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Activation-Induced Cytidine Deaminase Alters the Subcellular Localization of Tet Family Proteins

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Abstract

Activation-induced cytidine deminase (Aid), a unique enzyme that deaminates cytosine in DNA, shuttles between the nucleus and the cytoplasm. A recent study proposed a novel function of Aid in active DNA demethylation via deamination of 5-hydroxymethylcytosine, which is converted from 5-methylcytosine by the Ten-eleven translocation (Tet) family of enzymes. In this study, we examined the effect of simultaneous expression of Aid and Tet family proteins on the subcellular localization of each protein. We found that overexpressed Aid is mainly localized in the cytoplasm, whereas Tet1 and Tet2 are localized in the nucleus, and Tet3 is localized in both the cytoplasm and the nucleus. However, nuclear Tet proteins were gradually translocated to the cytoplasm when co-expressed with Aid. We also show that Aid-mediated translocation of Tet proteins is associated with Aid shuttling. Here we propose a possible role for Aid as a regulator of the subcellular localization of Tet family proteins.

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Introduction

DNA methylation is a stable epigenetic feature that is involved in gene silencing and the maintenance of long-lasting cell memories [1]. Dynamic regulation of the DNA methylation pattern is crucial for mammalian development, as well as differentiation and reprogramming [2,3]. In particular, the active loss of 5-methylcytosine (5mC) independent of cell division is considered to be a major initial event in the epigenetic reprogramming of early mammalian embryos [4]. It has been demonstrated that the loss of 5mC at the paternal pronucleus of a zygote is linked to the accumulation of 5-hydroxymethylcytosine (5hmC) [5–7]. The 5hmC is converted from 5mC by the ten-eleven translocation (Tet) family of proteins [8], and therefore 5hmC is considered to be an intermediate formed during the active DNA demethylation process in early embryos.

A recent study proposed a novel model for the removal of 5hmC, wherein activation-induced cytidine deaminase (Aid) induces the deamination of 5hmC, which is followed by base excision repair (BER), resulting in the conversion of 5hmC into unmethylated cytosine [9]. Based on this model for active DNA demethylation, coordinated actions of both the production and removal of 5hmC may regulate the conversion of 5mC into unmethylated cytosine. However, little is known how these proteins involved in the production and removal of 5hmC affect each other.

Aid is a well-known enzyme that converts cytosine into uracil in single-stranded DNA, causing somatic hypermutation and class switch recombination [10,11]. Aid is mainly localized in the cytoplasm under steady state conditions, but has the ability to shuttle between the nucleus and the cytoplasm [12,13]. Previous studies suggested that the changeable localization of Aid, which is mediated by its shuttling, plays a role in controlling its activity as a DNA modifier [14,15]. Considering that 5hmC is localized at the nucleus, the shuttling of Aid may also contribute to the modulation of 5hmC removal. In addition, Tet family proteins show translocation into the nucleus from the cytoplasm during the early developmental stage, when the rapid generation of 5hmC is observed [16]. Therefore, it is possible that distinct subcellular localization of the Tet family and Aid controls the production and removal of 5hmC, leading to the regulation of active DNA demethylation. In the present study, we examined the relationship between the Tet family and Aid from the view of their subcellular localization. We herein demonstrate that Aid has an effect on the subcellular localization of the Tet family, and that this is associated with Aid shuttling.

Materials and Methods

DNA constructs

Mouse Tet1 (GU079948, DDBJ), Tet2 (GU079949, DDBJ), Aid (NM_009645.2, NCBI), Apobec1 (NM_031159.3, NCBI) and Apobec2 (NM_009694.3, NCBI) were cloned from mouse embryonic stem cells, and Tet3 (NM_183138.2, NCBI) was obtained from mouse embryonic fibroblasts by PCR amplification with KOD plus Neo (TOYOBO) using primers as described in Table S1. The Tet family fragments were subcloned into pcDNA4HisMax (Life Technologies), and the Aid, Apobec1 and Apobec2 fragments were subcloned into pcDNA4MycHis (Life Technologies). Plasmids encoding the Xpress-tagged catalytic

domain (CD) of Tet1 (1367–2039 amino acids: aa), Tet2 (1044–1921aa) and Tet3 (697–1668aa) were generated by subcloning of the DNA fragments into BamHI and NotI sites for Tet1, EcoRI and XhoI sites for Tet2 or EcoRI and NotI sites for Tet3 of pcDNA4HisMax. Plasmids encoding Xpress-tagged mutants deficient in the catalytic domain (ΔCD) of Tet1 (1–1366aa), Tet2 (1–1043aa) and Tet3 (1–696aa) were also generated. The Aid mutants, AidΔNES (1–187aa) and AidΔN26 (27–198aa), were subcloned into BamHI/XhoI-digested pcDNA4MycHis. We used the KOD plus mutagenesis kit (TOYOBO) to generate a point mutant for Aid (F193A) and mutants for the Tet family catalytic domain, which include Tet1CDm (D1652Y, D1654A), Tet2CDm (H1304Y, D1306A) and Tet3CDm (H950Y, D952A).

Cell culture and cDNA transfection

Human embryonic kidney cells (HEK293FT) (Invitrogen) and human colon cancer cells (DLD-1) (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum. Both of them were transiently transfected with plasmid DNA by FuGENE6 (Promega) according to the manufacturer's instructions, followed by immunofluorescence or co-immunoprecipitaion 48 h post-transfection, unless otherwise noted.

Immunofluorescence

The cells were fixed and permeabilized with cold 100% methanol for 10 min on ice. For 5hmC staining, permeabilized cells were treated with 4 N HCl for 10 min, followed by 1.5 M Tris-HCl (pH 8.8) treatment for 10 min, before being blocked with 1% BSA. The cells were incubated with primary antibodies; anti-Xpress mouse monoclonal antibody (mAb) (Life Technologies), anti-Myc rabbit polyclonal antibody (MBL), anti-5hmC rabbit polyclonal antibody (Active Motif) or anti-Aicda rabbit polyclonal antibody (Abcam) overnight at 4°C, followed by Alexa Fluor-conjugated secondary antibodies (Life Technologies) for 1 h, and DAPI staining for 5 min at room temperature. After washing with PBS containing with 0.05% Tween 20, the samples were mounted by using the Prolong Gold Antifade Reagent (Life Technologies), followed by curing on a flat surface in the dark overnight at 4°C. For four color staining, a Zenon Alexa Fluor labeling kit (Life Technologies) was used. The images were captured by a confocal laser microscope (OLYMPUS, FV1000). To score the subcellular localization in DLD-1 cells, we counted all of the fluorescence positive cells on 4-well chamber dishes (BD). When using HEK293FT cells, we counted cells in randomly acquired fields on the 4-well chamber dishes. In the case of co-transfection, co-expressed cells were counted and scored according to the Tet localization. Scoring of the subcellular localization was performed as indicated in Fig. S1.

Immunoprecipitation and immunoblotting

Transfected HEK293FT cells were lysed in EBC buffer (50 mM Tris-HCl pH 8.0, 120 mM NaCl and 0.5% NP40 for detecting Xpress-tagged protein, or 1.0% NP40 for detecting Myc-tagged protein) with a protease inhibitor cocktail (SIGMA). The cell lysates were incubated for 3 h at 4°C with Dynabeads M280 sheep anti-mouse IgG (VERITAS) which had been pre-treated with an anti-Xpress mAb or an anti-Myc mAb (Enzo life science) for 1 h. After washing the immunoprecipitates four times with EBC buffer, the beads were boiled with Laemmli SDS-sample buffer. This supernatant was separated by SDS-PAGE and transferred to a PVDF membrane (Millipore). For immunoblotting of the Xpress-tagged protein, after the membrane were blocked with 2% nonfat dry milk in PBS containing 0.05% Tween20, they were incubated with an anti-Xpress mAb followed by anti-mouse IgG antibody conjugated to HRP specific for naive IgG (Novagen). For Myc-tagged protein blotting, after being blocked, the membrane was incubated with anti-Myc antibody conjugated to HRP (MBL). Each antibody was diluted in Can Get Signal for immunoblotting (TOYOBO). Protein bands were visualized using the Pierce ECL plus Western Blotting Substrate (Thermo), and detected with a LAS4000 instrument (GE HealthCare).

Statistic analysis

The statistical significance of differences between two groups was determined by the Mann-Whitney U test. A value of $p<0.05$ was considered to be statistically significant. The numbers of samples are referred to as "n" in each graph.

Results

Aid alters the subcellular localization of Tet1 from the nucleus to the cytoplasm

A previous study showed that Tet family proteins generate 5hmC, whereas Aid facilitates the conversion of 5hmC into cytosine [9]. In this study, we investigated the effect of simultaneous expression of Aid and Tet on their subcellular localization. We transfected C-terminally Myc-tagged Aid or N-terminally Xpress-tagged Tet1 into HEK293FT or DLD-1 cells, and examined the subcellular localization of ectopically expressed proteins. Aid was observed mainly in the cytoplasm, whereas Tet1 was predominantly localized in the nucleus when the single proteins were overexpressed. When cells were co-transfected with expression plasmids for Aid and Tet1, the Tet1 localization was altered from the nucleus to the cytoplasm in the co-transfected cells, whereas Aid remained in the cytoplasm (Fig. 1).

To determine the domain responsible for the altered localization of Tet1, we performed a subcellular localization analysis using a series of deletion constructs for Tet1, as previously reported [17]; full length (FL) (1–2039 amino acids: aa) which was used in the experiment shown in Fig. 1, the catalytic domain (CD) (1367–2039aa), and the N-terminal domain (ΔCD) (1–1366aa), which lacks CD (Fig. 2A). The Tet1FL plasmid and these mutants were transfected individually with or without the plasmid for Aid. At 48 hrs after transfection, the subcellular localization of Tet1 and Aid was examined by confocal microscopy (Figs. 2B and S2). In the case of single transfection, all Tet1 mutants were predominantly localized in the nucleus, and Aid was mainly localized in the cytoplasm. However, when Tet1FL or Tet1CD was co-expressed with Aid, Tet1 was translocated to the cytoplasm (N: 0%, N+C: 9%, C: 91% for FL, and N: 18%, N+C: 35%, C: 47% for CD, respectively). In contrast, Tet1ΔCD remained in the nucleus even when co-expressed with Aid (N: 75%, N+C: 19%, C: 6%), suggesting that the catalytic domain of Tet1 plays a role in the altered localization of the protein (Figs. 2B and C). Since a previous study indicated that the subcellular localization of Aid is affected by the position of the tag [18], we also carried out co-transfection experiments using untagged Aid protein. It was confirmed that untagged Aid, as well as C-terminal-tagged Aid, also affected the localization of Tet1CD (Fig. S3), supporting the notion that Aid expression alters the subcellular localization of Tet1.

