously described [19, 20]. Briefly, cases showing the allelic imbalance factor greater than 1.5 or less than 0.5 for at least one marker were judged as LOH.

Survival analysis and statistical analysis

Correlation between clinicopathological factors and immunohistochemical data was analyzed using the χ^2 test or Fisher's exact test. To elucidate the prognosis, we performed Kaplan-Meier survival analysis and log-rank tests. A p value of less than 0.05 was considered to be statistically significant.

Results

Mutation analysis

Sanger sequencing of the *ATM* gene was performed for 27 GAED specimens which were not analyzed in our previous NGS study [10]. In all, 51 cases were analyzed; however, an *ATM* mutation was detected only in 1 of 51 cases of GAED (Fig. 1A). This case was accompanied by a *TP53* nonsense mutation and LOH, leading to the loss of p53 expression as determined using immunohistochemistry (Table 1).

Methylation analysis

Methylation-specific PCR (MS-PCR) for the *TP53* gene was performed for 50 GAED cases; one case was excluded due to the exhaustion of the DNA sample. Promoter methylation of *TP53* was detected in 18% (9/50) of the cases (Fig. 1B). In this series, there was only one case with a *TP53* missense mutation; however, it did not show p53 overexpression. This case showed promoter methylation of *TP53* upon performing MS-PCR; however, promoter methylation of *TP53* was not associated with the immunohistochemical status of p53 ($p = 0.152$).

LOH analysis

Fifty-one GAED cases were analyzed for LOH of the *TP53* gene. We chose two polymorphic microsatellite markers at the *TP53* genetic locus (TP53 and AFM238). We found that the frequency of LOH at the *TP53* locus was 37.2% (19/51) (Fig. 1C, D). Upon comparison with The Cancer Genome Atlas (TCGA) database [21], the frequency of LOH of *TP53* was significantly higher in GAED than that in CGA (19/51, 37.2% vs. 3/295, 1.01%, $p < 0.01$) (Fig. 1E). In this series of cases, 10 cases showed TP53 non-sense mutations, and all of them did not show p53 overexpression as determined using immunohistochemistry. Among these 10 cases, 5 cases showed LOH at the *TP53* locus, thus were expected to show complete loss of function of p53. Furthermore, one case from a specimen with wild-type *TP53* showed LOH of the *TP53* locus and promoter methylation. This case was negative for immunohistochemical staining for p53.

Correlation between genetic alterations and p53 immunohistochemical staining

As we previously reported, totally, 35 (68.6%) out of 51 cases harbored *TP53* mutations, of which 23 (45.1%) were missense mutations and 10 (19.6%) were nonsense mutations [10]. Compared with the Cancer Genome Atlas (TCGA) database, the *TP53* mutation rate was significantly higher in GAED than that in conventional gastric adenocarcinomas (CGA) (35/51, 68.6% vs. 138/295, 46.8%, $p < .01$). Among 51 cases, 40 cases harbored at least one of these alterations in *TP53* such as mutation, LOH, and promoter methylation. *TP53* missense mutation-p53 IHC was significantly correlated very well. We had only one case with *TP53* missense mutation but without showing

