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Adenomatous polyposis coli 1A is likely to be methylated as a passenger in human gastric carcinogenesis

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ABSTRACT

Many promoter CpG islands (CGIs) are methylated as a consequence of or in association with carcinogenesis (passenger), in addition to being a cause of carcinogenesis (driver). In gastric cancers, promoter 1A of the adenomatous polyposis coli (*APC*) gene is frequently methylated, and is often discussed as a driver. However, the actual role of 1A methylation is unclear because the same *APC* protein is coded by two transcripts from two promoters, 1A and 1B, and their relative expression levels in gastric mucosae have not been quantified. To clarify this issue, we first identified detailed transcription start sites of 1A and 1B transcripts. We then confirmed that, among nine gastric cancer cell lines, 1A methylation, if present, could repress 1A transcription while 1B was expressed and not methylated. In primary samples, 1B expression was 15-fold higher than 1A expression in gastric mucosae of healthy volunteers, and was decreased markedly in non-cancerous gastric mucosae of cancer patients. Quantitative methylation analysis showed that promoter 1A was methylated at similar levels (20–40%) in healthy individuals and non-cancerous gastric mucosae of cancer patients, and promoter 1B was never methylated in any samples, including gastric cancers. These findings strongly indicated that methylation of *APC* promoter 1A is a passenger, and suggested that marked down-regulation of 1B expression could be related to formation of a field predisposed to gastric cancers.

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1. Introduction

Aberrant DNA methylation of promoter CpG islands (CGIs) is frequently causally involved in human carcinogenesis by inducing permanent silencing tumor-suppressor genes (driver methylation) [1]. At the same time, recent genome-wide studies have shown that a large number of CGIs are methylated in cancer cells [2–5]. Most of the methylated genes have no or little expression in normal precursor cells, and a significant fraction of them are considered to have been methylated as a consequence of or in association with carcinogenesis (passenger methylation) [2,4,6]. The presence of driver and passenger methylation is also true for gastric cancers, a major cancer in Asian countries and in which *H. pylori* infection is deeply involved [7,8]. It was recently shown that *H. pylori* infection induces methylation of various genes, both driver and passenger, in gastric epithelial cells [9,10], and that accumulation of aberrant DNA methylation is associated with gastric cancer development (an epigenetic field for cancerization) [11–13].

The adenomatous polyposis coli (*APC*) tumor suppressor gene, a negative regulator of WNT signaling [14–16],

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is known to be methylated in 34–83% of gastric cancers [17–21] while its mutations are very rare [22]. "*APC* methylation" in most studies deals with methylation of one of its two promoters, 1A and 1B, although transcripts from both promoters encode the same *APC* protein [23]. Promoter 1A is reported to be methylated not only in gastric cancers, but also in the normal mucosae with *H. pylori* infection [21]. On the other hand, promoter 1B is never methylated in gastric cancers and cancer cell lines, and neither in normal gastric tissue [17]. These points indicate that, if 1A is the major transcript in gastric mucosae, its methylation can be involved in gastric carcinogenesis as a driver. However, expression levels of 1A and 1B have not been quantified, and which of 1A and 1B is dominant has not been clarified yet.

In this study, we aimed to clarify the role of promoter 1A methylation in gastric carcinogenesis. To this end, we first confirmed transcription start sites (TSSs) of *APC* 1A and 1B, and analyzed the effect of promoter 1A methylation on 1A expression. We then quantified expression and methylation levels of 1A and 1B in gastric mucosae of healthy volunteers, non-cancerous gastric mucosae of cancer patients, and gastric cancer tissues.

2. Materials and methods

2.1. Cell lines and their 5-aza-2′-deoxycytidine and/or trichostatin A treatment

Four human gastric cancer cell lines, KATOIII, MKN28, MKN74, and NUGC3 were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan), and AGS was obtained from the American Type Culture Collection (Manassas, VA). Three gastric cancer cell lines, HSC39, HSC44, and HSC57 were gifted by Dr. K. Yanagihara, National Cancer Center Research Institute, Tokyo, Japan. TMK1 was gifted by Dr. W. Yasui, Hiroshima University, Hiroshima, Japan.

AGS and KATOIII cells were seeded on day 0, and media containing 0.3 μM 5-aza-2′-deoxycytidine (5-aza-dC; Sigma, St. Louis, MO) added on days 1 and 3, followed by addition of 1 μM trichostatin A (TSA, Sigma) on day 4, and harvested on day 5. Cells were also treated with mock, 5-aza-dC alone, and TSA alone. This dose of 5-aza-dC suppressed cellular growth to approximately half of non-treated cells. High molecular weight DNA was extracted by the phenol/chloroform method. RNA was extracted with ISOGEN (Nippon Gene, Tokyo, Japan) and purified with an RNeasy Mini kit (QIAGEN, Valencia, CA).