Next, to examine whether this effect is specific to Aid, we carried out the same experiments by using Apobec1 and Apobec2, instead of Aid, both of which show the similar enzymatic activity to Aid [19,20]. In particular, Apobec1 has been shown to shuttle between the nucleus and the cytoplasm [21]. We observed that overexpressed Apobec1 and Apobec2 were localized at both the

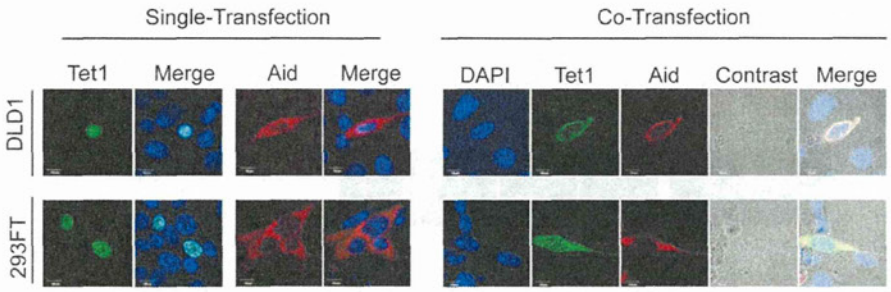

Figure 1. Overexpressed Aid alters the subcellular localization of Tet1. Images of cells transiently expressing N-terminally Xpress-tagged Tet1 or C-terminally Myc-tagged Aid. Tet1 was predominantly localized in the nucleus, whereas Aid was mainly localized in the cytoplasm 48 h after transfection in both DLD-1 and HEK293FT cells. When cells were co-transfected with a plasmid expressing Aid, the Tet1 subcellular localization was altered to the cytoplasm, where Aid was mainly localized. The scale bar is 10 μm.
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nucleus and the cytoplasm in DLD-1 cells regardless of the presence or absence of Tet1CD (Figs. S4). In contrast to the translocation of Tet1CD in the presence of Aid, Tet1CD always remained in the nucleus even when co-expressed with Apobec1 or Apobec2 (Figs. S4B and D, $p = 0.11$, with vs without Apobec1, $p = 0.38$, with vs without Apobec2). These results suggest that the altered subcellular localization of Tet1CD is not attributable to the artificial effects due to Aid overexpression.

Tet1 translocation is independent of its enzymatic activity

We observed that the subcellular localization of Tet1 was altered in the presence of Aid, but that Tet1ΔCD remained in the nucleus, implying that Tet1 enzymatic activity for the conversion of 5mC to 5hmC is associated with the translocation of Tet1. To test this, a Tet1CD mutant construct (CDm), which has mutations in the catalytic domain (D1652Y and D1654A) and lacks enzymatic activity, was generated (Fig. 3A) [17]. When Tet1CDm was solely transfected into DLD-1 cells, it was localized in the nucleus. However, the enzyme activity, which was detected by immunostaining for 5hmC, was not observed at all in Tet1CDm-expressing cells while it was evident in Tet1CD- and Tet1 FL-expressing cells (Fig. 3B). We also confirmed that Tet1ΔCD and Aid had no ability to produce 5hmC (Figs. 3B and S5A).

We next examined the subcellular localization of Tet1CDm when it was co-expressed with Aid in DLD-1 cells. Despite the lack of enzymatic activity in Tet1CDm, simultaneous expression of Aid and Tet1CDm caused the altered localization of Tet1CDm, and no significant difference in the localization of Tet1CD and Tet1CDm was observed when they were co-expressed with Aid ($p = 0.144$, CD vs CDm) (Figs. 2C and 3C). We obtained the similar observation using HEK293FT cells (Fig. S2). These results

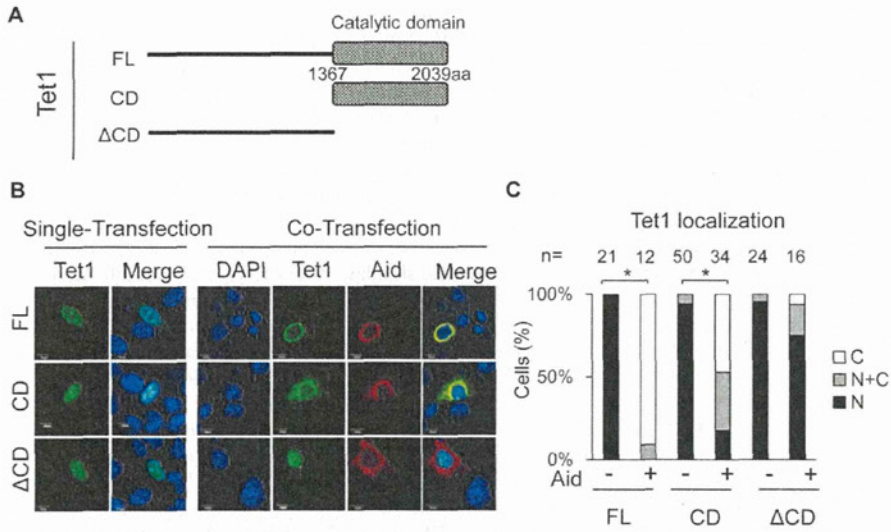

Figure 2. Tet1 translocation requires its catalytic domain. (A) A schematic representation of the Tet1 structure and its mutants used in this study. (aa = amino acid). (B) Confocal images of DLD-1 cells transiently expressing N-terminally Xpress-tagged Tet1 mutants with or without C-terminally Myc-tagged Aid. All Tet1 constructs (FL, CD and ΔCD) were localized in the nucleus when solely expressed in DLD-1 cells. When co-expressed with Aid, Tet1FL and Tet1CD were translocated to the cytoplasm, whereas Tet1ΔCD remained in the nucleus. (C) Each bar represents the proportion of cells with the different localizations of Tet1. The number (n) of cells indicated above each bar was scored according to their subcellular localization. N (black); nuclear localization, N+C (gray); both nuclear and cytoplasmic localization, C (white); cytoplasmic localization in multiple microscope fields. The scale bars in images are 10 μm. *$p<0.01$.
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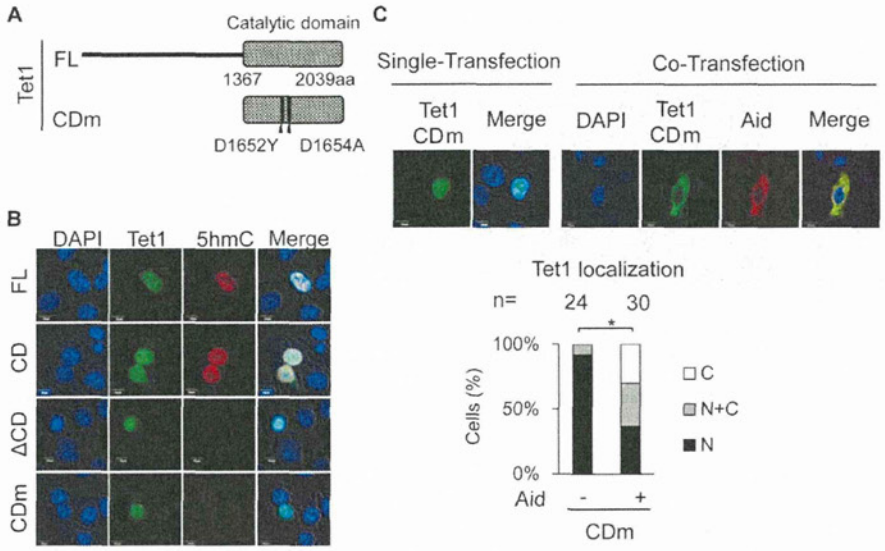

Figure 3. Tet1 translocation in the presence of Aid is independent of the Tet1 enzymatic activity. (A) A schematic representation of the Tet1CD mutant (CDm) used in this study. Tet1CDm were tagged with N-terminal Xpress. (B) Tet1FL and CD had enzyme activity and produced 5hmC, but Tet1ΔCD and CDm did not. (C) C-terminally Myc-tagged Aid expression altered the subcellular localization of Tet1CDm, which lacks the enzymatic activity. The upper panels are representative images of DLD-1 cells transiently expressing Tet1CDm with or without simultaneous expression of Aid. The lower graph shows the percentage score of the examined transfected cells (indicated as a number). The scale bars are 10 μm. *$p<0.01$. N (black); nuclear localization, N+C (gray); both nuclear and cytoplasmic localization, C (white); cytoplasmic localization in multiple microscope fields.
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indicate that, although the catalytic domain of Tet1 is important for the Aid-mediated translocation of Tet1, the translocation occurs independently of its enzymatic activity.

Co-expression of Aid has similar effects on the subcellular localization of other Tet family proteins

The Tet family proteins include Tet1, Tet2 and Tet3. We therefore examined whether the effects of Aid co-expression were also observed for other Tet family members. To perform these studies, FL, CD, ΔCD and CDm constructs for both Tet2 and Tet3 were generated (Fig. 4A) based on a previous report [17], and their subcellular localization in the presence or absence of Aid was examined. In both Tet2 and Tet3, the FL and CD proteins exhibited enzymatic activity, whereas the ΔCD and CDm mutants did not (Fig. S5B). Single expression of Tet2FL or its mutants led to the localization of the proteins primarily in the nucleus (Figs. 4B and C). However, in solely Tet3-expressing cells, Tet3ΔCD showed cytoplasmic localization, even in the single transfectants, although Tet3FL, Tet3CD and Tet3CDm were localized in both the nucleus and the cytoplasm (Figs. 4D and E), indicating that the catalytic domain of Tet3 is responsible for the nuclear localization of Tet3. Simultaneous expression of Aid and either of Tet FL, CD or CDm resulted in the altered subcellular localization of both Tet2 and Tet3 into the cytoplasm (Figs. 4B–E). These findings suggest that Aid alters the subcellular localization of all three Tet family proteins, and that this occurs independently of enzyme activity to produce 5hmC.

Translocation of Tet1 by Aid is associated with Aid shuttling

We next addressed how nuclear Tet1 is translocated into the cytoplasm by Aid. To understand the mechanism, we first examined the localization of both Tet1CD and Aid at different time points (10 h, 24 h, 48 h) after simultaneous transfection into HEK293FT cells. At 10 h after transfection, the subcellular localization of Tet1CD was mainly in the nucleus (N: 90%, N+C: 10%, C: 0%) while Aid was primarily expressed in the cytoplasm, showing the same localization pattern in the single transfected cells. At 24 h after transfection, the proportion of cells with cytoplasmic Tet1CD increased (N: 41%, N+C: 52%, C: 7%), and at 48 h after transfection, most of the Tet1CD were co-localized with Aid in the cytoplasm (N: 3%, N+C: 30%, C: 67%) (Figs. 5A and B). In contrast, Aid could be detected in the cytoplasm throughout this experiment.

