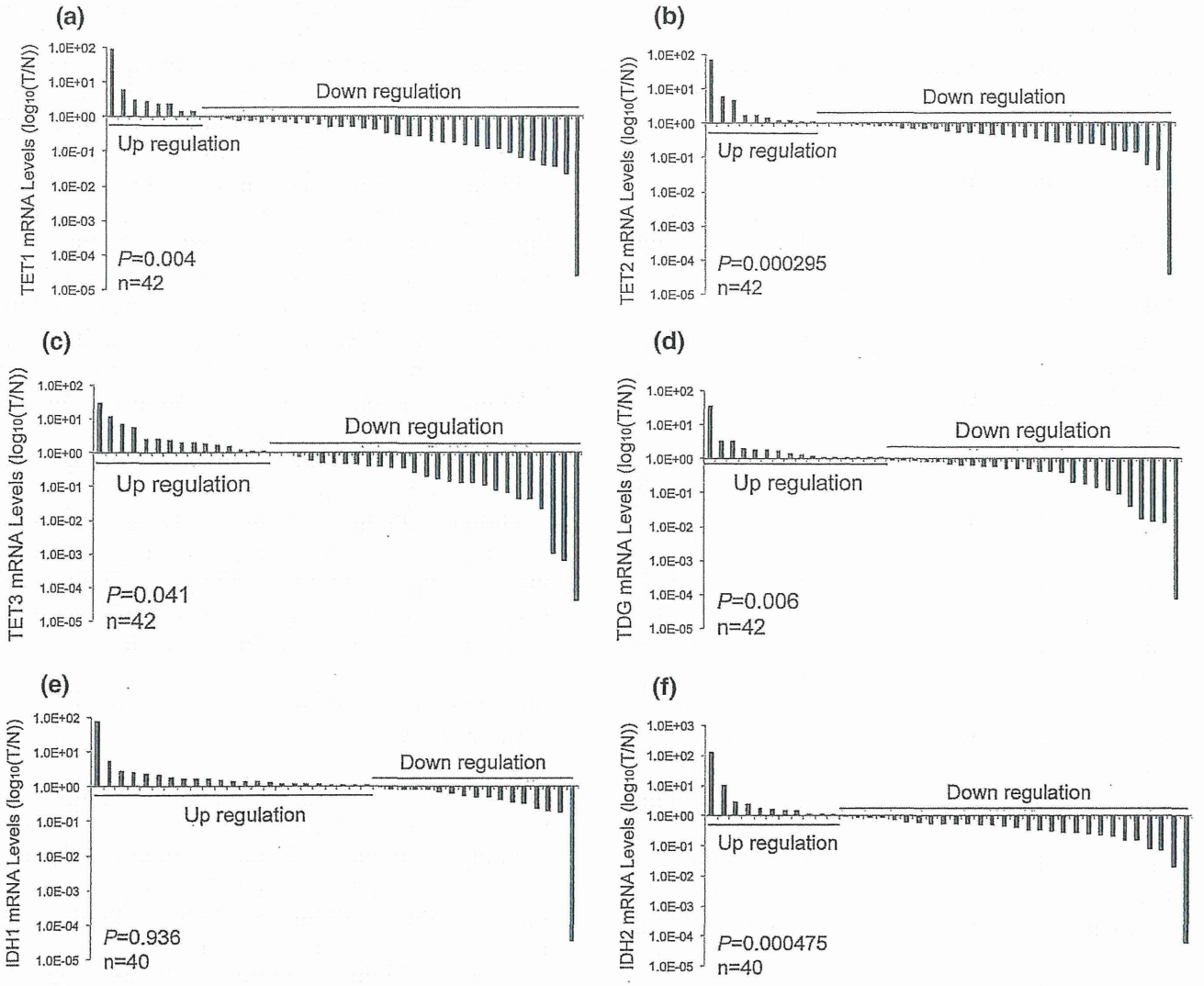

Fig. 1 Gene expression levels in gastric cancer tissue. Measurement of *TET1* (a), *TET2* (b), *TET3* (c), *TDG* (d), *IDH1* (e) and *IDH2* (f) mRNA expression levels in GCs and adjacent normal samples using real-time RT-PCR. *P* values were analyzed using the Mann-Whitney *U* test. $P < 0.05$ was considered statistically significant

to the corresponding non-tumor tissue (Fig. 1, *TET1*, $P = 0.004$; *TET2*, $P = 0.000295$; *TET3*, $P = 0.041$; *TDG*, $P = 0.006$; *IDH2*, $P = 0.000475$) while the expression of *IDH1* was not significantly decreased ($P = 0.936$). Down-regulation of *TET1* in the GCs was correlated with the tumor location (Table S3, $P = 0.036$). Furthermore, down-regulation of *IDH2* was positively correlated with the tumor classification N ($P = 0.002$) and the tumor stage ($P = 0.027$) (Table 2). However, no significant association between *TET2*, *TET3*, *TDG* or *IDH1* down-regulation and any of the clinicopathological factors was seen in our samples (data not shown).

5-hmC levels are correlated with *TET1* gene expression

A linear regression analysis showed that *TET1* expression was significantly and positively correlated with 5-hmC levels ($P = 0.037$, $R = 0.428$; Pearson's correlation coefficient). However, no correlation was seen between 5-hmC levels and the expressions of *TET2*, *TET3*, *TDG*, *IDH1* or *IDH2* in the gastric mucosa (Fig. 2).