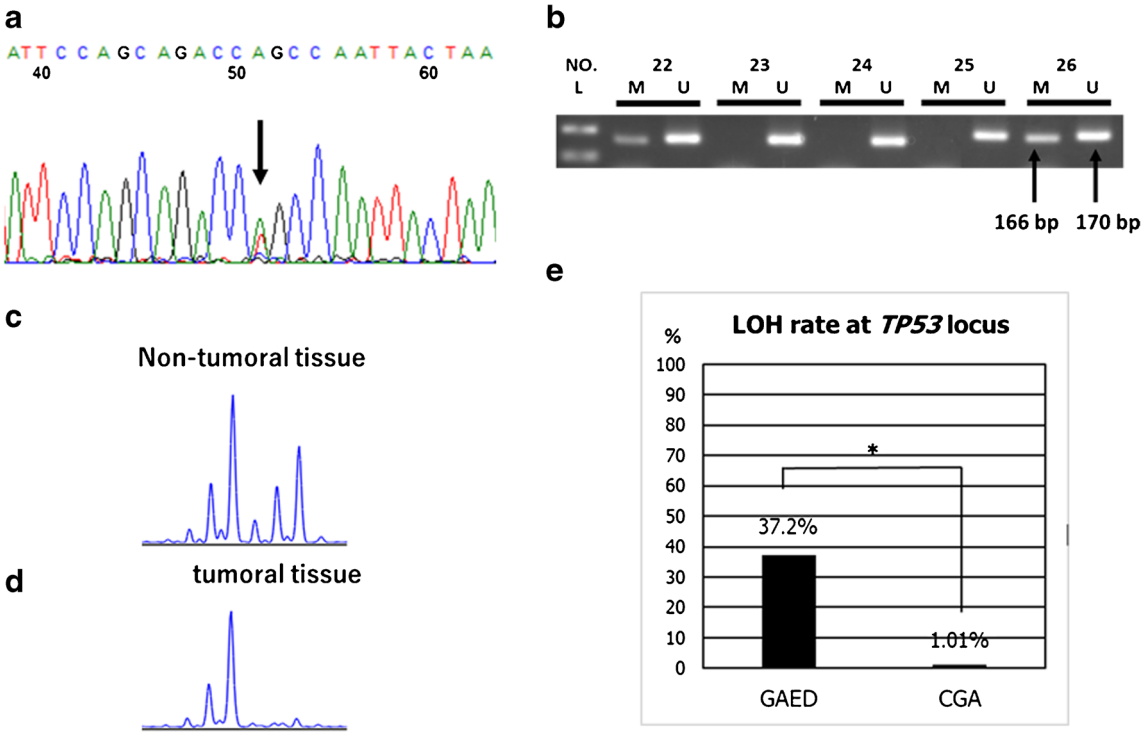

Fig. 1 **A** An *ATM* mutation was detected in only 1 case. This case was accompanied by a *TP53* nonsense mutation and LOH, leading to the loss of p53 expression as detected using immunohistochemistry. **B** Gel electrophoresis using *TP53* methylation-specific polymerase chain reaction primers. *TP53* promoter methylation was detected in 9 of 50 cases (18.0%). L: size marker, M: methylated, U: unmethylated. **C**, **D** Loss of the long allele was observed in a tumor sample (**D**) As compared to the corresponding normal tissue (**C**), indicative of loss of heterozygosity (LOH) (TP53). **E** LOH rate at the *TP53* locus in gastric adenocarcinoma with enteroblastic differentiation (GAED) was 37.2%. As compared to The Cancer Genome Atlas database, the LOH rate of the *TP53* locus in GAED was significantly higher than that in conventional gastric adenocarcinoma (CGA). *$p < 0.01$

p53 overexpression (Case#14). In addition, all cases with *TP53* nonsense mutations did not show p53 overexpression by IHC; however, there was no clear relationship between LOH, MS-PCR, and p53 immunohistochemical findings (Table 1, Supplementary Table 3).

Association between clinicopathological factors and immunohistochemistry for TET1 and 5-hmc

Clinicopathological factors were referred to from the previous study [10]. Reduced TET1 expression was found in 29 cases (56.9%, Fig. 2C, D). These cases were significantly associated with advanced stage ($p = 0.01$), lymph node metastasis ($p = 0.04$), and macroscopic type ($p = 0.01$) and tended to be associated with AFP expression in GAED ($p = 0.07$, Table 2). Reduced 5-hmc expression was found in 28 cases (54.9%, Fig. 2E, F). These cases were significantly associated with advanced stage ($p = 0.01$), gender ($p = 0.01$), tumor location ($p = 0.03$), tumor size ($p = 0.01$), and lymph node metastasis ($p = 0.01$) and tended to be associated with lymphatic invasion of GAED ($p = 0.07$, Table 2). Among 9 cases with *TP53* promoter methylation, reduced expression of TET1 was observed in 6 cases, and reduced expression of 5-hmc was observed in 5 cases; however, TET1 and 5-hmc expression were not associated with TP53 promoter methylation and p53 expression.