It is worth noting that Aid is a shuttling protein that is translocating between the nucleus and the cytoplasm [12,13]. Since a gradual increase in the number of cells with cytoplasmic Tet1CD was observed, we evaluated whether Tet1 translocation is associated with Aid shuttling. We performed immunofluorescence experiment by using full length Aid (Aid FL) and its mutants which are impaired in nuclear-cytoplasmic shuttling; Aid lacking NES (AidΔNES_1-187aa), Aid having a single point mutation in the NES (Aid F193A) [22] [23] or Aid lacking the N terminus of Aid, which loses the important sequences for nuclear entry (AidΔN26_27-198aa) [12] (Fig. 6A). As expected, AidΔNES transfected cells revealed an increased number of cells with nuclear localization of Aid (ΔNES; N: 27%, N+C: 33%, C: 40%) when compared with Aid FL- transfected cells (N: 3%, N+C: 28%, C: 69%) (Figs. 6B and C). In addition, Aid F193A showed an increased localization of Aid at the nucleus (N: 23%, N+C: 43%, C: 34%) than Aid FL did. (Figs. 6B and D). Next, we co-transfected these Aid mutants with Tet1CD in DLD-1 cells and examined the effect of the expressions of Aid mutants on the Tet1CD subcellular localization (Figs. 6E and F). Co-expression with AidΔNES resulted in a decrease in the number of Tet1CD-translocated cells (N: 55%, N+C: 20%, C: 25%) compared with

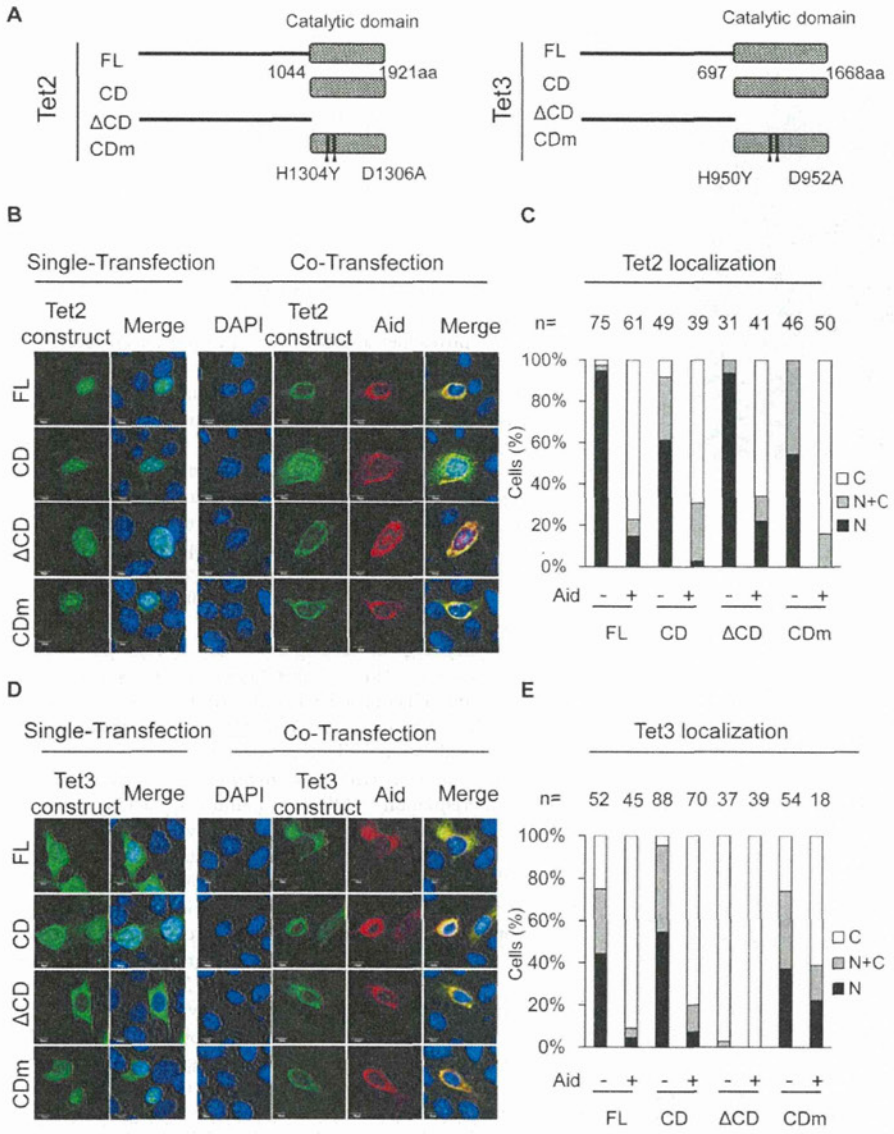

Figure 4. The subcellular localization of Tet2 and Tet3 is altered by Aid expression. (A) A schematic representation of the Tet2 and Tet3 structures and their mutants used in this study. (B) N-terminally Xpress-tagged Tet2 or its mutants with or without Aid tagged with C-terminal Myc were imaged by confocal microscopy in transiently transfected DLD-1 cells. (C) The number (n) of cells indicated above each bar was scored according to Tet2 subcellular localization. All Tet2 mutants were translocated to the cytoplasm in the presence of Aid ($p<0.01$, vs in the absence of Aid). (D, E) Simultaneous expression of N-terminally Xpress-tagged Tet3 and Aid-Myc. Tet3FL, CD and CDm were translocated to the cytoplasm when co-expressed with Aid ($p<0.01$, single-expression vs co-expression). Tet3ΔCD was localized in the cytoplasm regardless of the Aid expression. The scale bars are 10 μm. N (black); nuclear localization, N+C (gray); both nuclear and cytoplasmic localization, C (white); cytoplasmic localization in multiple microscope fields.
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that induced by with Aid FL (N: 18%, N+C: 35%, C: 47%) (Figs. 2C and 6E). Similarly, when co-expressed with a single point mutant AidF193A, the proportion of Tet1CD-translocated cells were decreased (N: 57%, N+C: 17%, C: 26%) in comparison to those when co-transfected with Aid FL (Figs. 2C and 6F). In addition, we performed the similar experiment using AidΔN26, which has defect in nuclear entry. AidΔN26 was predominantly localized at the cytoplasm in the case of single expression (N: 0%, N+C: 5%, C: 95%) (Fig. 6G). When co-expressed with AidΔN26, Tet1CD remained in the nucleus (N: 72%, N+C: 24%, C: 4%) (Figs. 2C and 6H). Taken together, these findings imply that Aid shuttling, which is mediated by the N-terminus and C-terminus domains of Aid, is associated with the Aid-mediated translocation of Tet1, and suggest that Tet1 translocation is dependent on the subcellular localization of Aid.

Interaction between Aid and Tet1

In this study, we found that nuclear Tet1 is translocated to the cytoplasm by Aid, and that the translocated Tet1 is co-localized with Aid. We next examined whether Aid interacts with Tet1

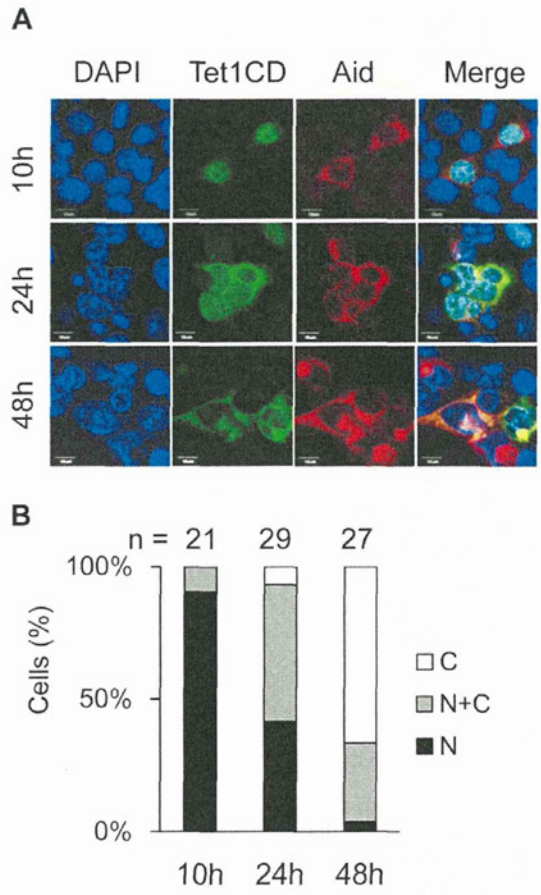

Figure 5. Nuclear Tet1 is gradually translocated into the cytoplasm by the simultaneous expression of Aid. (A) Confocal images of HEK293FT cells transiently co-expressing Tet1CD tagged with N-terminal Xpress and Aid tagged with C-terminal Myc at different time points (10 h, 24 h and 48 h) after co-transfection. The scale bars in images are 10 μm. (B) The number (n) of cells indicated above each bar was scored according to the Tet1CD subcellular localization. The number of cells with cytoplasmic Tet1CD gradually increased after co-transfection. N (black); nuclear localization, N+C (gray); both nuclear and cytoplasmic localization, C (white); cytoplasmic localization in multiple microscope fields.
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during this Aid-mediated translocation of Tet1. We carried out co-immunoprecipitation (co-IP) – immunoblotting (IB) using HEK293FT cells transfected with either or both the Xpress-Tet1CD and Aid FL-Myc vectors. Empty vectors were used as a negative control. Aid FL-Myc was co-precipitated with an anti-Xpress mAbs for Xpress-Tet1CD, and this association was confirmed by reciprocal IP with anti-Myc mAbs (Fig. 7A). Moreover, we observed a decreased interaction of AidΔNES with Tet1CD, although both AidΔNES and Tet1CD were localized in the nucleus (Fig. 7B). This result indicates that the NES domain of Aid is associated with the interaction between Aid and Tet1.

Discussion

Aid shuttles between the nucleus and the cytoplasm, interacting with several molecules, such as RNA polymerase II [24], CTNNBL-1 [25] and GANP [26], in order to target the IgV region and/or the S region DNA. Previous studies proposed that the shuttling of Aid plays a role in preventing excessive DNA mutation in the nucleus [18,27]. In the present study, we showed that the simultaneous expression of Aid and Tet family enzymes causes the altered subcellular localization of the Tet family proteins. Furthermore, the translocation of Tet was affected by Aid shuttling between the nucleus and the cytoplasm. These results suggest that Aid shuttling might have another function; altering the subcellular localization of Tet family members. However, it should be also noted that the level of Aid induced in this experiments seems to be substantially higher than that of physiological condition. Considering such artificial experimental system, further analyses for endogenous proteins are required to conclude the physiological function of Aid in the translocation of Tet family enzymes.

Although the physiological relevance of our findings remains to be established, it is important to note that the expression of both Tet family proteins and Aid is restricted to specific cell types. It was reported that Aid is highly expressed in oocytes [28], while Tet3 is expressed at high levels in oocytes and zygotes [6], thus indicating that both Tet3 and Aid are abundantly expressed in oocytes. Of note, Tet3 is localized in the cytoplasm in oocytes, but it translocates into the male pronucleus of zygotes shortly after fertilization [16]. Therefore, it seems that there is dynamic regulation of the subcellular localization of Tet family members during the early stage of development. Considering that simultaneous expression of Aid and Tet family members caused the translocalization of Tet proteins into the cytoplasm in this study, it is possible that endogenously expressed Aid contributes to the cytoplasmic localization of Tet3 in oocytes.

DNA methylation is critical for mammalian development and cellular differentiation [29]. In mammals, active genomic demethylation contributes to the genome-wide erasure of the DNA methylation observed in preimplantation embryos and primordial germ cells (PGCs) [30,31]. However, the mechanisms underlying active DNA demethylation in mammals have been highly controversial, although multiple mechanisms have been proposed [32–34]. Recently, an additional model was reported, wherein Aid facilitates the conversion of 5hmC into cytosine [9,35], and forms several complexes with thymine DNA glycosylase and GADD45a, which are involved in active DNA demethylation [36]. Our findings may also support the notion that Aid plays a role in DNA demethylation while interacting with several related factors.