2.2. Tissue samples

Normal gastric mucosae were obtained by endoscopic biopsy from 43 healthy volunteers (32 males and 11 females; 20 with *H. pylori* infection and 23 without; average age = 47.9). Eleven and 32 samples were used for expression and methylation analysis, respectively. Non-cancerous gastric mucosae were obtained by endoscopic biopsy from 45 gastric cancer patients (35 males and 10 females; 29 with *H. pylori* infection and 16 without; average age = 66.5), and were used for methylation analysis. All of the biopsy specimens were obtained with informed consents. *H. pylori* infection status was analyzed by a serum anti-*H. pylori* IgG antibody test (SRL, Tokyo, Japan), rapid urease test (Otsuka, Tokushima, Japan), or culture test (Eiken, Tokyo, Japan). Gastric epithelial cells were separated from stromal cells by the gland isolation technique using non-cancerous gastric mucosae of 10 gastric cancer patients (10 males; average age = 59.8) who underwent gastrectomy due to gastric cancers. Peripheral leukocytes were obtained from eight healthy volunteers (seven males and one female; average age = 35.5).

Gastric cancer tissues were obtained from 47 gastric cancer patients (40 males and seven females; average age = 64.3) who underwent gastrectomy due to gastric cancers. All cancers were histologically diagnosed according to the Japanese classification of gastric carcinoma, and classified according to the Lauren classification system [24]. Genomic DNA and total RNA were isolated in the same way as the cell lines.

2.3. Quantitative reverse transcription-PCR (qRT-PCR)

cDNA was synthesized from 1 μg of total RNA using a Superscript III kit (Invitrogen, Carlsbad, CA) with a random primer. qRT-PCR was performed by real-time PCR using SYBR® Green I (BioWhittaker Molecular Applications, Rockland, ME) and an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The number of molecules in a sample was determined by comparing its amplification with those of standard DNA samples that contained known numbers of molecules (10^1–10^6 molecules). The standard samples were prepared by serial dilution of PCR products quantified after purification using Zymo-Spin I™ Columns (Zymo Research, Orange, CA). The amount of the standard samples was measured by the QIAxcel system (QIAGEN). The mRNA quantity of each gene was normalized to that of β2-microglobulin. The primers and PCR conditions are shown in Supplemental Table 1.

2.4. Bisulfite treatment, methylation-specific PCR (MSP) and quantitative MSP (qMSP)

Bisulfite modification was performed using 1 μg of *Bam*HI-digested genomic DNA as previously described [25], and the modified DNA was suspended in 30 μl of TE buffer. MSP was performed with a primer set specific to the methylated or unmethylated sequence (M or U set), using 0.5 μL of the sodium bisulfite-treated DNA. DNA methylated with *Sss*I methylase (New England Biolabs, Beverly, MA) and DNA amplified by a GenomiPhi DNA amplification kit (GE Healthcare Bio-Sciences, Buckinghamshire, England) were used as fully methylated and unmethylated control DNA, respectively.

qMSP was performed by real-time PCR, using 1 μL of the sodium bisulfite-treated DNA. Although the same primer set as MSP was used for qMSP, a specific annealing temperature in the presence of SYBR® Green I was re-determined using the fully methylated and unmethylated DNA. The primers and PCR conditions are shown in Supplemental Table 2. The standard samples for real-time PCR were pro-

duced by serial dilution of PCR products quantified after purification. Based on the numbers of molecules measured by the M and U primers, a methylation level was calculated as a fraction of methylated molecules in the total number of DNA molecules.

2.5. Rapid amplification of 5′ complementary DNA ends (5′ RACE)

5′ RACE was performed using a GeneRacer™ kit (Invitrogen) on RNA from KATOIII, which is known to have no genetic alteration of *APC* [26]. The PCR product was cloned into a pGEM-T Easy Vector (Promega, Madison, WI), and a total of 31 clones were sequenced using an ABI310 DNA sequencer (Applied Biosystems, Foster City, CA). The TSSs derived from multiple clones and located at the upstream of the *APC* translational start site were searched.

3. Results

3.1. Determination of APC transcriptional start sites by 5′ RACE

DNA methylation of the nucleosome-free region immediately upstream of a TSS is critical for gene silencing, and accurate determination of TSSs is important to evaluate involvement of DNA methylation in gene silencing [1,2,27]. The TSS of *APC* 1A in a database of TSSs (DBTSS) is located 2 bp upstream of the TSS in NCBI (described as +1 here), and no other TSSs of 1A are known. In contrast, *APC* 1B is reported to have three variants, B1 B2 and B3 (Fig. 1A) [23]. The TSS of 1B in DBTSS is located 2 bp downstream of the TSS in NCBI (described as +1 here) based on the report by Horii et al. [23].