Comparison of 5-mC oxidation activities of TET1, TET2 and TET3 proteins *in vitro*

Expressions of *TET1-3* were significantly decreased in GCs but only *TET1* expression was notably correlated with 5-hmC levels. To further examine the relationships between 5-hmC and TET proteins in human cells we compared the 5-mC oxidation activities of the TET1, TET2 and TET3 proteins. Thus, full-length *TET1*, *TET2* and *TET3* plasmids were transfected in HEK293T cells. Then LC-MS/MS and real-time RT-PCR were used to quantitate the 5-hmC and gene expression levels in these cell lines.

Table 2 Correlation between IDH2 gene expression and clinicopathological attributes

Variables	Cases	Down-regulation	Up-regulation	*P*-value
Age				
<60	6	5	1	1.000
>60	34	24	10	
Gender				
Male	27	21	6	0.451
Female	13	8	5	
Tumor location				
Lower	8	4	4	0.378
Middle	14	10	4	
Upper	10	8	2	
Others*	8	-	-	
T classification				
T1	9	5	4	0.568
T2	4	3	1	
T3	15	11	4	
T4	12	10	2	
N classification				
N0	13	7	6	0.002
N1	5	1	4	
N2	10	10	0	
N3	12	11	1	
Stage				
I	10	5	5	0.027
II	9	5	4	
III	21	19	2	
Histological type				
Intestinal	23	18	5	0.285
Diffuse	15	9	6	
Mixed	2	-	-	

P values were evaluated using the Pearson chi-square or the Fisher exact test. $P < 0.05$ was considered statistically significant. * 8 cases in which the tumor covered all the lower, middle, and upper parts. Down-regulation: Tumor/Normal <1.0; Up-regulation: Tumor/Normal >1.0

As shown in Fig. 3a, the forced expressions of *TET1* and *TET2* significantly increased the 5-hmC level ($P < 0.01$) while *TET3* moderately increased the 5-hmC level. Notably, TET1 had the highest efficiency in global DNA 5-mC oxidation among the TET family proteins.

Sequencing analysis of *TET1-3* in the GCs

We were interested in whether 5-hmC reduction in tumors was associated with the somatic mutation of *TET1-3* in GCs. Therefore, sequencing was performed to analyze 18 pairs of GCs. All the GC samples were confirmed to have significantly decreased 5-hmC levels in the tumor portions as compared to the adjacent non-tumor portions when examined using LC-MS/MS.

According to the Japanese Single Nucleotide Polymorphism (JSNP) database and the National Center for Biotechnology (NCBI) (updated on January 31, 2014), 6 SNPs (c.485A>G, c.577T>A, c.767C>T, c.2751G>A, c.3053A>G, and c.3369A>G) in *TET1*, 6 SNPs (c.86C>G, c.652G>A, c.3117G>A, c.4140T>C, c.5162T>G, and c.5284A>G) in *TET2*, and 2 SNPs (c.1520C>G and c.2131T>C) in *TET3* are currently known. Using a sequencing analysis we found 2 known missense variations (c.5162T>G (p.L1721W) and c.5284A>G [p.I1762V]) in the *TET2* catalytic domain and 1 novel variation (c.3000C>T) in *TET3*. No somatic tumor-specific mutations were found in any of our samples. Two of these SNPs (c.3117G>A and c.4140T>C) in *TET2* were previously detected among Japanese children with essential thrombocythemia [20] but their pathogenic significance was unclear. Since our sequencing study was performed using a relatively small number of available samples we decided to include the known functional variants Y1148* [21] and F1706fs*13 [22] in our transfection experiments.

Comparing 5-mC oxidation activity of 7 human TET2 variant proteins

We constructed and tested the 5-hmC-generating activities of TET2 catalytic domain (1123-2002 aa) variants in HEK293T cells identified in this study or in previous studies [21–23]. As shown in Fig. 3b, c, using the DNA dot blot and LC-MS/MS (Figure S4), TET2 proteins obviously increased the number of 5-hmC adducts in human cells. The 5-mC oxidation activities of Y1148* [21] and F1706fs*13 [22] proteins were severely impaired but the functions of A1476V [21], V1836M [21], L1240V [23], L1721W (newly found in this study) and I1762V (newly found in this study) proteins seemed to be retained. Moreover, LC-MS/MS data seems to be consistent with the results obtained from the dot blot analysis.

Discussion

Herein we applied LC-MS/MS for the quantification of 5-mC, 5-hmC, 5-fC, and 5-caC levels in both the tumorous and non-tumorous portions of human gastric mucosa resected. 5-mC was moderately decreased in GCs. 5-caC and 5-fC are also important metabolites of these dynamics of cytosine modification, as shown in Figure S1. Considering reports on the existence of these molecules are limited in normal brain and brain tumor tissues [24, 25] our attempt was to first try to detect these modified cytosines in human non-neural tissue. We assumed that our detection limits