To determine whether the altered subcellular localization of Tet contributes to the altered production of 5hmC, we performed immunodetection for 5hmC in dually-transfected cells (expressing both Tet1 and Aid), where Tet1 was translocated into the cytoplasm. Although Tet1 CD had already been translocated into the cytoplasm, 5hmC was still detectable in the nucleus (Fig. S6). Therefore, we could not conclude whether the translocation of Tet can affect the production of 5hmC in cells expressing both proteins. One of the possible explanations for our observation is that 5hmC is, to some extent, stable after its production, which made it difficult to detect an alteration in the 5hmC levels under our experimental conditions.

In summary, our present findings indicate that Aid regulates the subcellular localization of Tet family proteins, and that this is associated with Aid shuttling. The subcellular localization of proteins is crucial for their functional activity and is associated with their functional diversity [37,38]. Since both Aid and the Tet family proteins are involved in the modification of 5hmC, the coordinated action of these proteins might control epigenetic modifications by affecting the subcellular localization of Tet family

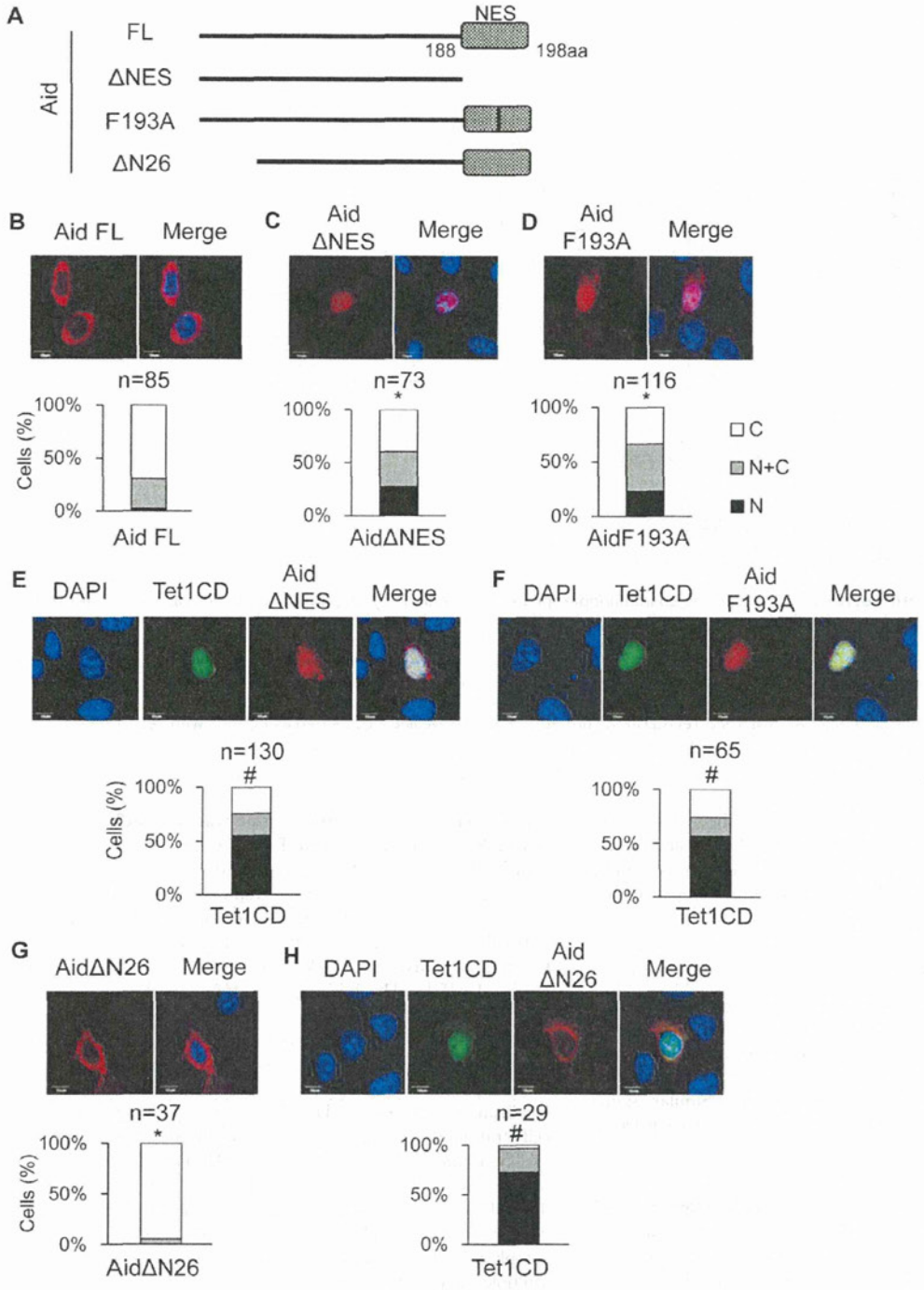

Figure 6. Aid shuttling is associated with Aid-mediating transcloation of Tet1. (A) A schematic representation of the Aid structure and its mutants used in this study. All Aid constructs were tagged with C-terminal Myc. (B–D) The upper figures are representative confocal images of DLD-1 cells transiently expressing only Aid FL (B), ΔNES (C) or F193A (D). The lower figure represents the proportion of cells with different subcellular localization of Aid. Aid mutants defect in NES showed the increased nuclear localization. *$p<0.05$ vs Aid FL. (E, F) The upper figures are representative confocal images of DLD-1 cells transiently co-expressing AidΔNES and Tet1CD (E), or AidF193A and Tet1CD (F). Tet1CD were tagged with N-terminal Xpress. The lower figure shows the proportion of cells with different localizations of Tet1 (E; AidΔNES and Tet1CD, F; AidF193A and Tet1CD). Aid mutants, which exhibit impaired shuttling between the nucleus and the cytoplasm, failed to alter the subcellular localization of Tet1. #$p<0.05$ vs with Aid FL. (G) The upper figures are representative confocal images of DLD-1 cells transiently expressing only AidΔN26. The lower figure represents the proportion of cells with different subcellular localization of AidΔN26. *$p<0.05$ vs Aid FL. (H) The upper figures are representative confocal images of DLD-1 cells transiently co-expressing AidΔN26 and Tet1CD. The lower figure shows the proportion of cells with different localizations of Tet1. #$p<0.05$ vs with AidFL. The scale bars are 10 μm. N (black); nuclear localization, N+C (gray); both nuclear and cytoplasmic localization, C (white); cytoplasmic localization in multiple microscope fields.
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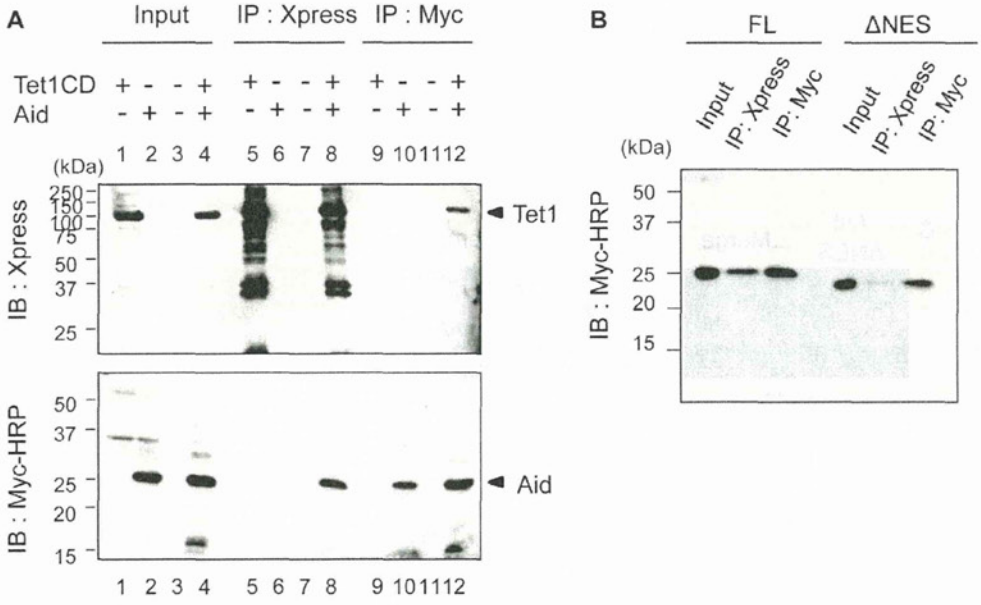

Figure 7. Aid interacts with Tet1CD. (A) Tet1CD was co-immunoprecipitated with Aid FL. Lysates from HEK293FT cells transfected with N-terminally Xpress-tagged Tet1CD, C-terminally Myc-tagged AidFL or both of them were immunoprecipitated (IP) by anti-Xpress mAbs or anti-Myc mAbs. Immunoblotting (IB) was performed by using an anti-Xpress Abs or anti-Myc-HRP antibody. Lane nos. 1, 5 and 9 were single transfections of Tet1CD. Lane nos. 2, 6 and 10 were single-transfections of Aid. Lane nos. 3, 7 and 11 were for mock transfection. Lane nos. 4, 8 and 12 shows the results for the co-transfection of Tet1CD and Aid. (B) The Co-IP experiment was performed by using lysates from HEK293FT cells co-transfected with N-terminally Xpress tagged-Tet1CD and C-terminally Myc-tagged Aid FL, or with N-terminally Xpress-tagged Tet1CD and C-terminally Myc-tagged AidΔNES. Despite the similar localization of AidΔNES and Tet1CD in the nucleus, AidΔNES revealed a decreased association with Tet1CD compared to Aid FL.
doi:10.1371/journal.pone.0045031.g007

proteins as we described in this study. Further studies are warranted to uncover the functional and physiological significance of the Aid-mediated translocation of Tet, which may eventually extend our understanding of the regulation of 5hmC production and active DNA demethylation.