To determine TSSs of *APC*, we performed 5′ RACE, and identified five novel TSSs, all of which were in exon 1B (Fig. 1B). Therefore, we analyzed the methylation status of the immediate upstream regions of the TSSs of 1A and 1B in NCBI (200 bp or less) as promoter 1A and 1B. The activity of promoter 1A was assessed by quantification of *APC* 1A using PCR primers on exons 1A and 2. The activity of promoter 1B was assessed by quantification of *APC* B1 plus B2 using primers on the 3′ region of exon 1B (not transcribed in B3) and exon 2 since expression levels of B1 plus B2 paralleled that of B3 among 28 samples of various origins (Fig. 1C).

3.2. Effect of APC 1A methylation on its silencing

To examine the effect of methylation of promoters 1A and 1B on their silencing, their methylation was first analyzed in nine human gastric cancer cell lines. Promoter 1A was completely methylated in six cell lines, completely unmethylated in two, and in a mixed status in one (Fig. 2A). Promoter 1B was completely unmethylated in all the nine cell lines analyzed. By quantitative mRNA expression analysis of individual 1A and 1B transcripts, it was found that 1A was consistently repressed in the six cell lines with 1A methylation (Fig. 2A). 1B was expressed in all of the nine cell lines.

When 1A methylation was removed by a demethylating agent, 5-aza-dC, in two cell lines with its complete methylation (AGS and KATOIII), 1A expression was restored (Fig. 2B). Addition of TSA significantly (48-fold in AGS and 17-fold in KATOIII) enhanced 1A restoration by 5-aza-dC in both cells. In contrast, 1B expression was not restored by treatment with 5-aza-dC alone or TSA alone. Only in AGS, slight (2.7-fold) up-regulation of 1B was observed by the combined treatment with 5-aza-dC and TSA. This showed that, if promoter 1A is methylated, it leads to 1A silencing, but that promoter 1B was consistently unmethylated and expressed.

3.3. APC 1B is the major transcript in normal human gastric mucosae

To examine which of *APC* 1A and 1B is the major transcript in gastric mucosae, we quantified their expression levels using the primers described above in 11 gastric mucosae of healthy volunteers (five with *H. pylori* infection and six without), 10 non-cancerous gastric mucosae of gastric cancer patients, and 19 gastric cancers from which high-grade RNA was isolated (Fig. 3A). In the gastric mucosae of *H. pylori*-negative healthy individuals, the average 1B expression level was 15-fold higher than that of 1A. In the gastric mucosae of *H. pylori*-positive healthy individuals, the average 1B expression level decreased to 52% of that of *H. pylori*-negative individuals, but was still 11-fold higher than that of 1A. In the non-cancerous gastric mucosae of cancer patients, the average 1B expression level further decreased to 9% of that of *H. pylori*-negative individuals. In the 19 gastric cancers, the average 1B expression level was 5% of that of *H. pylori*-negative healthy individuals. The 1A expression level was consistently low among these four groups.

To exclude the possibility that the abundant 1B expression was derived from gastric stromal cells, gastric epithelial cells and stromal cells were separated by the gland isolation technique. For this technique, several cm^2 areas of gastric mucosae were necessary, and we were able to analyze only non-cancerous gastric mucosae of cancer patients (surgical specimens). Isolation of gastric epithelial cells was confirmed by the shape of the glands obtained (Fig. 3B). 1B showed similar expression levels between the isolated gastric epithelial cells and the remaining stromal cells (Fig. 3C). 1A showed lower expression levels in the epithelial cells than in the remaining stromal cells. These findings supported that the abundant 1B expression in gastric mucosae was not due to contamination of stromal cells.

3.4. High methylation level of promoter 1A irrespective of H. pylori infection status in gastric mucosae, and its presence in gastric cancers

In non-cancerous tissues, which are polyclonal, quantification of the DNA methylation level is essential to assess the fraction of cells with methylation. Therefore, methylation levels of promoters 1A and 1B were quantified in gastric mucosae of 32 healthy volunteers (normal mucosae, 14 with *H. pylori* infection and 18 without), and 45 non-cancerous mucosae of gastric cancer patients (29 with *H. pylori* infection and 16 without) obtained by endoscopic biopsy. Methylation levels were also quantified in 47 gastric cancers. The methylation level of promoter 1A was 20–40% in the normal mucosae and non-cancerous mucosae, regardless of *H. pylori* infection statuses (Fig. 4A). In cancer tissues, it ranged from 0% to 73%, reflecting the monoclonal nature of cancer tissues. In sharp contrast, promoter 1B was not methylated at all in any of the samples (Fig. 4A).