Association between TET1 and 5-hmc expression in GAED

Immunostaining results for TET1 and 5-hmc significantly correlated with one another. Fisher's exact test revealed that reduced TET1 expression was significantly associated with reduced 5-hmC expression ($p = 0.01$).

Prognostic impacts of TET1 and 5-hmc in GAED

The 3-year overall survival rate for GAED was 53.9%, and was updated from our previous report [10], and the 3-year recurrence-free survival (RFS) rate 44.9%. Survival analysis revealed that GAED patients with reduced TET1 and 5-hmc expression showed poor OS and RFS, than that seen in patients with preserved expression of these proteins (Fig. 3A–D). P53 overexpression, promoter methylation of TP53 and LOH status of the *TP53* locus did not affect OS and RFS.

Table 1 Association between genetic alterations and p53, TET-1 and 5-hmc immunohistochemistry (IHC)

Case	Type of *TP53* mutation	*TP53* mutation	*ATM* mutation	LOH at *TP53* locus	Metylation status of *TP53*	p53 overexpression by IHC	TET1 IHC	5-hmc IHC
1	m	c.524G>A p.R175H, c.807C>A p.S269R	None	+	−	+	−	−
2		None	None	−	−	−	−	+
3		None	None	−	+	+	+	−
4	n	c.880G>T p.E294*	None	+	−	−	+	+
5	n	c.610G>T p.E204*	None	+	−	−	+	+
6	m, intronic	c.734G>A p.G245D, c.559+1G>A	None	−	+	+	+	+
7	n	c.586C>T p.R196*	None	−	−	−	+	+
8	Intronic	c.559+8A>G	None	+	−	−	+	+
9		None	None	−	+	+	+	+
10	m	c.721T>G p.S241A	None	−	−	+	−	+
11	m	c.853G>A p.E285K	None	−	−	+	+	+
12	m	c.817C>T p.R273C	None	−	−	+	+	+
13	m	c.536A>G p.H179R	None	−	NA	+	+	+
14	m	c.802A>T p.N268Y	None	−	+	−	−	−
15	m	c.749C>T p.P250L	None	+	−	+	+	+
16	n	c.796G>T p.G266*	None	−	−	−	+	−
17	m	c.745A>T p.R249W	None	+	−	+	+	+
18		None	None	−	−	−	+	−
19		None	None	−	−	−	−	−
20	m	c.725G>C p.C242S	None	−	−	+	−	+
21	m	c.332T>C p.L111P	None	−	+	+	−	+
22	m	c.713G>A p.C238G	None	+	+	+	−	+
23		None	None	+	−	+	−	−
24		None	None	+	−	−	−	−
25	n	c.586C>T p.R196*	c.7952A>T p.Q2651L	+	−	−	−	+
26		None	None	+	+	+	−	−
27		None	None	−	−	−	−	−
28	n	c.610G>T p.E204*	None	−	−	−	+	+
29		None	None	−	−	−	−	−
30		None	None	+	+	−	−	−
31	m	c.733G>A p.G245S	None	−	−	+	+	+
32	m	c.524G>A p.R175H	None	−	−	+	+	+
33	m	c.838A>G p.R280G	None	+	+	+	−	−
34	Intronic	c.376-2A>G	None	−	−	+	−	−
35	m	c.821T>G p.V274G	None	+	−	+	−	−
36		None	None	−	−	+	−	−
37	n	c.378C>G p.Y126*	None	+	−	−	+	+
38	m	c.524G>A p.R175H	None	−	−	+	−	−
39	m	c.817C>T p.R273C	None	+	−	+	−	−
40		None	None	−	−	−	+	−
41	n	c.1043T>A p.L348*	None	−	−	−	+	+
42	m	c.743G>C p.R248P	None	−	−	+	+	−
43	m	c.733G>A p.G245S	None	+	−	+	+	−
44	n	c.499C>T p.Q167*	None	−	−	−	−	+
45	m	c.842A>G p.D281G, c.707A>C p.Y236S	None	−	−	+	−	−