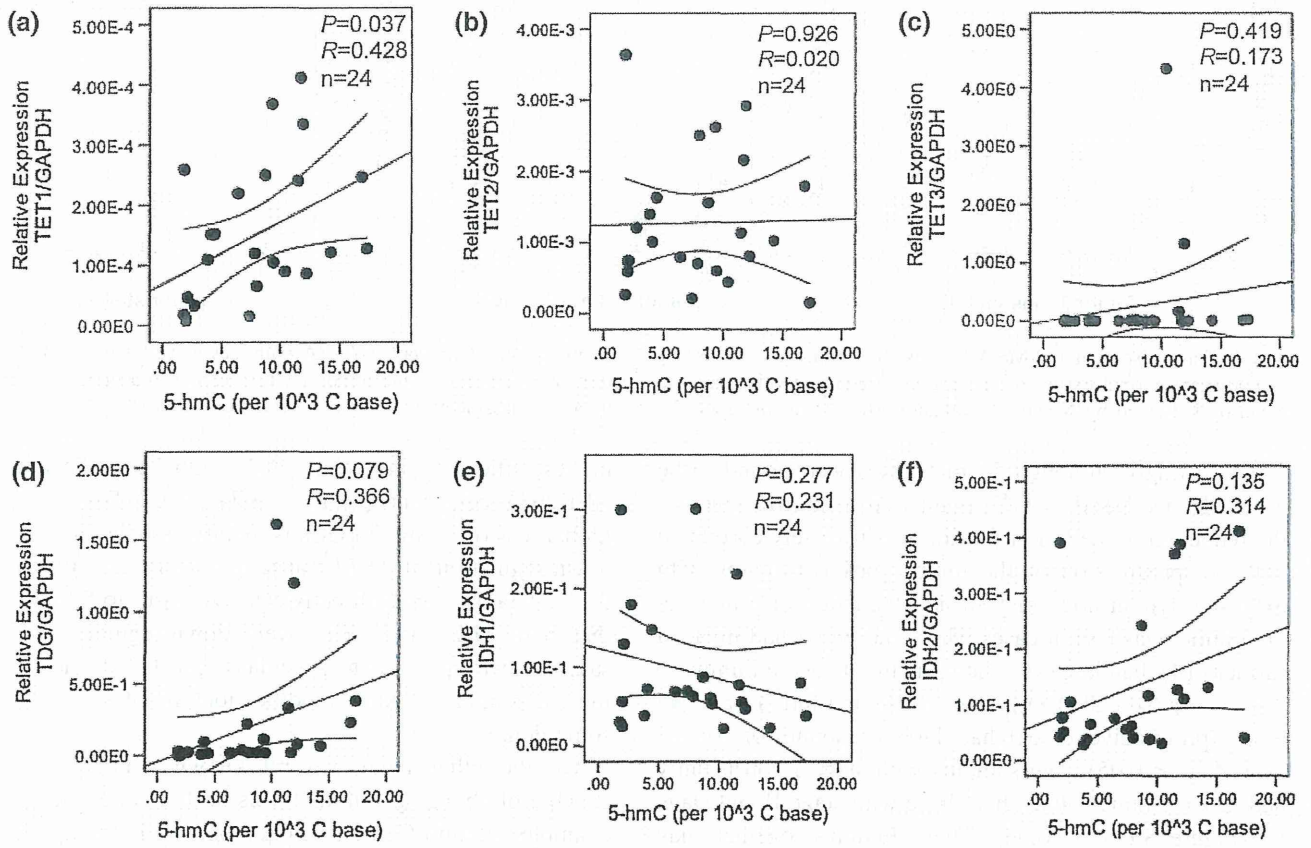

Fig. 2 Linear regression analyzing the relationships between 5-hmC levels and *TET1* (a), *TET2* (b), *TET3* (c), *TDG* (d), *IDH1* (e) and *IDH2* (f) mRNA expressions in GCs and adjacent normal samples. *P* values were analyzed using a two-tailed Pearson correlation. $P < 0.05$ was considered statistically significant

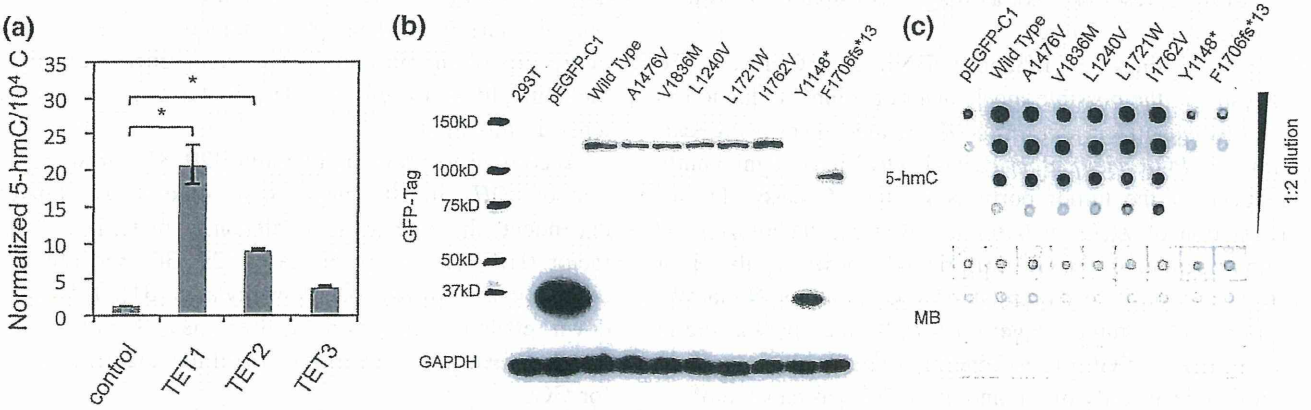

Fig. 3 Overexpression of TET proteins in HEK293T cells. (a) Comparison of 5-mC oxidation activity of TET proteins using LC-MS/MS. 5-hmC values were divided by the corresponding gene mRNA levels and the standard error. One-way ANOVA with Tukey's *post-hoc* test, $*P < 0.01$; (b) Western blot for detecting the expression of 7 variants of GFP-tagged TET2 catalytic domain proteins (1123-2002 aa). GAPDH was used as a control; (c) Comparison of 5-mC oxidation activities of 7 variants of TET2 catalytic domain proteins using a DNA dot blot. Methylene blue staining was used as a total genomic DNA loading control. MB: methylene blue