To examine in what cell types promoter 1A was methylated, we quantified 1A methylation levels in gastric epithelial cells and stromal cells prepared from non-cancerous gastric mucosae of three gastric cancer patients and in peripheral leukocytes of eight healthy volunteers. Promoter 1A was methylated at higher levels in the gastric epithelial cells than in the stromal cells, and was not methylated in peripheral leukocytes (Fig. 4B). This showed that promoter 1A methylation detected in gastric mucosae was due to methylation in gastric epithelial cells, in addition to stromal cells.

The high fraction of cells with 1A methylation among gastric epithelial cells should have affected its overall 1A expression level if its expression in cells without methylation had been high enough. However, in our observation, the 1A expression level was not correlated with 1A methylation levels among 10 samples of gastric epithelial cells (Spearman's rank-order correlation coefficient = 0.44, Fig. 4C). Also, the 1A expression level observed ($0.5–1 \times 10^{-4}$/β2MG) was considered to be $0.5–1 \times 10^{-2}$ mRNA molecules in a cell, on the assumption that 1 μg total RNA can be isolated from 10^5 cells. This also supported that *APC* 1A was expressed only at a trace level, or not expressed with biological significance, even in cells without 1A methylation.

4. Discussion

APC 1B was the major transcript in normal gastric mucosae. Promoter 1A was methylated at similar levels in gastric mucosae of healthy individuals (with and without *H. pylori* infection) and non-cancerous gastric mucosae of gastric cancer patients. Although promoter 1A methylation could silence its expression, the fraction of gastric epithelial cells with methylation did not influence the overall 1A expression level, showing that 1A was expressed only at trace levels in cells without methylation. It is becoming clear that genes with low expression levels are susceptible