Table 1 (continued)

Case	Type of *TP53* mutation	*TP53* mutation	*ATM* mutation	LOH at *TP53* locus	Metylation status of *TP53*	p53 overexpression by IHC	TET1 IHC	5-hmc IHC
46	n	c.586C>T p.R196*	None	+	−	−	−	−
47		None	None	−	−	−	−	−
48	m	c.733G>A p.G245S	None	−	−	+	−	−
49		None	None	−	−	−	−	−
50	m	c.1009C>T p.R337C	None	+	−	+	−	−
51		None	None	−	−	−	−	−

m missense mutation, *n* nonsense mutation

Discussion

Based on the finding from our previous study, that *TP53* mutations frequently occurred in GAED, we further sought for other inactivation mechanisms of p53 in GAED. *ATM* has been shown to activate p53 by phosphorylation of serine 15, rendering an inhibitory effect by degradation via MDM2 [22, 23]. Furthermore, it has also been shown that ATM-dependent phosphorylation of MDM2 on serine 395 attenuates the p53-inhibitory potential of MDM2 [24]. In this series of cases, there were 5 cases without *TP53* missense mutation; however, they showed p53 overexpression. Thus, we hypothesized whether wild-type *TP53* cases might show an *ATM* mutation, because loss of p53 function was expected to play an important role in tumorigenesis in GAED; however, we found only one case with an *ATM* mutation (already detected in our previous study), and this case was accompanied with a *TP53* nonsense mutation and LOH, and was expected to show loss