Supporting Information

Figure S1 Representative images for each subcellular localization. Representative images of each subcellular localization are shown. Dominant immunofluorescent signals in the nucleus and cytoplasm were regarded as nuclear localization (N) and cytoplasmic localization (C), respectively. Similar signal intensity in both the nucleus and the cytoplasm was regarded as C+N. Scale bars are 10 μm.
(TIF)

Figure S2 Aid alters the subcellular localization of Tet1 in HEK293FT cells. HEK293FT cells expressing Aid also revealed translocation of Tet1CD and CDm. Consistent with the results in DLD-1 cells, Tet1ΔCD was retained in the nucleus even in the presence of Aid. The scale bars are 10 μm.
(TIF)

Figure S3 Untagged Aid expression results in the subcellular translocation of Tet1. Untagged Aid was detected by an anti-Aid polyclonal antibody. Untagged Aid was mainly localized in the cytoplasm, which was the same as Myc-tagged Aid. Simultaneous expression of untagged Aid and Tet1CD caused the altered localization of Tet1CD in the cytoplasm. The scale bars are 10 μm.
(TIF)

Figure S4 Apobec family has not an effect on the subcellular localization of Tet1. (A) The upper figures were confocal images of DLD-1 cells transiently expressing C-terminally Myc-tagged Apobec1. The lower graph represents the proportion of cells with different subcellular localization of Apobec1. (B) The upper figures were images of DLD-1 cells transiently co-expressing C-terminally Myc-tagged Apobec1 and N-terminally Xpress-tagged Tet1CD. The lower graph represents the percentage of the different subcellular localization of Tet1CD on the co-expressing cells. (C) The upper was images of DLD-1 cells transiently expressing C-terminally Myc-tagged Apobec2. The lower represents the proportion of cells with different subcellular localization of Apobec2. (D) The upper were images of DLD-1 cells transiently co-expressing C-terminally Myc-tagged Apobec2 and N-terminally Xpress-tagged Tet1CD. The lower showed the proportion of cells with different subcellular localization of Tet1CD on the co-expressing cells. The scale bars are 10 μm. N (black); nuclear localization, N+C (gray); both nuclear and cytoplasmic localization, C (white); cytoplasmic localization in multiple microscope fields.
(TIF)

Figure S5 Detection of 5hmC by immunostaining using in DLD-1 cells. (A) Aid alone could not produce 5hmC. (B) The FL and CD had enzymatic activity, whereas the ΔCD and CDm proteins did not in both Tet2 and Tet3. Aid was tagged with C-terminal Myc and Tets were with N-terminal Xpress. The scale bars are 10 μm.
(TIF)

Figure S6 5hmC remains in the nucleus even after Tet1CD transfer to the cytoplasm in HEK293FT cells. The 5hmC could be still detected in the nucleus, even though Tet1CD was translocated from the nucleus to the cytoplasm in the presence of Aid. Aid was tagged with C-terminal Myc and Tets were with N-terminal Xpress. The scale bars are 20 μm.
(TIF)

Table S1 Primer sets for cloning Tet family and Aid used in this study. F: forward primer, R: reverse primer
(DOC)

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Author Contributions

Conceived and designed the experiments: YA AW KS YY. Performed the experiments: YA. Analyzed the data: YA AW YY. Wrote the paper: YA AW YY.

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Gastric Cancer
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ORIGINAL ARTICLE

Altered mucosal DNA methylation in parallel with highly active *Helicobacter pylori*-related gastritis

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Abstract
Background Chronic inflammation triggered by *Helicobacter pylori* causes altered DNA methylation in stomach mucosae, which is deeply involved in gastric carcinogenesis. This study aimed to elucidate the correlation between altered mucosal DNA methylation levels and activity of *H. pylori*-related gastritis, because inflammatory activity shows particular correlations with the development of diffuse-type cancer.
Methods Methylation levels in stomach mucosae of 78 healthy volunteers were determined by real-time methylation-specific PCR or bisulfite pyrosequencing. Examined loci were the promoter CpG islands of six genes (*FLNc*, *HAND1*, *THBD*, *p41ARC*, *HRASLS*, and *LOX*) and the CpG sites of non-coding repetitive elements (Alu and Satα) that are reportedly altered by *H. pylori* infection. Activity of *H. pylori*-related gastritis was evaluated using two serum markers: *H. pylori* antibody titer and pepsinogen II.
Results Methylation levels of the six CpG islands were consistently increased, and those of the two repetitive elements were consistently decreased in a stepwise manner with the activity of gastric inflammation as represented by serum marker levels. Each serum marker level was well correlated with the overall DNA methylation status of stomach mucosa, and these two serologic markers were additive in the detection of the mucosa with severely altered DNA methylation.
Conclusions Alteration in mucosal DNA methylation level was closely correlated with activity of *H. pylori*-related gastritis as evaluated by serum markers. The observed correlation between altered DNA methylation levels and activity of *H. pylori*-related gastritis appears to be one of the relevant molecular mechanisms underlying the development of diffuse-type cancer.

Keywords Gastric cancer · *Helicobacter pylori* · Carcinogenesis · Active gastritis · DNA methylation

Abbreviations

UC	Ulcerative colitis
H. pylori	*Helicobacter pylori*
PG	Pepsinogen
CGI	CpG island

Introduction

Epidemiological studies have revealed that long-lasting inflammation in specific organs is associated with increased risks of cancers, such as ulcerative colitis (UC)-associated colon cancer [1], liver cancer [2], and gastric cancer [3]. Accumulation of genetic and epigenetic alterations induced by chronic inflammation appears to contribute to cancer development [4]. In addition, activity of chronic inflammation has been shown to be associated with the incidence of cancer development [5–7]. In long-standing UC, patients with highly active inflammation during the disease course are more likely to develop

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dysplasia or cancer than those without [5]. Likewise, in viral hepatitis, patients with high serum alanine aminotransferase levels are more likely to develop hepatocellular carcinoma than those without [6, 7].

In stomach mucosae, chronic inflammation is triggered by *Helicobacter pylori* (*H. pylori*) infection and causes a predisposition toward gastric cancer development [8]. In terms of correlations between activity of *H. pylori*-related gastritis and incidence of gastric cancer, two serum markers have been evaluated: *H. pylori* antibody and pepsinogen (PG) II [9, 10]. Both the titer of serum *H. pylori* antibody, which is usually used for determining the current status of the bacterial infection, and PG II, an aspartic protease also known as an inactive precursor of pepsin C produced mainly by the stomach mucosae, have been shown to correlate with the activity of *H. pylori*-related gastritis [11, 12]. In addition, our previous reports have indicated that individuals with *H. pylori* antibody titers >500 U/ml or PG II levels >30 ng/ml are at high risk of developing gastric cancer, particularly diffuse-type cancer according to Lauren's histopathological classification [10, 13]. Ito et al. [14] also reported that patients with PG II >30 ng/ml show an increased risk of diffuse-type gastric cancer.

DNA methylation is a covalent chemical modification resulting in the addition of a methyl group at the C5 position of cytosine in the sequence context 5′-CG-3′ [15]. This epigenetic effect plays pivotal roles in carcinogenesis [16, 17]. In the context of cancer development and progression, two types of DNA methylation have been identified: CpG island (CGI) hypermethylation and global hypomethylation [18]. CGI hypermethylation occurs within promoter CGIs and is often associated with a loss of protein expression by transcription repression [19]. On the other hand, global hypomethylation, which is observed in repetitive elements such as Alu, LINE-1, and Satα has been shown to be associated with genomic instability [20, 21].

DNA methylation is also deeply involved in chronic inflammation-mediated carcinogenesis. As shown in our previous studies [22, 23], aberrant DNA methylation has been observed in *H. pylori*-infected stomach mucosae as well as liver with viral infection [24] or colorectal mucosae with UC [25]. Altered DNA methylation in noncancerous inflammatory tissues has been considered to be one of the early steps toward neoplastic change. The accumulation of DNA methylation along with inflammation contributes to the development of cancer, according to the hypothesis of 'an epigenetic field for cancerization' [17, 26].

A recent study showed a correlation between highly active inflammation and altered mucosal DNA methylation in UC [27]. That report suggested that higher methylation levels induced by active inflammation were associated with inflammation-related cancer development. In contrast, correlations between activity of gastritis and altered mucosal DNA methylation levels have not been reported. Activity of gastritis is reportedly correlated with the development of diffuse-type gastric cancer [10, 13]. We therefore hypothesized that the degree of altered DNA methylation in stomach mucosae is correlated with activity of gastritis, since methylation observed in inflammatory stomach mucosae would directly or indirectly induce diffuse-type cancer development.

This study therefore aimed to clarify whether aberrant DNA methylation levels are correlated with inflammation activity in *H. pylori*-infected stomach mucosae. To this end, we measured and evaluated methylation levels of promoter CGIs of six genes (*FLNc*, *HAND1*, *THBD*, *p41ARC*, *HRASLS*, and *LOX*), and two repetitive elements (Alu and Satα), all of which have been shown to be hyper- or hypomethylated in *H. pylori*-related gastritis in our previous reports [22, 28] in human stomach mucosae according to the inflammation status evaluated by *H. pylori*-antibody titers and serum PG II levels.

Materials and methods

Cases, samples, and DNA extraction

Seventy-eight healthy volunteers were recruited after providing informed consent during an endoscopic screening program for gastric cancer under the approval of the Institutional Review Board of Wakayama Medical University. Fast blood samples were collected as part of the routine laboratory tests. Aliquots of the separated sera were stored below −20 °C until use. All study subjects were confirmed to be free from any previous history of gastric cancer, surgical resection of the stomach, or chronic renal failure. Subjects likewise had no history of *H. pylori* eradication therapy and had not been prescribed medications that might affect gastrointestinal function, such as proton pump inhibitors, adrenocortical steroids, or nonsteroidal antiinflammatory drugs. Stomach mucosae samples were collected by endoscopic biopsy of the antral and the corpus regions in the lesser curvature with sterilized biopsy forceps (Olympus, Tokyo, Japan), immediately frozen in liquid nitrogen, and stored at −80 °C until extraction of genomic DNA. High molecular weight DNA was extracted using the phenol/chloroform method.

Serologic analysis

Anti-*H. pylori* immunoglobulin (Ig)G antibody titers were measured using an enzyme-linked immunosorbent assay (ELISA) (MBL, Nagoya, Japan). Subjects with antibody titers >50 U/ml were classified as *H. pylori* positive, and

those with antibody titers 50 U/ml were regarded as infection negative. The *H. pylori*-positive group was further divided into two groups according to the antibody titer: 500 U/ml as a low-titer group and >500 U/ml as a high-titer group.

Serum pepsinogen levels were measured using PG I/PG II RIA-based kits (Dainabbot, Tokyo, Japan), which involved a modified radioimmunoassay method that we previously established [29]. Subjects were divided into three groups according to PG II level: group II-0, PG II ≤10 ng/ml; group II-10, PG II >10 ng/ml and ≤30 ng/ml; group II-30, PG II >30 ng/ml. These thresholds were determined in a previous study according to the risk of gastric cancer [13].

Sodium bisulfite modification, quantitative real-time methylation-specific PCR, and bisulfite pyrosequencing

Bisulfite modification was performed using 1 μg of *Bam*HI-digested genomic DNA as previously described [30]. The modified DNA was suspended in 40 μl of Tris-EDTA buffer, and an aliquot of 1 μl was used for quantitative real-time methylation-specific PCR (qMSP) and bisulfite pyrosequencing.

The qMSP was performed as described previously [22]. Briefly, real-time PCR with a primer set specific to methylated (M) or unmethylated (U) sequences was undertaken using SYBR® Green I (BioWhittaker Molecular Applications, Rockland, ME, USA) and an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). Standard DNA was prepared by cloning PCR products into the pGEM-T Easy vector (Promega, Madison, WI, USA). The number of molecules in a sample was determined by comparing its amplification with those of standard DNA that contained exact numbers of molecules (10^1–10^6 molecules). Based on the numbers of M molecules and U molecules for a genomic region, a methylation level of the region was calculated as the fraction of M molecules among the total number of DNA molecules (no. of M molecules + no. of U molecules).

Bisulfite pyrosequencing was performed as described previously [28]. Briefly, biotinylated PCR product with designed bisulfite PCR primers was purified and made single stranded. PCR products were bound to streptavidin-coated Sepharose beads, purified, washed, and denatured using a 0.2 ml/l of NaOH solution. Thereafter, those products were annealed to 0.2 μM pyrosequencing primers, and pyrosequencing was undertaken using the PSQ 96 Pyrosequencing System (Qiagen, Valencia, CA, USA). A methylation level was obtained using PSQ Assay Design software (Qiagen). The primers used in qMSP and bisulfite pyrosequencing have been described in previous reports [22, 28].