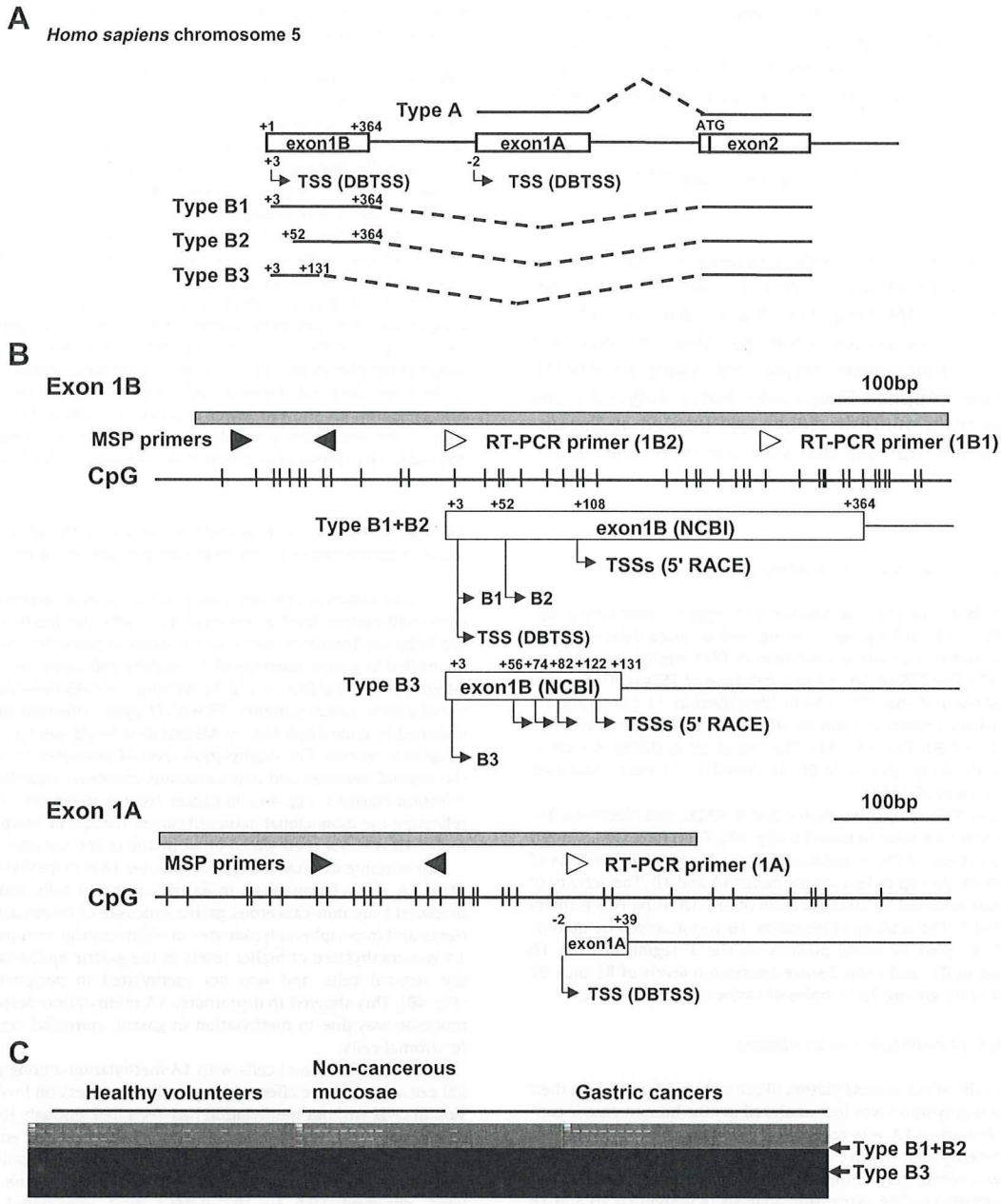

Fig. 1. Genomic and mRNA structures of *APC* 1A and 1B. (A) Splicing forms of 1A and 1B. One form of 1A and three variants of 1B are known to be produced from individual promoters. (B) CpG maps of promoters 1A and 1B and their TSSs. The TSS of *APC* 1A in a database of TSSs (DBTSS) is located 2 bp downstream of the TSS in NCBI (NC_000005.8 112101483, described as +1 here). The TSS of 1B in DBTSS is located 2 bp downstream of the TSS in NCBI (NC_000005.8 112071115, described as +1 here) based on D13981 [23]. Three TSSs, B1 B2 and B3, were based on the report by Horii et al. [23]. Five TSSs were identified in this study by 5′ RACE. *Vertical lines*, individual CpG sites; *Gray boxes*, the CGI regions; *Arrows*, TSSs; and *Arrowheads*, positions of MSP and RT-PCR primers. (C) Similar expression levels of *APC* B1 plus B2 and *APC* B3. Quantitative RT-PCR analysis of all the three variants (B1, B2, and B3) using one set of primers was impossible due to different lengths of PCR products. It was confirmed that the expression level of B1 plus B2 paralleled that of B3 among 28 samples.

to DNA methylation [2,4,6]. Our results and current knowledge strongly indicated that *APC* 1A was methylated as a passenger during gastric carcinogenesis.

This conclusion was not in agreement with many previous reports that discussed *APC* 1A methylation as a driver [17–21]. Our conclusion was attained by accurate quantitative expression and methylation analysis, which has become popular recently, and most previous reports did not adopt quantitative analysis. Quantitative methylation analysis revealed that *APC* 1A was methylated in normal gastric mucosae of healthy individuals, regardless of *H. pylori* infection status, and that the methylation level was not correlated with age ($N = 32$, $r = -0.02$). Therefore, it was considered that *APC* 1A methylation was physiologically present in human gastric mucosae as a simple fluctuation in the methylation status of a non-expressed gene or as a