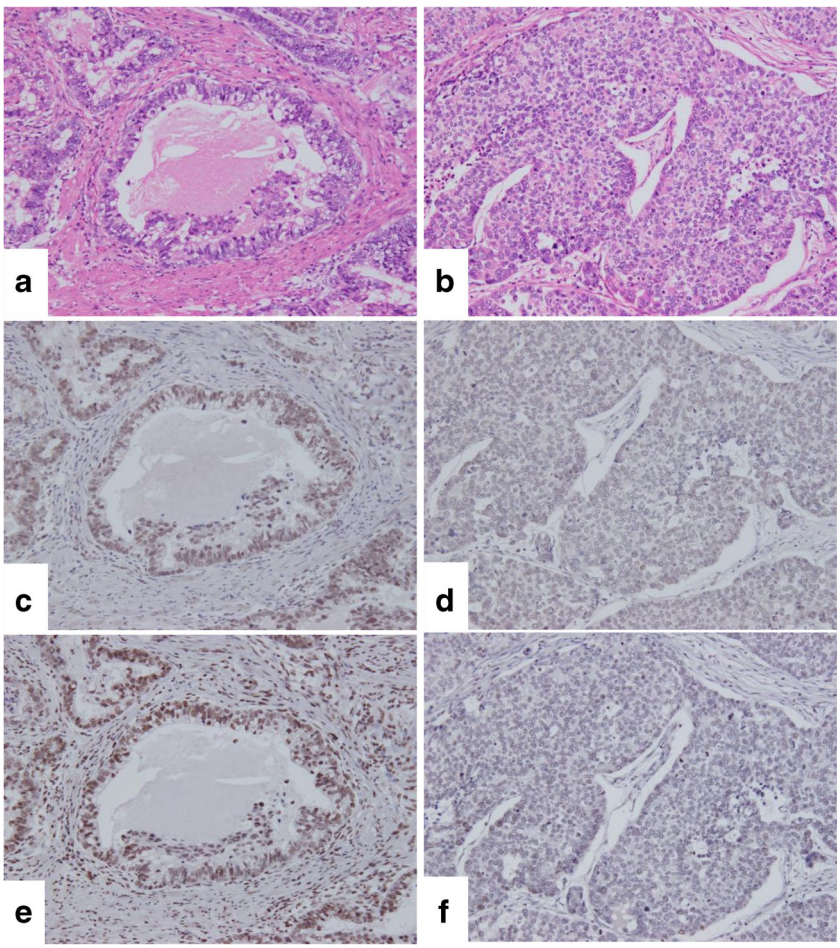

Fig. 2 **A**, **B** Hematoxylin and eosin staining of gastric adenocarcinoma with enteroblastic differentiation (GAED) tissue. Preserved Ten-eleven translocation 1 (TET1) expression was observed in a case of GAED (**C**: corresponding to **A**). Reduced TET1 expression was observed in a case of GAED (**D**: corresponding to **B**). Preserved 5-hydroxymethylcytosine (5-hmc) expression was observed in a case of GAED (**E**: corresponding to **A**). Reduced 5-hmc expression was observed in a case of GAED (**F**: corresponding to **B**). Original magnification (**A**–**D**), 200×

Table 2 The expression of TET1 and 5-hmc in GAED with clinicopathological factors ($n = 51$)

Parameter	TET1		p value	5-hmc		p value
	Reduced ($n = 29$)	Preserved ($n = 22$)		Reduced ($n = 28$)	Preserved ($n = 23$)	
Age (years) (mean ± SD)	72.1 ± 9.20	69.9 ± 9.29	1	69.4 ± 9.58	73.9 ± 8.58	0.4
Gender (male/female)	25/4	17/5	0.47	27/1	15/8	0.01
Tumor location (U/M/L)	11/7/11	4/5/13	0.24	12/7/9	3/5/15	0.03
Tumor size (mm) (mean ± SD)	48.1 ± 31.3	35.5 ± 30.7	0.47	53.5 ± 34.3	29.6 ± 21.6	0.05
Therapy method (operation/ESD)	27/2	17/5	0.22	27/1	17/6	0.04
Macroscopic type (elevated/depressed)	3/2	0/13	0.01	2/3	1/12	0.17
Macroscopic type (type 1/2/3/4/5)	1/13/10/0/0	1/7/0/1/0	0.03	2/11/9/0/1	0/9/1/0/0	0.15
Invasion depth (early/advanced)	4/25	13/9	< 0.01	4/24	13/10	< 0.01
TNM stage (I/II, III, IV)	3/26	12/10	< 0.01	4/24	11/12	0.01
Lymphatic invasion (+/−)	22/7	12/10	0.14	22/6	12/11	0.07
Venous invasion (+/−)	21/8	14/8	0.55	21/7	14/9	0.37
Lymph nodes metastasis (+/−)	24/5	12/10	0.04	24/4	12/11	0.01
Liver metastasis (+/−)	12/17	5/17	0.23	11/17	6/17	0.38
Growth patterns (Sllid/Tubulo-papillary type)	10/19	6/16	0.762	10/18	6/17	0.55
Immunohistochemical analysis						
AFP (+/−)	6/23	10/12	0.07	8/20	8/15	0.76
GPC3 (+/−)	22/7	20/2	0.27	23/5	19/4	1
SALL-4 (+/−)	23/6	18/4	1	24/4	17/6	0.32