were 0.103 fmol (5-fC) and 0.133 fmol (5-caC) based on calibration curves using internal controls; actually, no signal peaks were observed in human gastric tissues. Thus, we interpreted that there were no 5-fC and 5-caC in human gastric tissues. On the other hand, 5-hmC was reduced by about 3-fold in the tumors of GC cases. In line with recent studies [9, 26], our data also show that 5-hmC values varied widely among individuals.

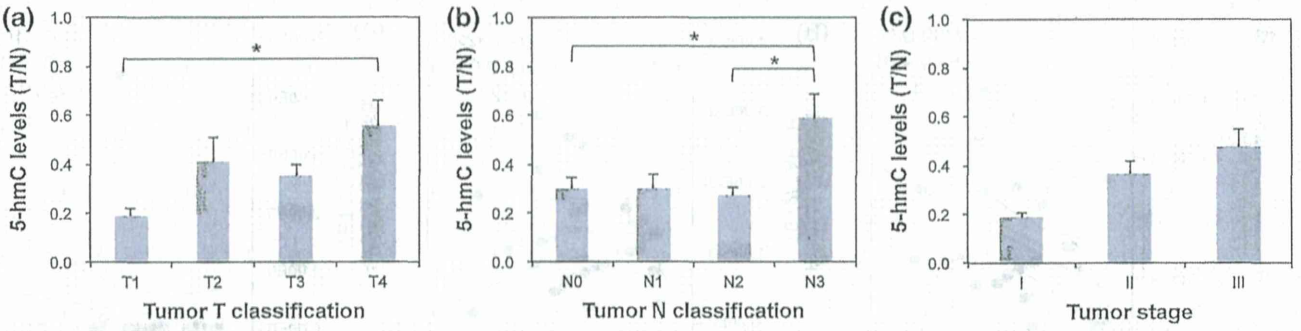

Fig. 4 Comparisons of LC-MS/MS values of 5-hmC in GC tissues and adjacent normal tissues of GC patients. Indicated values are the mean of T divided by N and the standard error. T: tumor tissue; N: normal tissue. One-way ANOVA with Tukey's post hoc test, $*P < 0.05$. **a** 5-hmC levels and tumor T classification; **b** 5-hmC levels and tumor N classification; **c** 5-hmC levels and tumor stage

Interestingly, although 5-hmC levels were found to be generally decreased in GCs compared to gastric mucosa, the reduction in the tumor portion was inversely correlated with the progression of the tumor portion in cases with extensive lymph node metastasis. Reduction of 5-hmC in the tumors was less in cases where the tumor had invaded further (T4) than in cases where the tumors were limited to the mucosa (so-called early cancer T1). In short, T4 and N3 cases (progressive cases) had larger amounts of 5-hmC (Fig. 4, $P < 0.05$). The samples with stage I consistently had lower 5-hmC values than those with stage II and stage III (Figure S5, $P < 0.05$). These findings together may suggest that the loss of 5-hmC in human GCs occurred in the early stage, at least in our cohort. More extensive studies using multiple cohorts will be needed to determine whether the loss of 5-hmC could become one of the markers representing some epigenetic nature of human gastric cancer in future.

Several enzymes influence DNA 5-hmC levels. To investigate the possible mechanism of 5-hmC reduction in GCs we quantified transcripts of 7 related genes using real-time RT-PCR. *TET1-3*, *TDG* and *IDH2* were significantly reduced in the tumor portions in the GC cases. Down-regulation of *TET1* in particular was correlated with the tumor location of the GC. Surprisingly, down-regulation of *IDH2* was positively correlated with the tumor N classification and the tumor stage; thus, *IDH2* may play a role in the progress of GC. These findings are partially consistent with a recent study of melanoma. *TET2* was capable of re-establishing the 5-hmC landscape in human melanoma cells. IDH2 increased the 5-hmC levels and prolonged tumor-free survival in a zebrafish melanoma model [11]. This evidence suggests that a deficiency of these enzymes may lead to one of the mechanisms of global 5-hmC reduction in GC. The next important finding was that the global 5-hmC levels were significantly and positively correlated with *TET1* expression using a linear regression analysis, but not with *TET2-3*, *TDG* or *IDH1-2*. Additionally, our *in vitro* study confirmed that *TET1* has the highest efficiency in global DNA 5-hmC modification in HEK293T cells among *TET* family genes. Together, the global loss of 5-hmC in GCs is mainly associated with the down-regulation of *TET1* transcripts. Considering that all the TET family genes directly catalyze 5-mC to 5-hmC and that both *TET2* and *TET3* were down-regulated in our samples, we continue to speculate that TET2 and TET3 may also impact 5-hmC modifications in GCs, at least to some degree.