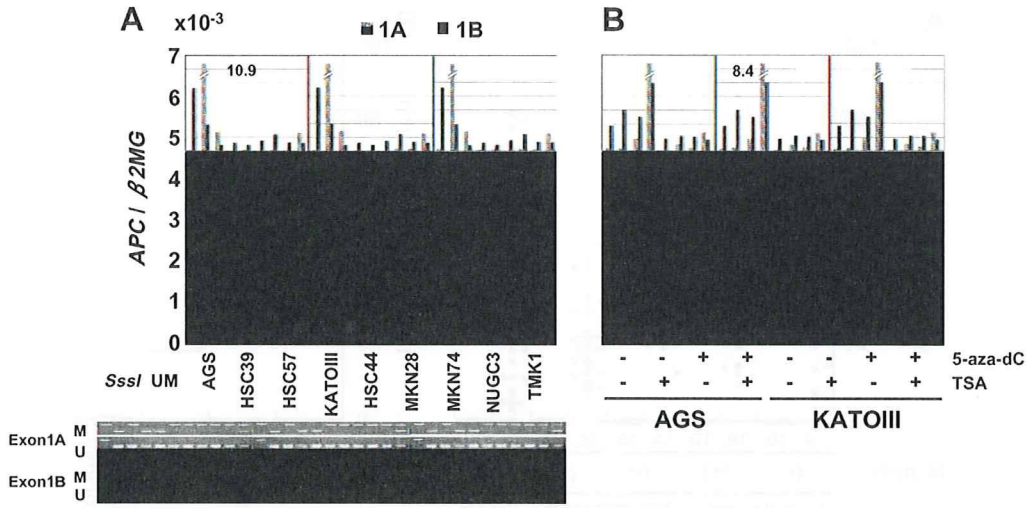

Fig. 2. Methylation-silencing of 1A in human gastric cancer cell lines. (A) qRT-PCR of *APC* 1A and 1B, and MSP of promoters 1A and 1B in nine cell lines. The mRNA quantity of each gene was normalized to that of β2-microgloburin (β2MG). It was found that 1A was consistently repressed in the six cell lines with 1A methylation. *Sss*I, fully methylated DNA (genomic DNA methylated with *Sss*I methylase); and UM, fully unmethylated DNA (DNA amplified by a GenomiPhi DNA amplification kit). (B) Expression changes of *APC* 1A and 1B after 5-aza-dC and/or TSA treatment. Two cell lines with complete methylation of 1A were treated with 5-aza-dC (0.3 μM) and/or TSA (1 μM), and 1A and 1B expression levels were quantified by qRT-PCR. Restoration of the 1A expression in AGS and KATOIII by the 5-aza-dC treatment was observed. Addition of TSA significantly enhanced the 1A restoration by the 5-aza-dC treatment. 1B expression was not restored by treatment with 5-aza-dC alone or TSA alone. Only in AGS, slight up-regulation of 1B was found by combination treatment with 5-aza-dC and TSA.

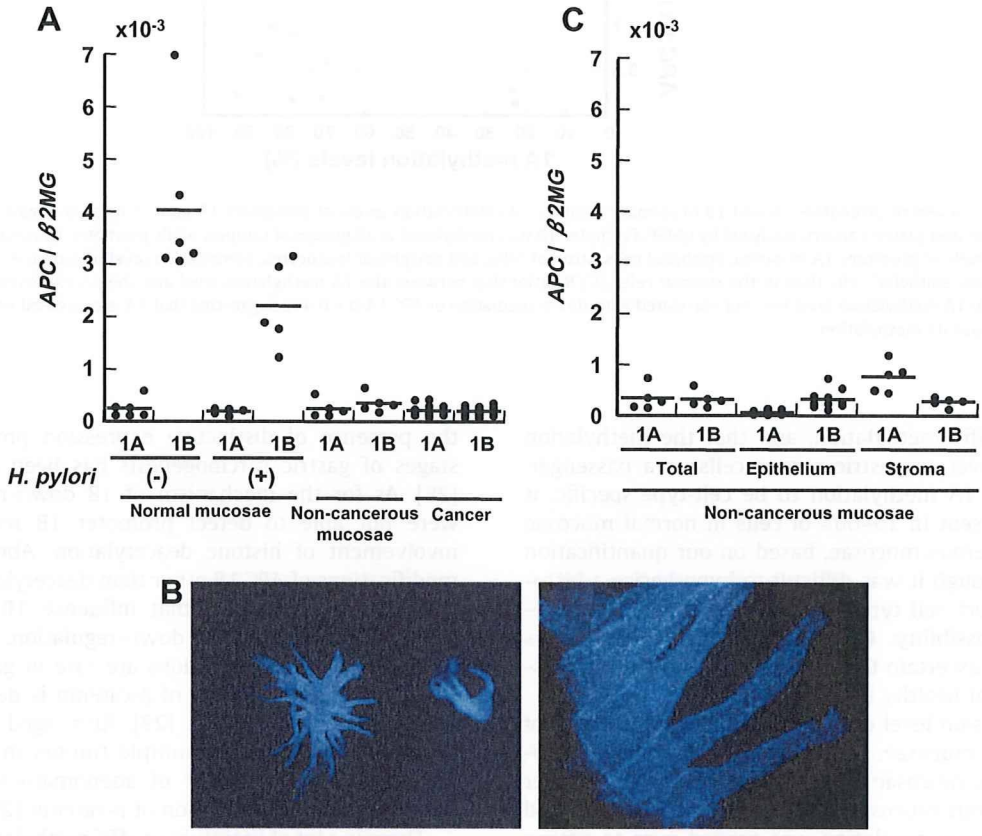

Fig. 3. Expression levels of *APC* 1A and 1B in primary samples. (A) Expression levels of 1A and 1B in normal gastric mucosae, non-cancerous mucosae, and gastric cancers. In *H. pylori*-negative gastric mucosae of healthy volunteers, the average 1B expression level was 15-fold higher than that of 1A. The 1B expression level was down-regulated in *H. pylori*-positive gastric mucosae, and further in non-cancerous gastric mucosae. (B) DAPI staining of isolated gastric glands. (C) Expression levels of 1A and 1B in gastric epithelial and stromal cells obtained from non-cancerous gastric mucosae of cancer patients. 1B showed similar expression levels between the isolated gastric epithelial cells and the remaining stromal cells.