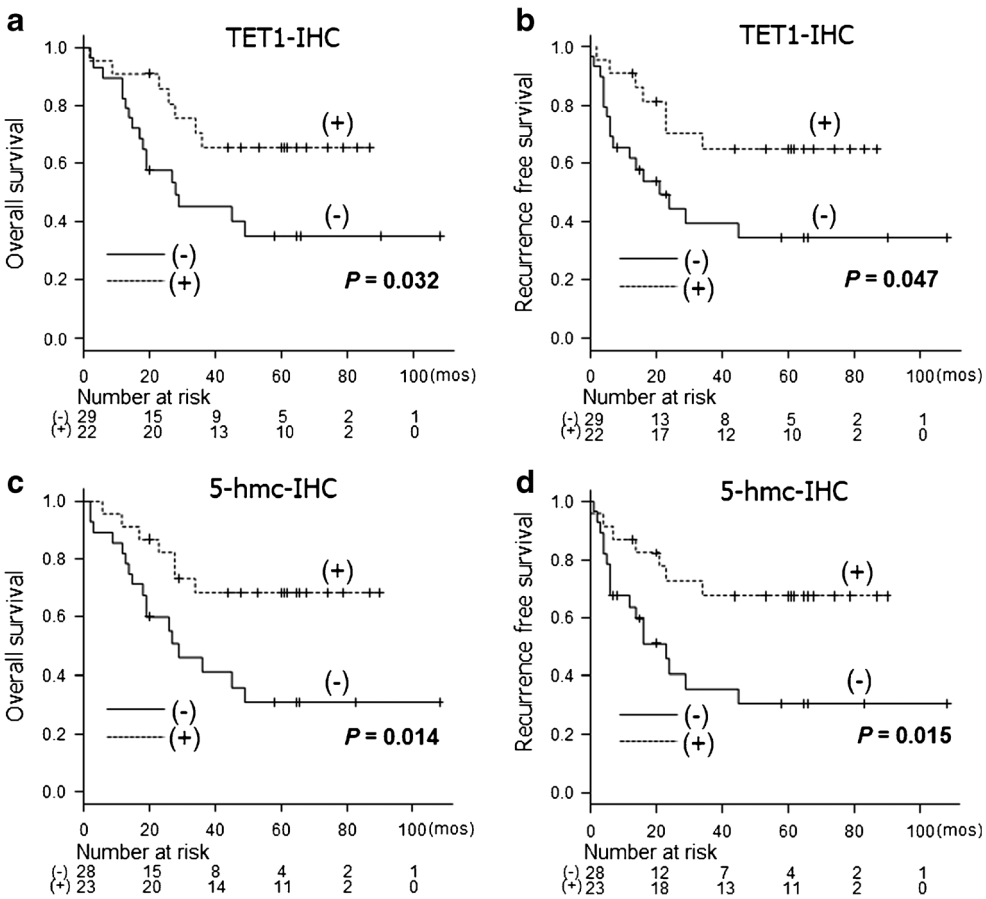

Fig. 3 Kaplan-Meier survival curves. The 3-year overall survival (OS) and the 3-year recurrence free survival (RFS) rates according to the expression of Ten-eleven translocation 1 (TET1) (**A**, **B**) and 5-hydroxymethylcytosine (5-hmc) expression in gastric adenocarcinoma with enteroblastic differentiation (GAED) (**C**, **D**). Group with preserved 5-hmc and TET1 expression showed better OS rate (**A**, **C**) and RFS rate (**B**, **D**) as compared to that with reduced expression. (+): Preserved expression, (−): Reduced expression

of p53 function. These findings suggest that *ATM* mutation is not very significant in the loss of p53 function in GAED. LOH of the *TP53* locus was frequently detected in GAED as compared to that seen in CGA. In addition, in this series, there were 10 cases with *TP53* non-sense mutations, all of which did not show p53 overexpression as determined using immunohistochemistry. Five out of these 10 cases also showed LOH at this locus, and were expected to show complete loss of function of p53. These findings suggest that the inactivation of p53 plays an important role in the tumorigenesis of GAED.

Contrastingly, we found promoter methylation of *TP53* in 18% of the cases. The frequency of promoter methylation of *TP53* in CGA has not been adequately evaluated; however, one report described that none of the 43 cases harbored promoter methylation of *TP53* in CGA [17]. Promoter methylation of *TP53* in a small subset of GAED patients might be relatively rare; however, it is one of the epigenetic events that characterizes this tumor. *TP53* missense mutation-p53 overexpression was significantly correlated very well as observed in ovarian high-grade serous carcinoma [25]. We had only one case with *TP53* missense mutation but without showing p53 overexpression (Case#14). In this case, MS-PCR for *TP53* promoter was positive; therefore, we speculated that *TP53* promoter methylation might have occurred in the mutated allele. In addition, all cases with *TP53* nonsense mutations did not show p53 overexpression by IHC. This finding suggests that promoter methylation is partly involved in loss of p53 expression in GAED; however, considering that epigenetic silencing by promoter methylation is a reversible event and its relatively lower frequency (18%) compared to the LOH ratio (37.2%), it would be considered that the LOH is the second mechanism of p53 inactivation.