On the other hand, several known confounders are capable of changing 5-hmC levels in the human body; for example, vitamin C and hydroquinone are both capable of increasing 5-hmC levels in vitro [27, 28]. Lifestyle factors, such as food and medicine intake as well as cosmetics use, also cannot be excluded from factors that may influence the 5-hmC level in the human stomach. Estimations of the intake of particular diets based on a food frequency questionnaire and other descriptive methods used in epidemiology are often difficult, but a comprehensive analysis including lifestyle information will be a necessary challenge in this field.

According to previous literature [29–31], down-regulation of *IDH2* in GC may inhibits other 2-oxoglutarate-dependent dioxygenases, particularly hypoxia-inducible factor (HIF) prolyl hydroxylases [29, 30] and the JmjC domain containing histone demethylases [31]. Thus, *IDH2* down-regulation in gastric cancer may provide various insights regarding the biology of and therapeutic strategies for GCs.

Another finding, the down-regulation of *TDG* in gastric cancer, is likely to attract many investigators in the future. The functions of *TDG*, with the exception of its DNA glycosylase activity, are elusive and these functions are speculated to be relevant to *p53* [32] and Wnt Signaling [33].

Very recently, Yang et al. [12] reported that 5-hmC levels were markedly and negatively correlated with tumor invasion; that is, with the TNM stage and the number of lymph node metastases in GCs. They measured the 5-hmC level using a DNA dot blot assay and the down-regulation

of *TET1* transcripts was notably associated with a reduction of 5-hmC levels in GCs. The positive correlation between *TET1* expression and 5-hmC levels found in our correlation analysis was consistent with their clinical findings and was reasonable considering the function of *TET1*. Some inconsistencies were present in that the correlation between the reduction of 5-hmC and progression indicators (N or T factors) of cancer was not clear in our subjects. This inconsistency might be related to the different analysis technologies, the limited number of cases, differences in the patient populations and other confounding factors. Our studies not only showed down-regulation of *TET1* in our samples but also that of *TET2*, *TET3*, *TDG* and *IDH2*.

DNA mutation is another factor that influences protein activity. *TET2* is mutationally inactivated in about 15 % of human myeloid cancers [34]. However, a few mutations of *TET1-3* have been found in solid cancers and no somatic mutation in *TET1-3* has been found to be associated with the loss of 5-hmC in our samples. Given that our sequencing study was performed using a relatively small number of available samples we also validated our findings using the Catalogue of Somatic Mutations in Cancer (COSMIC) [35]. As of January 31, 2014, the mutation rate of *TET1* was 0 % (0/47), that of *TET2* was 0 % (0/47) and that of *TET3* was 2.23 % (1/47; p.L711L) in GCs.

Despite the small number of samples, our robust measurements of the DNA 5-hmC adduct and the *in vitro* analysis validated a decrease in 5-hmC in GCs, mainly due to *TET1* down-regulation. In addition to the biological significance of *TDG* and *IDH2* reduction in gastric cancer information on the local status of 5-hmC in the genome will be the next important topic of research on GCs in terms of acquired DNA changes, including adductomics [36]. Further studies in a larger cohort are required to evaluate the relationship among 5-hmC, TET1, IDH2 and clinical characteristic such as the stage of GC.

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PLK4 overexpression and its effect on centrosome regulation and chromosome stability in human gastric cancer

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Abstract Polo-like kinase 4 (PLK4) is a centrosomal protein that is involved in the regulation of centrosome duplication. This study aimed to determine whether the genetic abnormality of PLK4 is involved in human gastric cancer. First, we examined the status of PLK4 mRNA expression in 7 gastric cancer cell lines and 48 primary gastric cancers using an RT-PCR analysis. The upregulation of PLK4 mRNA expression was detected in 57.1 % (4/7) of the gastric cancer cell lines, and a novel PLK4 variant with exon 4, but without exon 5, was identified. In the primary gastric cancers, the upregulation of PLK4 mRNA expression in the cancerous cells was detected in 50.0 % (24/48) of the cases, and this upregulation was statistically significant (P value = 0.0139). Next, we established AGS gastric cancer cells capable of inducibly expressing PLK4 using the piggyBac transposon vector system and showed that PLK4 overexpression induced centrosome amplification and chromosome instability using immunofluorescence and FISH analyses, respectively. Furthermore, PLK4 overexpression suppressed primary cilia formation. Our current findings suggested that PLK4 is upregulated in a subset of primary gastric cancers and that PLK4 overexpression induces centrosome amplification and chromosome instability and causes the suppression of primary cilia formation.

Keywords Centrosome amplification · Chromosome instability · Gastric cancer · PLK4 · Primary cilia

Introduction

Chromosome instability (CIN) is a genetic feature observed in human cancer, and a number of studies have shown that centrosome amplification is one of the factors that contributes to CIN in cancerous cells [1–5]. The centrosome, a major microtubule-organizing center, is composed of a pair of centrioles and surrounding protein aggregates called pericentriolar material, and each cell is maintained so that it has one or two centrosomes during the cell cycle [6, 7]. When centrosome amplification (≥3 centrosomes in a cell) occurs, it causes an increase in aberrant mitotic spindle formation, lagging chromosome formation, merotelic kinetochore-microtubule attachment errors, and chromosome segregation errors, all of which are thought to be possible causes of CIN [1–5, 8, 9].