The methylation score was determined as 1, 2, or 3 at each locus according to the methylation level: 1, degree of altered methylation within the lowest one-third of all subjects; 3, degree of altered methylation within the highest one-third, with the remaining categorized as 2. Comprehensive methylation status of each individual was determined by accumulating 'the methylation score' of six CGIs and three loci of two repetitive elements.

Statistical analysis

Correlations between serum *H. pylori* antibody titers and serum PG II levels were analyzed using Spearman's rank correlation coefficient. Differences in median methylation levels among the three groups according to serum levels of *H. pylori* antibody titer or PG II were analyzed using the Kruskal-Wallis test. When the Kruskal-Wallis test showed a significant difference, a multiple comparison was performed using the Steel-Dwass test. All analyses were performed using JMP version 9.0 software (SAS Institute, Cary, NC, USA), and the results were considered significant for values of $p < 0.05$.

Results

Background characteristics of the individuals analyzed

Median age for the 78 examined individuals was 55 years (range 23–91 years), and 39 individuals (50 %) were male. Other background characteristics of the participants are presented in Table 1. *H. pylori* antibody titers and serum

Table 1 Background characteristics of the individuals

	All individuals ($N = 78$)
Age (years)[a]	55 (23–91)
Gender	
Male	39 (50 %)
Female	39 (50 %)
H. pylori antibody titer	
Negative (≤50 U/ml)	37 (47 %)
Low titer (>50, ≤500 U/ml)	28 (36 %)
High titer (>500 U/ml)	13 (17 %)
PG II level	
II-0 (≤10 U/ml)	27 (35 %)
II-10 (>10, ≤30 U/ml)	36 (46 %)
II-30 (>30 U/ml)	15 (19 %)

H. pylori Helicobacter pylori, *PG II* pepsinogen II

[a] Value is median (range)

PG II levels of subjects were measured. As both of these two markers were expected to reflect the activity of inflammation in the stomach mucosae, a significant correlation was observed between the two serologic markers in these 78 subjects (Spearman's rank correlation coefficient, 0.49; $p < 0.05$).

Mucosal DNA methylation levels in stomach and serum *H. pylori* antibody titers

Methylation levels in the stomach mucosae of the 78 subjects were determined. Six promoter CGIs (*FLNc*, *HAND1*, *THBD*, *p41ARC*, *HRASLS*, and *LOX*) and two non-coding

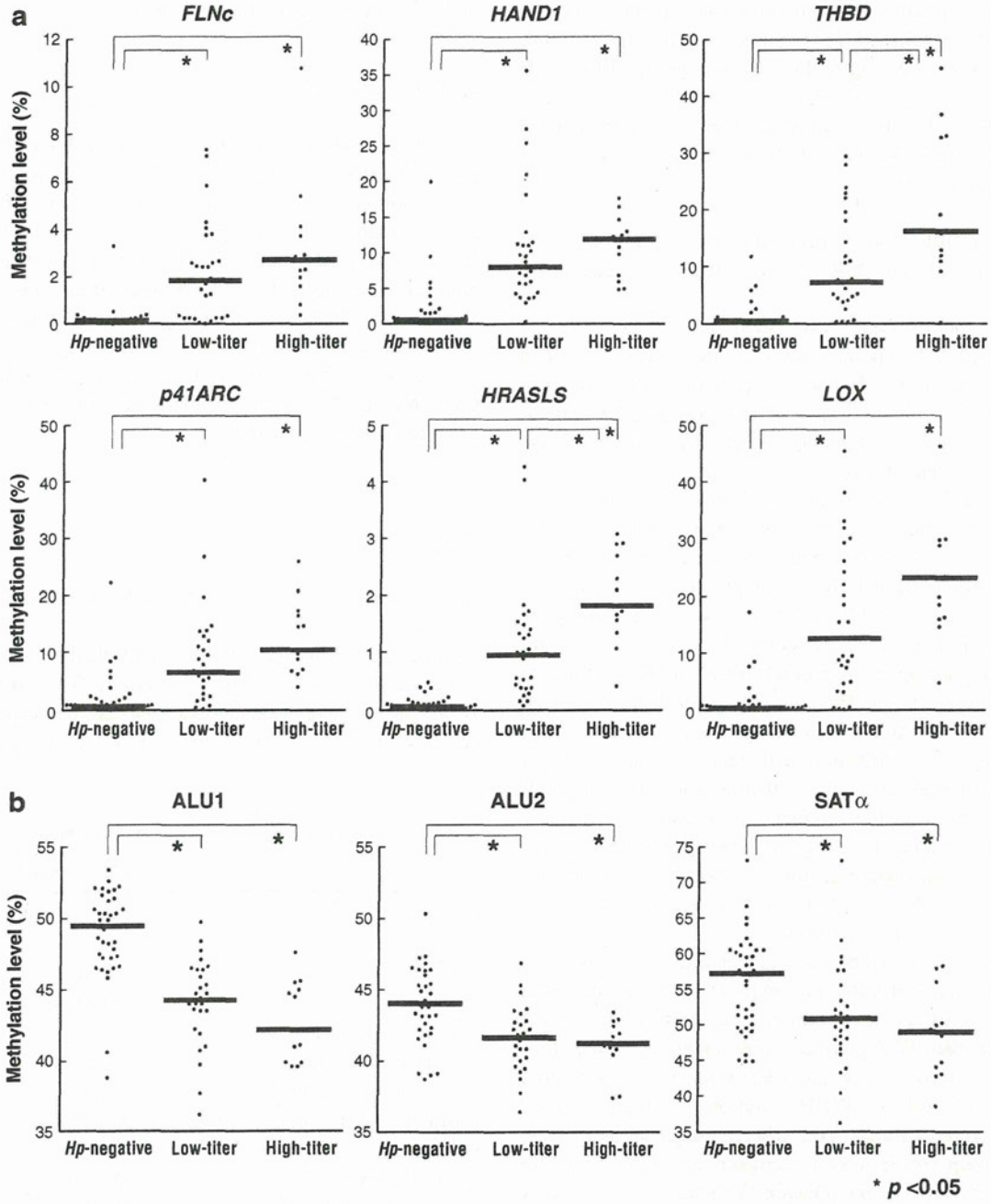


Fig. 1 Methylation levels of the promoter CpG islands of six genes (**a**) and two repetitive DNA elements (**b**) in the antral region of the gastric mucosae of the 78 subjects according to serum *H. pylori* antibody titer. Compared to the *H. pylori*-negative group, the methylation level was consistently increased (CpG island hypermethylation) or decreased (hypomethylation in repetitive elements) in a stepwise manner in the low- and high-titer groups ($p < 0.05$)

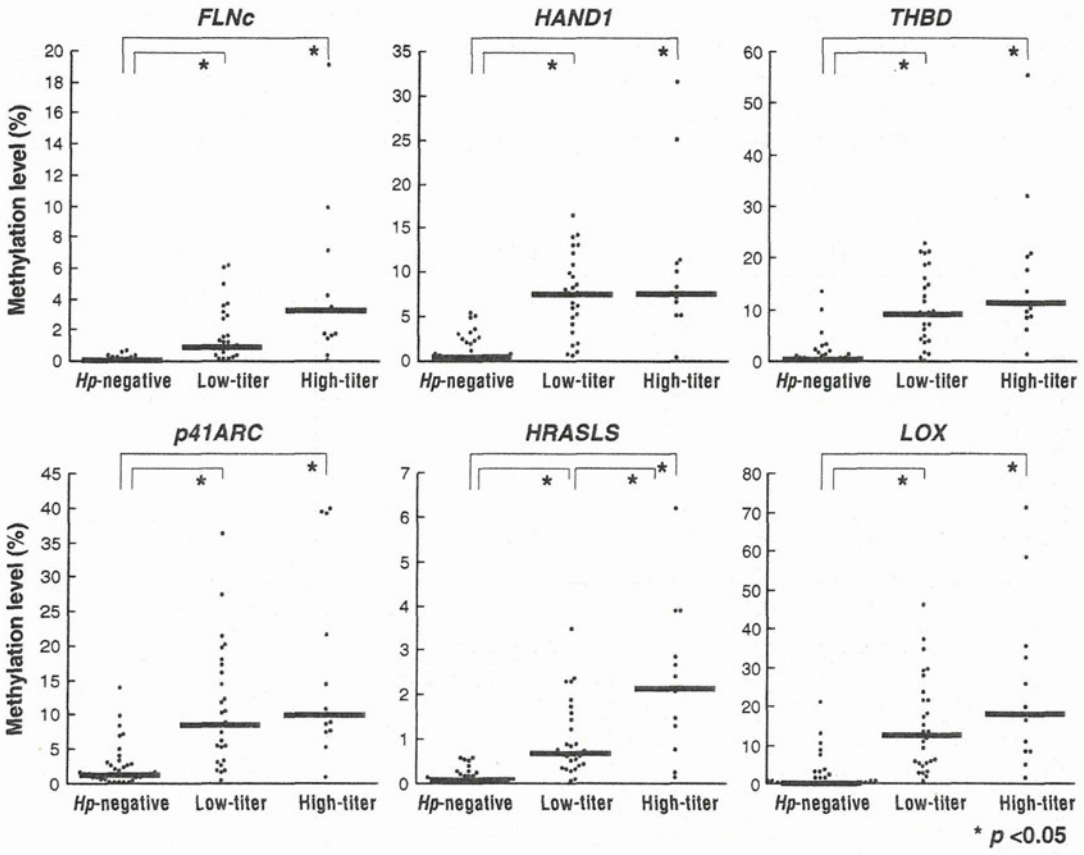


Fig. 2 Methylation levels of the promoter CpG islands of six genes in the corpus region of the gastric mucosae according to serum *H. pylori* antibody titer. Compared to the *H. pylori*-negative group, the methylation level was consistently increased in a stepwise manner in the low- and high-titer groups ($p < 0.05$)

repetitive elements (Alu and Satα) were examined by qMSP and bisulfite pyrosequencing, respectively.

Methylation levels of the CGIs were consistently increased in a stepwise manner with elevations of *H. pylori* antibody titer (Kruskal-Wallis test: $p < 0.05$) in both of the antral (Fig. 1a) and corpus regions (Fig. 2). At all examined loci, methylation levels in *H. pylori*-negative subjects were at or around 0 % (from 0.0 % at *FLNc* to 0.71 % at *p41ARC* in the antral region and from 0.0 % at *FLNc* to 1.23 % at *p41ARC* in the corpus region), while methylation levels in the low-titer group were significantly higher to variable extents (from 1.0 % at *HRASLS* to 12.4 % at *LOX* in the antral region and from 0.7 % at *HRASLS* to 12.3 % at *LOX* in the corpus region) (Steel-Dwass test: $p < 0.05$). In addition, methylation levels at each locus in the high-titer group were elevated 1.5- to 2.3-fold in the antral region and 1.0- to 3.5-fold in the corpus region compared to those in the low-titer group, although significant differences were only observed at *THBD* and *HRASLS* in the antral region and only at *HRASLS* in the corpus region (Steel-Dwass test: $p < 0.05$).