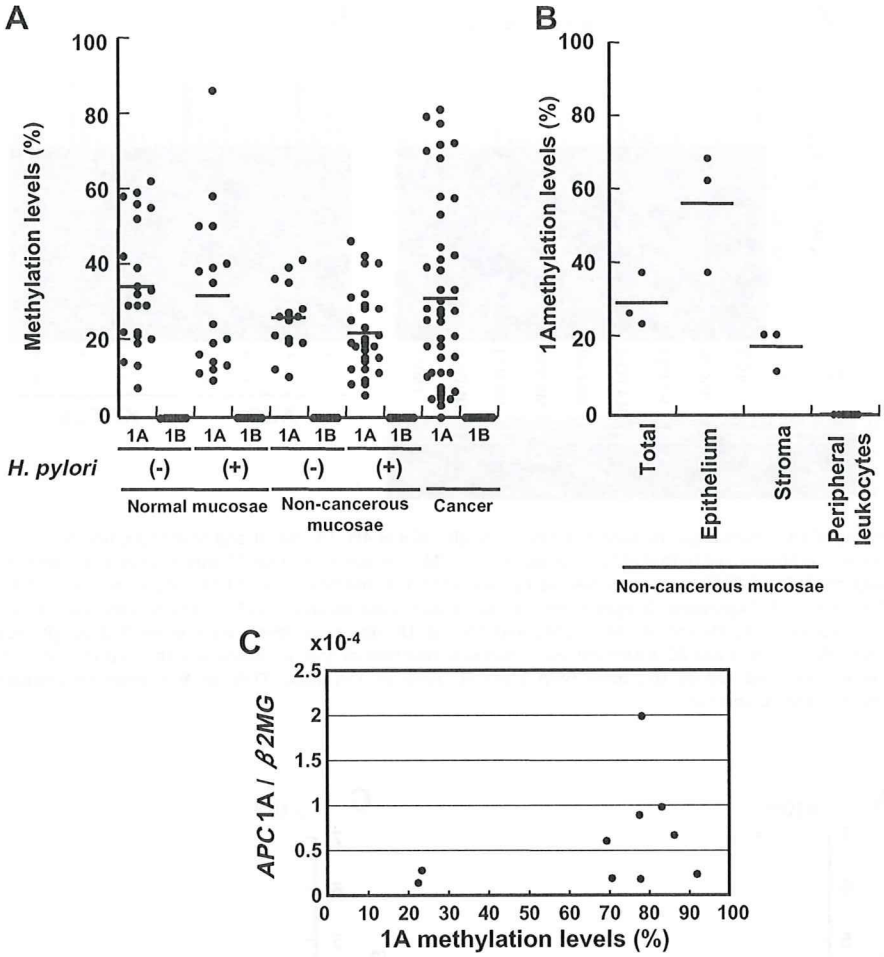

Fig. 4. Methylation levels of promoters 1A and 1B in primary samples. (A) Methylation levels of promoters 1A and 1B in normal gastric mucosae, non-cancerous mucosae, and gastric cancers, analyzed by qMSP. Promoter 1A was methylated in all groups of samples while promoter 1B was never methylated. (B) Methylation levels of promoter 1A in gastric epithelial cells, stromal cells, and peripheral leukocytes. Methylation level of promoter 1A was relatively higher in the gastric epithelial cells than in the stromal cells. (C) Relationship between the 1A methylation level and the 1A expression level in gastric epithelial cells. The 1A methylation level was not correlated with down-regulation of *APC* 1A ($r = 0.44$), suggesting that 1A is expressed only at a trace level even in cells without its methylation.

cell-type-specific methylation, and that the methylation was carried over to gastric cancer cells as a passenger. For promoter 1A methylation to be cell-type-specific, it should be present in 15–60% of cells in normal mucosae and non-cancerous mucosae, based on our quantification (Fig. 4A). Although it was difficult to hypothesize a histologically distinct cell type with this population, there remains the possibility. Quantitative expression analysis enabled us to ascertain that *APC* 1B was dominant in gastric mucosae of healthy individuals.

The expression level of *APC* 1B, the major transcript of *APC* in gastric mucosae, was down-regulated in *H. pylori*-positive gastric mucosae of healthy volunteers and further in non-cancerous mucosae of gastric cancer patients, and the marked down-regulation was carried over to gastric cancers. This suggested that down-regulation of *APC* and activation of the WNT/β-catenin pathway itself could be involved in early stages of human gastric carcinogenesis. In addition to the presence of epigenetic alterations [11], the presence of distinctive expression profiles in early stages of gastric carcinogenesis has been demonstrated [28]. As for the mechanism of 1B down-regulation, we were not able to detect promoter 1B methylation, or involvement of histone deacetylation. Aberrant histone modifications of *APC* 1B other than deacetylation or epigenetic changes of genes that influence 1B transcription could be involved in the down-regulation. It is reported that, although *APC* mutations are rare in gastric cancers, the nuclear accumulation of β-catenin is detected in 39% of human gastric cancers [29]. Also, aged $APC^{Min/+}$ mice spontaneously develop multiple tumors in the stomach, and such tumors consist of adenomatous glands with strong nuclear accumulation of β-catenin [29].