Polo-like kinase 4 (PLK4) is a centrosomal protein involved in regulating the number of centrosomes in human cells [10, 11]. The SCF component CUL1 and SCF slimb ubiquitin ligase function as suppressors of centriole multiplication by regulating the PLK4 protein level [12, 13], and PLK4 kinase activity also regulates its own stability [14, 15]. PLK4 interacts with CEP152 and controls centrosome duplication in human cells [16]. Clinically, PLK4 expression is upregulated in colon cancer [17], while it is downregulated in hepatocellular carcinoma [18]. However, no reports of PLK4 genetic alterations in other cancers have been made. Since CIN is frequently observed

The sequence data for a novel variant of PLK4 have been submitted to the DNA Data Bank of Japan (DDBJ) database (http://www.ddbj.nig.ac.jp/index-e.html) under accession number AB741646.

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in lung and gastric cancer [19, 20], we hypothesized that a PLK4 abnormality is one of the factors capable of inducing CIN in lung and gastric cancer. Therefore, in the present study, we first examined whether PLK4 is aberrantly expressed in human lung and gastric cancer cell lines. Since a higher level of PLK4 expression was detected in the gastric cancer cell lines and the relationship between centrosome abnormality and gastric cancer remains largely unknown, we further examined the PLK4 expression status in primary gastric cancers and investigated whether a PLK4 abnormality can cause CIN in a gastric cancer cell line.

Centrioles have the ability to function as basal bodies for the formation of primary cilia, which participate in the modulation of growth factor signaling pathways, and defects in the formation of primary cilia are associated with some types of human diseases including cancer [21, 22]. Since PLK4 is a centrosomal protein, we also investigated whether PLK4 overexpression has any effect on primary cilia formation.

Materials and methods

Total RNA samples

Human small airway epithelial cells (SAEC), 14 lung cancer cell lines (A549, ABC-1, H358, H460, H820, H2087, Lu65, LC-2/ad, RERF-LC–MS, RERF-LC-KJ, Lu130, Lu135, H526, and H1688), and 7 gastric cancer cell lines (AGS, TMK1, MKN1, MKN28, MKN45, MKN74, and KATO-III) were used, as reported previously [23–26]. Gastric cancer tissues and corresponding normal gastric tissues from primary gastric cancer patients ($n = 48$) were obtained from Hamamatsu University Hospital (Japan). The total RNA was extracted using an RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) and converted to cDNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). The study design was approved by the institutional review board of Hamamatsu University School of Medicine.

Semiquantitative reverse-transcription (RT)-polymerase chain reaction (PCR)

RT-PCR for the PLK4 transcript was performed in 20-μL reaction mixtures containing cDNA of cell lines, HotStarTaq DNA polymerase (Qiagen), and a set of PCR primer pairs (5′-CGG AGA ACC CAG GCC AGA GC-3′ and 5′-AAA GTG TGA GGT CCC GGT GTA GT-3′) under the following conditions: 30 s at 94 °C, 30 s at 62 °C and 60 s at 72 °C for 31 or 35 cycles. The relative amounts of PLK4 transcript were normalized to those of the GAPDH transcript. After the initial RT-PCR, the products were cloned into pGEM-T Easy Vector (Promega, Madison, WI), and several clones were sequenced using the BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Tokyo, Japan) and an ABI 3100 Genetic Analyzer (Applied Biosystems).

Quantitative RT-PCR (qRT-PCR)

The expression of the PLK4 mRNA transcript was measured using real-time qRT-PCR with a LightCycler instrument (Roche, Palo Alto, CA). PCR amplification of the PLK4 variants 1 and 3 transcripts and the GAPDH transcript was performed using cDNA and the QuantiTect SYBR Green PCR kit (Qiagen). The following PCR primers were used: 5′-ACC TGC ATC GGG GAG AAG AT-3′ and 5′-TTC CTG CTT TGT ACA TGG CTT TC-3′ for PLK4 variant 1 and 5′-ACA AGA GTA GGG AAG GCG GG-3′ and 5′-TTC CTG CTT TGT ACA TGG CTT TC-3′ for PLK4 variant 3. The PCR primer sequences for the GAPDH transcripts have been previously described [27]. The relative amounts of PLK4 transcript were normalized to those of the GAPDH transcript. The T/N values were calculated by dividing the normalized transcript amounts in the cancerous tissue of primary cancers by the amounts in the corresponding non-cancerous tissue.

Cell culture, transfection, and serum starvation

The human gastric cancer cell line AGS and the kidney cell line 293T were obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained at 37 °C in RPMI1640 medium supplemented with 10 % fetal bovine serum and penicillin/streptomycin under a 5 % CO_2 atmosphere. The GFP-PLK4 plasmid was kindly provided by Dr. E.A. Nigg. A plasmid vector was transfected into the cells using Lipofectamine 2000 reagent (Invitrogen) according to the supplier's recommendations. For the induction of primary cilia formation, the cells were serum-starved for 24 h.

Establishment of stable inducible cell lines

AGS and 293T cells were transfected with the cumate switch inducible vector for the expression of PLK4 variant 1 N-terminally fused with a Myc epitope tag together with the piggyBac transposase vector (System Biosciences, Mountain View, CA). To establish stable inducible cell lines, positively transposed cells were selected using puromycin (1 μg/mL: Clontech, Palo Alto, CA). Since the inducible piggyBac vector features a tight cumate switch combined with the EF1-CymR repressor-T2A-Puro cassette to establish stable cell lines, the addition of cumate

solution (System Biosciences) to the puromycin-selected cells led to the induction of Myc-PLK4 expression.