As for repetitive elements of Alu and Satα, methylation levels were consistently reduced in a stepwise manner with the elevation of *H. pylori* antibody titers in the antral region (Fig. 1b) (Kruskal-Wallis test: $p < 0.05$). Differences in methylation level at all these loci in the antral region were significant both between the *H. pylori*-negative and low-titer groups and between the *H. pylori*-negative and high-titer groups (Steel-Dwass test: $p < 0.05$). A similar trend was also observed with analysis using the samples of the corpus regions (data not shown), although the statistical significance could not be confirmed because of the smaller number of samples.

Mucosal DNA methylation levels in stomach and serum PG II levels

Correlations between DNA methylation levels and serum PG II levels were analyzed. Methylation levels at the CGIs were consistently increased in a stepwise manner with elevations in PG II level, as observed in the correlation with *H. pylori* antibody titers (Kruskal-Wallis test: $p < 0.05$) in both of the

antral (Fig. 3a) and corpus regions (Fig. 4). However, differences between group II-10 and group II-30 were inconspicuous and not significant at any of the six loci.

Methylation levels of repetitive elements at Alu and Satα were consistently reduced in a stepwise manner with elevations in PG II level, although the difference at Satα was not significant (Kruskal-Wallis test) (Fig. 3b).

Serum PG levels are generally used for the detection of *H. pylori*-infection related atrophic gastritis on the basis of the previously described PG test positive criteria (PG I

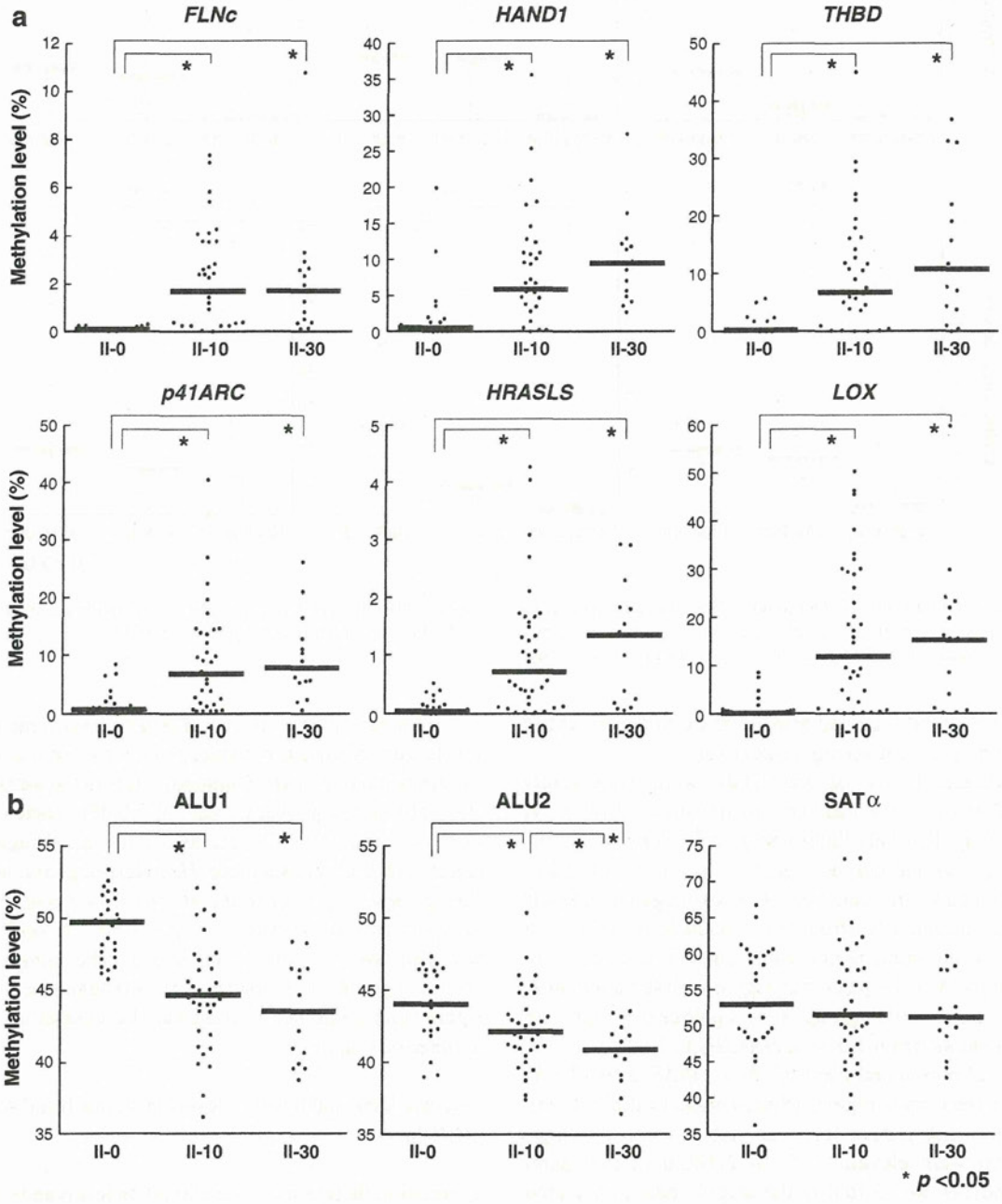


Fig. 3 Methylation levels of promoter CpG islands of six genes (**a**) and two repetitive DNA elements (**b**) in the antral region of the gastric mucosae of the 78 subjects according to serum pepsinogen II levels. Compared to group II-0, the methylation level was consistently increased (CpG island hypermethylation) or decreased (hypomethylation in repetitive elements) in a stepwise manner in group II-10 and group II-30 ($p < 0.05$)

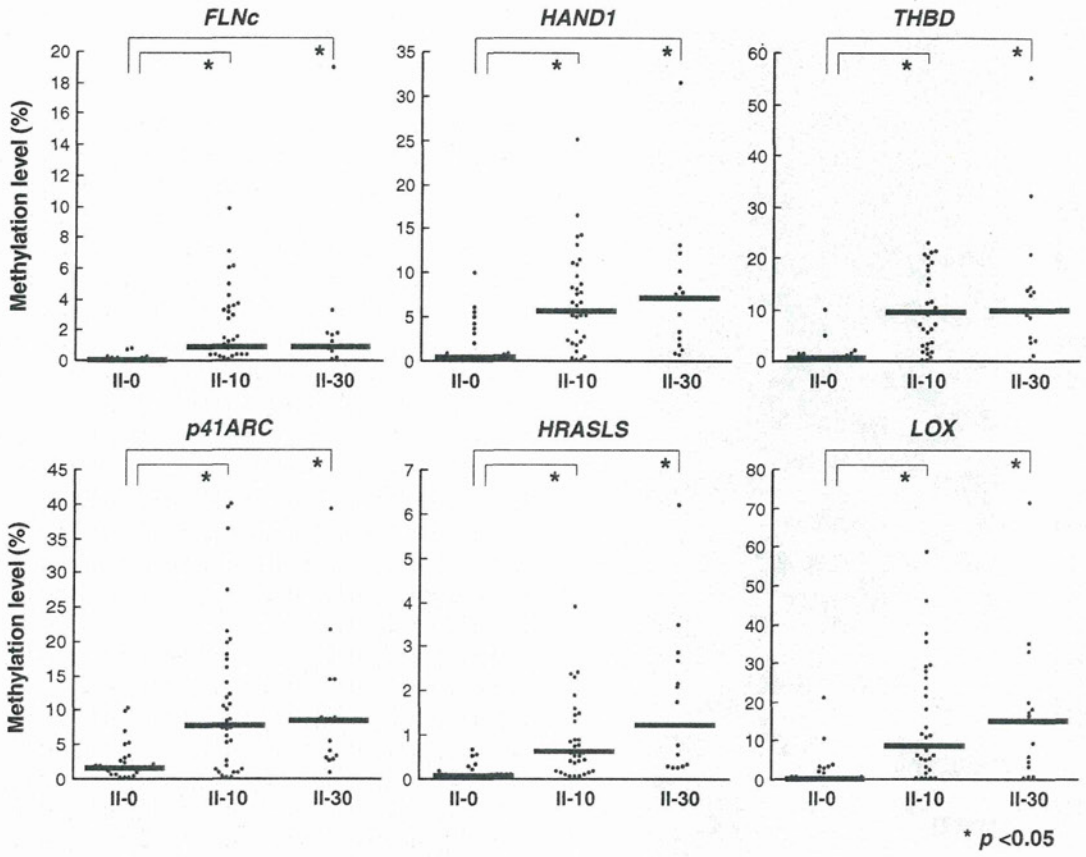


Fig. 4 Methylation levels of the promoter CpG islands of six genes in the corpus region of the gastric mucosae according to serum *H. pylori* antibody titer. Compared to the *H. pylori*-negative group, the methylation level was consistently increased in a stepwise manner in the low- and high-titer groups ($p < 0.05$)

≤70 ng/ml and PG I/II ratio ≤3.0) [10]. Then we compared with methylation levels between subjects with and without gastric atrophy according to the criteria. Among the 41 *H. pylori*-infected subjects (low-titer group and high-titer group), 19 subjects were classified as those with atrophic gastritis. The median methylation levels were not affected by the presence or absence of atrophy. The methylation levels in the high PG II subjects (group II-30) were also similar between the subjects with and without atrophy.

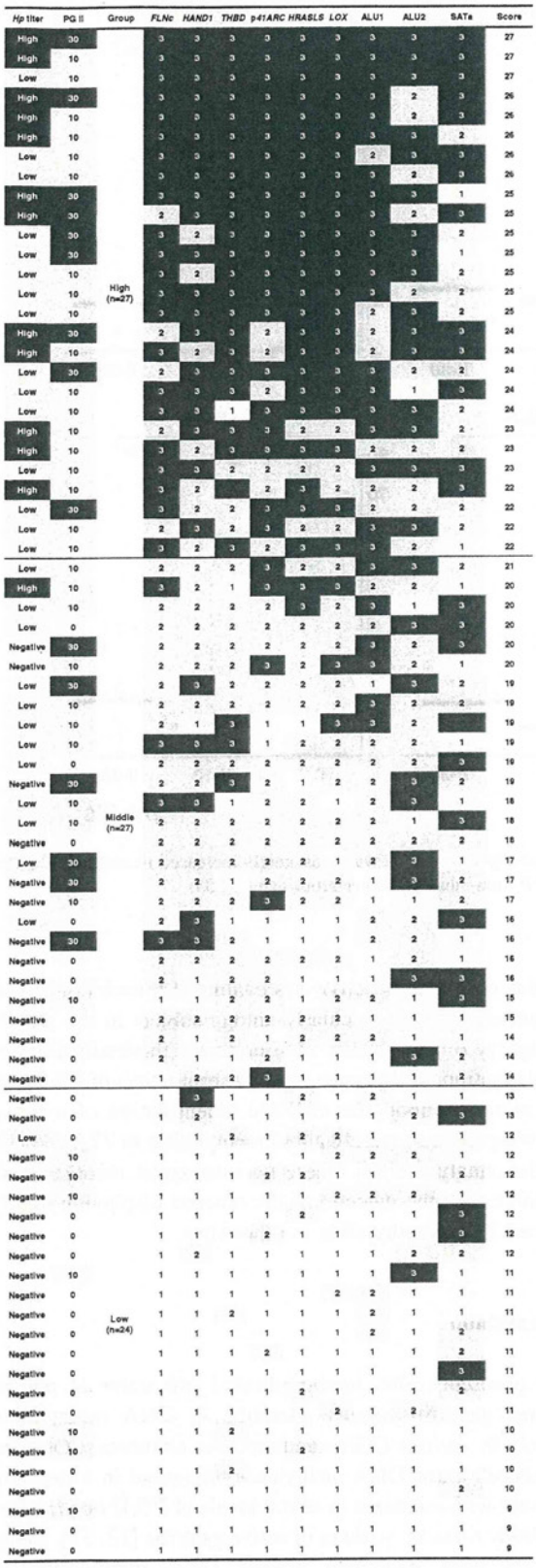
Spectrum of methylation status for individual subjects

Lastly, we comprehensively overviewed the methylation status and serum markers of all participating individuals. Methylation status was stratified proportionally into three categories (high, middle, and low) according to the accumulated methylation scores of the nine examined loci as mentioned in the "Materials and methods."