TET1 catalyzes the oxidation of 5-mC to 5-hmC, which results in demethylation of genomic DNA. Reduced 5-hmc and TET1 levels are seen in various cancers and have been considered to an epigenetic hallmark of cancers [13, 14]. Systematic review and meta-analysis by Zhaoli et al. showed that reduced 5-hmc levels were significantly associated with lymph node metastasis and advanced stage in various cancers including glioblastoma, gastric, hepatocellular, esophageal, prostate, ovarian, intrahepatic cholangio-, kidney, breast, and cervical cancers [14]. Moreover, reduced 5-hmc levels were significantly associated with poor prognosis (OS and RFS) in the above-mentioned cancers [14]. In addition, reduced 5-hmc levels were significantly associated with lymph node metastasis; tumor size; poor prognosis in lung cancer; advanced stage and poor prognosis in laryngeal cancer; and advanced stage and poor prognosis in malignant melanoma [26–28]. Previous studies have also demonstrated that reduced TET1 levels are significantly associated with lymph node metastasis; advanced stage; poor prognosis in endometrial cancer; metastasis and poor prognosis in colorectal cancer; and poor prognosis in kidney cancer [29–31]. In our study, reduced TET1 expression was found in 29 cases (56.9%) and reduced 5-hmC expression was found in 28 cases (54.9%) in GAED, and each of them was significantly correlated ($p = 0.01$), suggesting that reduced TET1 expression might be one of the mechanisms underlying reduced 5-hmc expression in GAED. Reduced expression of TET1 and 5-hmc in CGA and their prognostic impacts have been also demonstrated [15]. We did not find any difference between GAED and CGA in this respect.

In this study, p53 overexpression, promoter methylation of *TP53* and LOH status of the *TP53* locus did not affect OS and RFS. On the contrary, the survival analysis revealed that GAED patients with reduced TET1 and 5-hmc expression showed poor OS and RFS. Among 9 cases with *TP53* promoter methylation, reduced expression of TET1 was observed in 6 cases, suggesting that the *TP53* promoter might be one of the targets for demethylation by TET1, although the number of cases with *TP53* promoter methylation was too small to make a definite conclusion. These findings also raise the possibility that the effect of the reduced expression of TET1 and 5-hmc might be mediated by epigenetic regulation of other genes besides *TP53*. Regarding this point, Ras association domain family member 5 (*RASSF5*) has recently been identified as key downstream target of TET1 demethylation in ovarian cancer [16]. In addition, it has also been shown that FAM20C could be targeted by TET1 to promote odontoblastic differentiation potential of human dental pulp cells [32]. Furthermore, reduced expression of TET1 and 5-hmC was associated with adverse clinical outcomes in GAED, also suggesting that aberrant promoter methylation affects the acquisition of aggressive behavior of this tumor.

In summary, promoter methylation of *TP53* is partly involved in loss of p53 expression, and LOH of the *TP53* locus as was frequently detected in GAED as compared to that in CGA. Inactivation of p53 might play an important role in the tumorigenesis of GAED. Furthermore, aberrant methylation by reduced TET1 and 5-hmc may be involved in the acquisition of aggressive behavior in GAED.

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Author contribution Noboru Yatagai, Tsuyoshi Saito, Yoichi Akazawa, Takuo Hayashi, Takashi Murakami, and Takashi Yao designed the research project and evaluated the histological and immunohistochemical findings. Noboru Yatagai, Takuo Hayashi, and Tsuyoshi Saito analyzed the obtained data and wrote the main part of the manuscript and Sumio Watanabe, Akihito Nagahara, and Takashi Yao reviewed the draft with critical comments and wrote the manuscript. Noboru Yatagai, Yuya Yanai, Sho Tsuyama, Hiroya Ueyama, and Takashi Murakami performed molecular pathological part of the experiments.

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Conflict of interest The authors declare that they have no conflicts of interest.

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