Western blot analysis

Western blot analysis using an anti-Myc polyclonal antibody (Upstate Biotechnology, Lake Placid, NY), an anti-acetylated α-tubulin monoclonal antibody (6-11B-1; Sigma-Aldrich, St. Louis, MO), or an anti-β-tubulin monoclonal antibody (clone 2-28-33; Sigma-Aldrich) was performed as described previously [25]. Immunoreactivity was visualized using an ECL chemiluminescence system (GE Healthcare Bio-Science, Piscataway, NJ).

Indirect immunofluorescence analysis

Cells were fixed with methanol and permeabilized with 1 % Nonidet P-40 solution. After blocked with normal goat serum or bovine serum albumin, the cells were probed with anti-γ-tubulin monoclonal antibody (GTU88; Sigma-Aldrich) or anti-acetylated α-tubulin monoclonal antibody (6-11B-1; Sigma-Aldrich). Indirect immunofluorescence labeling was performed by exposure to Alexa Fluor 594-conjugated secondary antibody (Molecular Probes, Eugene, OR), and the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). The cells were examined under a fluorescence microscope (Olympus BX-51-FL; Olympus, Tokyo, Japan) equipped with epifluorescence filters and a photometric CCD camera (Sensicam; PCO Company, Kelheim, Germany) or a confocal laser scanning microscope (Fluoview FV1000, Olympus).

Fluorescence in situ hybridization (FISH) analysis

Trypsinized cells were treated with 0.075 M KCl hypotonic solution and fixed in Carnoy's fixative. The fixed cell suspension was spread onto glass slides, and the slides were then hybridized with a Spectrum Orange-labeled probe for the centromere locus on chromosome 16, centromere enumeration probe (CEP) 16 (Vysis, Downer Groves, IL, USA) as described previously [27]. DAPI was used for nuclear staining.

Statistical analysis

The statistical analysis was performed using an unpaired *t*-test, Chi square test, or Wilcoxon matched pairs test. JMP version 9.0 software (SAS Institute, Cary, NC, USA) was used for the analyses. *P* values less than 0.05 were considered statistically significant.

Results

Upregulation of PLK4 variant 1 mRNA expression in lung and gastric cancer cell lines

To investigate the status of PLK4 expression in human lung and gastric cancers, we first checked the transcript variants of human PLK4. Three PLK4 transcript variants, i.e., variants 1 (NM_014264), 2 (NM_001190799), and 3 (NM_001190801), arising from alternative splicing are known, according to the homepage of the National Center for Biotechnology Information (NCBI) (**http://www.ncbi.nlm.nih.gov/**) (Fig. 1a). Variant 1 encodes a 970-amino-acid-long protein, which is the largest among the three variant proteins, while variants 2 and 3 lack exons 4 and 1, respectively. When we semiquantitatively compared the amount of RT-PCR product between variants 1 and 2 using the PCR primers for the sequence on exons 1 and 6, the expression level of variant 1 was found to be much higher than that of variant 2 in three cell lines (Fig. 1b). Interestingly, a novel transcript variant containing exon 4, but not exon 5, (submitted to the DDBJ accession no. AB741646) was identified by TA-cloning of the RT-PCR fragment and subsequent DNA sequencing (Fig. 1b, c). Although this variant features a premature stop codon, resulting in the formation of a 105-amino-acid-long protein, the variant contains 30 amino acids at its C-terminal that are not homologous to those in variant 1 (Fig. 1c). Next, we compared the mRNA expression levels between variants 1 and 3 using real-time qRT-PCR analysis and found that the expression level of variant 1 was much higher than that of variant 3 in three human cell lines (Fig. 1d). All the above results suggested that PLK4 variant 1 is the most predominant transcript among the four variants, including the novel one. Therefore, we next examined the expression status of PLK4 variant 1 in 14 lung and 7 gastric cancer cell lines. The relative expressions of the cancer cell lines were calculated by comparing the expression of the non-cancerous cell line, SAEC (Fig. 1e). The relative PLK4 expression level was 0.24–2.60 (average, 1.41) in the lung cancer cell lines and 0.73–3.35 (average, 1.83) in gastric cancer cell lines, and an increased PLK4 expression level (≥1.5) was observed in 7 (50 %) of the 14 lung cancer cell lines and 4 (57.1 %) of the 7 gastric cancer cell lines, meaning that the expression of PLK4 variant 1 is upregulated in a subset of lung and gastric cancer cell lines. Since the average PLK4 expression level and the percentage of cell lines showing an increased PLK4 expression level were higher in the gastric cancer cell lines than in the lung cancer cell lines, we next examined the status of PLK4 expression in primary gastric cancer.