Methylation status based on the nine analyzed sites was in parallel with serum markers (Fig. 5). These results also indicated discrepancies in methylation levels between grades of gastritis activity as evaluated by each of the two serum markers, particularly among subjects in the middle category of methylation. Furthermore, the stratification of methylation levels at each site by serum levels of PG II and *H. pylori* antibody titer allowed identification of a group showing more severely altered methylation in 77.8 % (7/9) of the analyzed sites; these two serological markers were additive for the detection of the mucosa displaying highly altered DNA methylation in these sites.

Discussion

The present results clearly indicated that active *H. pylori*-related gastritis induces alteration in DNA methylation evenly in various CGIs and repetitive elements. Observed levels of altered DNA methylation increased in a stepwise manner with increases in serum levels of PG II or *H. pylori* antibody titers as markers of active gastritis [12, 31]. Many reports have examined altered DNA methylation in inflammatory tissues in terms of cancer risk in the fields of



◀ **Fig. 5** Comprehensive view of methylation status and the two serum markers among individual participants. As a whole, methylation status paralleled serum markers in each individual

viral hepatitis, UC, and *H. pylori*-related gastritis [32–34]. However, attention has rarely been paid to the correlations between the degree of altered DNA methylation and activity of inflammation, probably because of difficulties in quantitatively evaluating inflammation in tissues. The diagnosis of gastritis is based on histopathology of the stomach mucosa. However, since *H. pylori*-related gastritis is a multifocal process, it is difficult to accurately diagnose the degree of active inflammation in the whole stomach based on a few endoscopic biopsy samples. Furthermore, histological diagnosis of gastritis depends on subjective judgment without a gold standard [35]. The present study therefore used two serum tests as more objective surrogate markers of gastric inflammation, and this is the first report to show a close correlation between the activity of gastritis as reflected by these two serum markers and the degree of altered methylation.

The antibody titers of the two serum markers, PG II and *H. pylori*, were additive in the detection of mucosa with severely altered DNA methylation. In addition, discrepancies were apparent between grades of serum levels of the two markers, particularly among subjects with mucosal DNA methylation categorized as middle grade. These results suggest that differences exist in inflammatory status as identified by each of these two markers; while *H. pylori* antibody levels reflect the complex interactions between bacterial infection and immunological host response, PG II levels are considered to reflect local mucosal reaction. These two serum tests thus appear to reflect two different aspects of *H. pylori* infection, and subjects with high levels of both *H. pylori* antibody and PG II seem highly likely to have the highest activities of gastritis and highest degree of altered DNA methylation.

Regarding the *H. pylori*-related carcinogenesis, distinct pathways have been proposed for two histological types of gastric cancer: intestinal type and diffuse type [36]. Intestinal-type cancer is considered to develop in a multistep process starting from chronic active gastritis and progressing through chronic atrophic gastritis, intestinal metaplasia, and dysplasia [37]. In contrast, diffuse-type cancer develops in the stomach following chronic active inflammation without passing through the intermediate steps of atrophic gastritis or intestinal metaplasia. Of particular note, the activity of mucosal inflammation is proposed for a risk of diffuse-type cancer [9, 10, 38]. To date, studies about DNA methylation in gastric carcinogenesis have been mainly focused on the intestinal type [22, 23, 28, 39]. In reality, among *H. pylori*-negative subjects, including those with past infection,

subjects with gastric cancer, particularly those with multiple gastric cancers, have stomach mucosae with more highly altered DNA methylation than those without [22, 39]. The finding in this report, in contrast, showed that the degree of altered DNA methylation parallels the activity of gastritis and appears to be deeply involved in active inflammation-mediated carcinogenesis leading to the development of diffuse-type cancer. Furthermore, recent evidence indicates that alterations in the DNA methylation of various gene regions including the CDH1 (*E-cadherin*) promoter are frequently observed in diffuse-type cancer and are deeply involved in the development of this type of gastric cancer [40, 41].

Some limitations in the study methods must be considered when interpreting the present findings. First, differences in DNA methylation levels among all stratified groups were insufficient to show statistical significance because of the relatively small number of samples. However, methylation levels were consistently altered in the same manner among CGI hypermethylation or hypomethylation of repetitive elements along with increases in serum marker levels. We thus believe that DNA methylation levels are tightly correlated with both markers reflecting the activity of gastritis. Second, some clinical factors that affect DNA methylation alteration might not have been completely excluded. For example, Epstein-Barr virus infection is known to enhance the DNA methylation in gastric cancer tissues [23]. However, *H. pylori* infection is a major factor in the alteration of DNA methylation in stomach mucosae of healthy volunteers, as repeatedly shown in previous studies [22, 42]. Indeed, the CGI methylation level was almost zero among *H. pylori*-negative subjects in the present study. Third, the small amount of samples led to no histological evaluations on *H. pylori*-related gastritis including the degree of intestinal metaplasia or stromal inflammatory cells. Although those mucosal findings in biopsy samples could affect the alteration in DNA methylation [43], the degree of the alterations might be modest or dependent on genes as suggested in some previous reports [34, 44]. In contrast, alterations in DNA methylation in parallel with the severity of gastritis were considerable and consistently observed in the present study. Thus, we believe that bias due to those factors on the present results might be marginal.

In conclusion, the results of this study strongly indicate that DNA methylation levels in stomach mucosae are closely correlated with inflammatory activity in stomach mucosae. In terms of *H. pylori*-related stomach carcinogenesis, highly altered DNA methylation correlated with highly active gastritis appears to represent one of the relevant molecular mechanisms underlying the development of diffuse-type gastric cancer. The evaluation of altered DNA methylation in stomach mucosa is thus likely to be useful in predicting the risk of gastric cancer, particularly for diffuse-type cancer. We are now carrying out a long-term follow-up study of subjects from the present study for cancer development.

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Conflict of interest All authors disclose no financial or personal relationships with other people or organizations that could inappropriately influence the present work.

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REVIEWS IN BASIC AND CLINICAL GASTROENTEROLOGY AND HEPATOLOGY

Robert F. Schwabe and John W. Wiley, Section Editors

Inflammation-Associated Cancer Development in Digestive Organs: Mechanisms and Roles for Genetic and Epigenetic Modulation

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Chronic inflammation, regardless of infectious agents, plays important roles in the development of various cancers, particularly in digestive organs, including *Helicobacter pylori*–associated gastric cancer, hepatitis C virus–positive hepatocellular carcinoma, and colitis-associated colon cancers. Cancer development is characterized by stepwise accumulation of genetic and epigenetic alterations of various proto-oncogenes and tumor-suppressor genes. During chronic inflammation, infectious agents such as *H pylori* and hepatitis C virus as well as intrinsic mediators of inflammatory responses, including proinflammatory cytokines and reactive oxygen and nitrogen species, can induce genetic and epigenetic changes, including point mutations, deletions, duplications, recombinations, and methylation of various tumor-related genes through various mechanisms. Furthermore, inflammation also modulates the expressions of microRNAs that influence the production of several tumor-related messenger RNAs or proteins. These molecular events induced by chronic inflammation work in concert to alter important pathways involved in normal cellular function, and hence accelerate inflammation-associated cancer development. Among these, recent studies highlighted an important role of activation-induced cytidine deaminase, a nucleotide-editing enzyme essential for somatic hypermutation and class-switch recombination of the immunoglobulin gene, as a genomic modulator in inflammation-associated cancer development.

Keywords: H pylori; HCV; Mutation Induction; Epigenetics.

Nearly 150 years ago, Rudolf Virchow noted that inflammatory cells are present in tumor tissues and that tumors develop at sites of chronic inflammation; he suggested that chronic inflammation plays important roles in cancer development. Since then, many clinical and epidemiologic studies have confirmed a strong association between inflammation and cancer (Table 1).[1,2] For instance, epidemiologic studies have shown that approximately 10%–15% of cancers were related to chronic infections with viruses, bacteria, or parasites,[3–7] and, moreover, that up to 25% of all cancers were associated with chronic inflammation irrespective of the presence or absence of infection.[5–7]

In inflammation-associated cancer development, in addition to infectious agents such as *Helicobacter pylori* and hepatitis C virus (HCV), many intrinsic mediators of inflammation including proinflammatory cytokines, eicosanoids, growth factors, and reactive oxygen species (ROS) and reactive nitrogen species exert important effects in cancer development through various mechanisms. These include enhancement of cell growth and mobility, induction of angiogenesis, and inhibition of apoptosis. However, a hallmark of cancer development is the stepwise accumulation of various genetic and epigenetic alterations of the genome. Indeed, recent genomewide analysis of human cancer tissues revealed that a single cancer cell generally possesses approximately 100 mutations in coding regions, 10–20 of which are known as driver genes that contribute to cancer development,[8–10] and, moreover, that there are many somatic gene rearrangements, including duplications, deletions, and inversions in human cancer genomes.[11,12] In addition to genetic alterations, recent studies also have shown that chronic inflammation enhances epigenetic changes as represented by DNA methylation.[13] It is estimated that several hundreds to thousands of genes are methylated in a cancer cell,[14] and that

Abbreviations used in this paper: A, adenine; AID, activation-induced cytidine deaminase; C, cytosine; CDKN, cyclin-dependent kinase inhibitor; COX-2, cyclooxygenase 2; DNMT, DNA methyltransferase; DSS, dextran sulfate sodium; EZH2, enhancer of zeste homolog 2; G, guanine; H, histone; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IBD, inflammatory bowel disease; IL-1β, interleukin-1β; JNK, c-Jun N-terminal kinase; Let-7, lethal-7; LPS, lipopolysaccharide; miRNA, microRNA; MAPK, mitogen-activated protein kinase; MLH1, mutL homolog 1; mRNA, messenger RNA; MSH, mutS homolog; NF-κB, activation of transcription factor nuclear factor κB; NOS, nitric oxide synthase; PSC, primary sclerosing cholangitis; ROS, reactive oxygen species; STAT, signal transducer and activator of transcription; T, thymine; TNF-α, tumor necrosis factor-α; U, uracil.

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