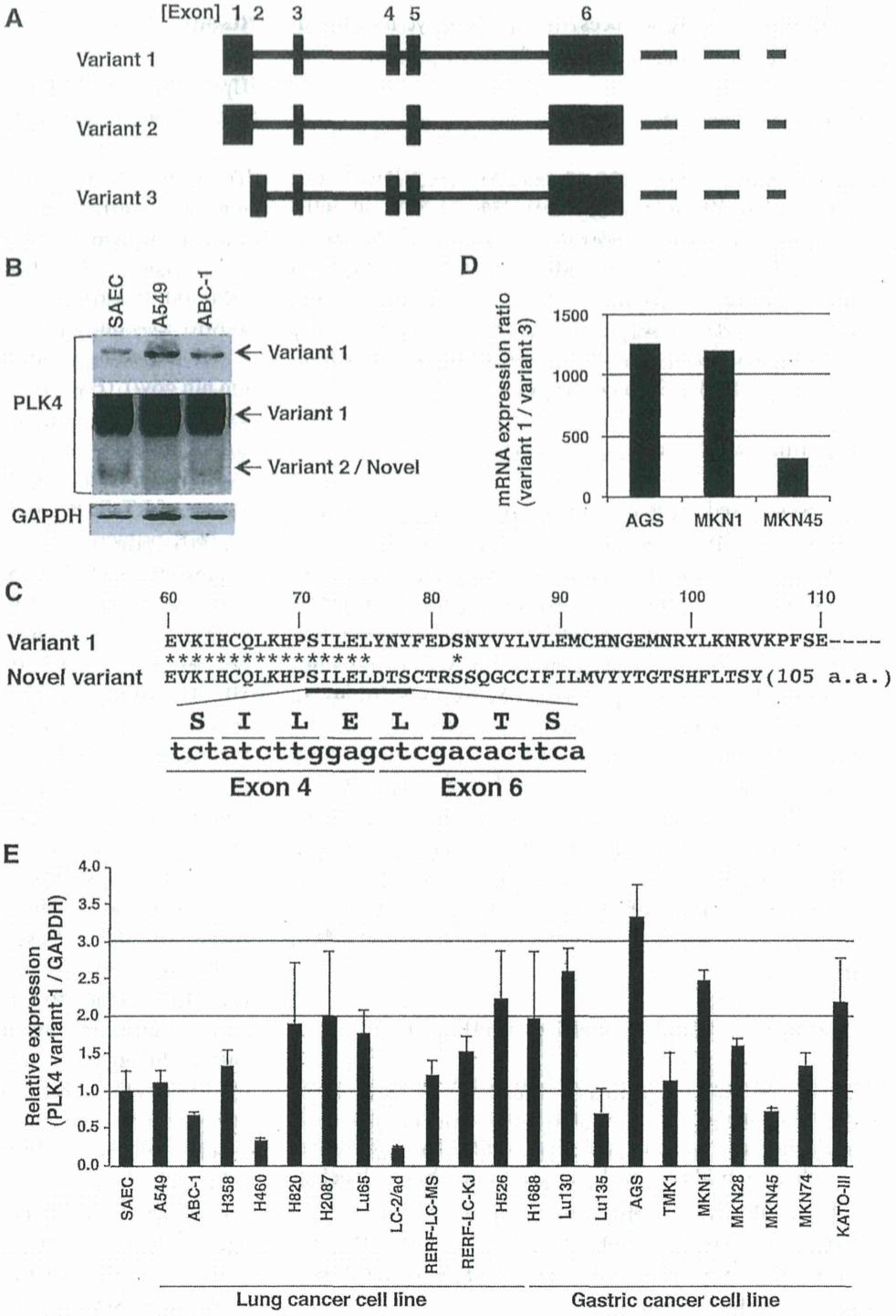

Fig. 1 Predominant mRNA expression level of human PLK4 variant 1 and upregulation of PLK4 expression in lung and gastric cancer cell lines. **a** Scheme showing the 5′ side region of *PLK4* coding transcript variants 1–3. The *filled boxes* represent exons. Exons 7–17 are omitted. **b** Detection of PLK4 mRNA transcript using RT-PCR and PCR primers for the sequence in exons 1 and 6. The *upper row* and the *second row* show the PLK4 transcripts amplified by 31 cycles of PCR and 35 cycles of PCR, respectively. GAPDH was analyzed as an internal control. "Novel" refers to a novel PLK4 transcript variant identified using TA-cloning of the RT-PCR fragment and subsequent DNA sequencing. The sizes of the PCR products of variant 1, variant 2, and the novel variant are 446, 350, and 331 bp, respectively. **c** Predicted amino acid sequence for the novel PLK4 transcript variant detected in **b**. Exon 4 is directly connected to exon 6 in this variant. This variant encodes a truncated protein that is 105 amino acids in length. **d** Determination of mRNA expression ratio of PLK4 variant 1 to variant 3 using real-time qRT-PCR analysis. **e** Measurement of the expression levels of PLK4 variant 1 transcripts in lung and gastric cancer cell lines using a semiquantitative RT-PCR analysis. The amounts of PLK4 variant 1 transcripts normalized to the amount of GAPDH transcripts are shown in the *graph*. The expression level of SAEC, a non-cancerous cell line, was also measured as a control and was defined as being equal to 1.0

Upregulation of PLK4 mRNA expression in human primary gastric cancer

We next attempted to examine whether the expression of PLK4 variant 1 is also upregulated in primary gastric cancer. We examined the expression of PLK4 variant 1 mRNA in 48 primary gastric cancers and calculated the ratio of the level of PLK4 variant 1 mRNA expression in the cancerous tissues to the level in the corresponding non-cancerous tissues (T/N ratio). An increased PLK4 expression level (T/N value $\geq$1.5) was observed in 24 (50 %) of the 48 primary gastric cancers (Fig. 2). Moreover, a significant difference was detected in the PLK4 expression level between the cancerous tissues and the corresponding non-cancerous tissues using a statistical analysis (P value = 0.0139, Wilcoxon matched pairs test). No