There is a lot of literature on *APC* methylation in cancers of tissues other than the stomach [30–34]. However, at least in some of these tissues, it remains to be clarified which of 1A and 1B is the major transcript. Since genes with low transcription are susceptible to DNA methylation

[2,4,6], the meaning of methylation should be carefully established. To establish that methylation of one of the two *APC* promoters is the driver of carcinogenesis, evidence of low or no expression from the other promoter is necessary.

In summary, the *APC* 1B expression level was significantly and much higher than the *APC* 1A expression level in human normal gastric mucosae. Therefore, methylation of the *APC* promoter 1A is likely to be a passenger in human gastric carcinogenesis.

Conflicts of interest

None declared.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2009.05.016.

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Association between frequent CpG island methylation and *HER2* amplification in human breast cancers

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The presence of frequent methylation of CpG islands (CGIs), designated as the CpG island methylator phenotype in some cancers, is associated with distinct clinicopathological characteristics, including gene amplification, in individual tumor types. Amplification of *HER2* in human breast cancers is an important prognostic and therapeutic target, but an association between *HER2* amplification and frequent CGI methylation is unknown. To clarify the association, we here quantified methylation levels of promoter CGIs of 11 genes, which are unlikely to confer growth advantage to cells, in 63 human breast cancers. The number of methylated genes in a cancer did not obey a bimodal distribution, and the 63 cancers were classified into those with frequent methylation (n = 16), moderate methylation (n = 26) and no methylation (n = 21). The incidence of *HER2* amplification was significantly higher in the cancers with frequent methylation (11 of 16) than in those with no methylation (2 of 21, P = 0.001). Also, the number of methylated genes correlated with the degree of *HER2* amplification (r = 0.411, P = 0.002). Correlation analysis with clinicopathological characteristics and methylation of *CDKN2A*, *BRCA1* and *CDH1* revealed that frequent methylation had significant correlation with higher nuclear grades (P = 0.001). These showed that frequent methylation had a strong association with *HER2* amplification in breast cancers and suggested that frequent methylation can be a determinant of various characteristics in a fraction of human breast cancers.

Introduction

Aberrant DNA methylation is deeply involved in the development and progression of human cancers (1–4). Methylation of CpG islands (CGIs) in promoter regions is a major mechanism for inactivation of tumor suppressor genes. At the same time, maintenance of appropriate DNA methylation levels is known to be important for maintenance of genome integrity. DNA hypomethylation can lead to genomic instability and increased tumor incidence in mice (5,6) and is associated with loss of heterozygosity in human cancers (7,8). On the other hand, aberrant DNA methylation precedes loss of heterozygosity in human liver cancers (9).

The presence of frequent methylation of CGIs in a cancer was first described in colorectal cancers and designated as the CpG island methylator phenotype (CIMP) (10). Depending upon tumor tissue types, the presence of frequent CGIs methylation, or CIMP, can be clearly observed and is associated with distinct clinicopathological features. For example, by careful selection of marker genes and their quantitative methylation analysis, CIMP in colorectal cancers was shown to be strongly associated with *BRAF* mutations (11). In neuroblastomas, both in Japan and Germany, CIMP was observed as a distinct entity associated with poor prognosis and *MYCN* amplification (12,13). Remarkably, all the cases with *MYCN* amplification had frequent methylation, with only one exception. Cases with CIMP but without *MYCN* amplification had a better prognosis than those that had both and a worse prognosis than those that had neither. This complete containment of tumors with *MYCN* amplification within CIMP-positive tumors suggested that CIMP could precede gene amplification or that at least the presence of frequent aberrant DNA methylation was associated with gene amplification.

Gene amplification of *HER2*, which is a member of the epidermal growth factor receptor family (14), is very important in human breast cancers. Initially, *HER2* amplification was found to be present in 15–30% of newly diagnosed breast cancer cases and to be associated with increased metastatic potential and decreased overall survival (15). Suppression of *HER2* activity was shown to have antitumor activity, and antibodies against HER2 were developed as a therapeutic agent against breast cancers. Now, it is well known that a humanized antibody against HER2, such as trastuzumab, is very effective against breast cancers with *HER2* amplification (16,17). Nevertheless, inducers of *HER2* amplification remain unknown.

In this study, we aimed to clarify whether or not the presence of frequent CGI methylation was associated with *HER2* amplification in human breast cancers. For this end, from the genes silenced in human cancers (18,19), we selected genes whose silencing is unlikely to confer growth advantage and avoided selection bias of cells with methylation. Also, we performed quantitative methylation analysis of their putative nucleosome-depleted regions (20), which are most resistant to DNA methylation (21). Association between frequent CGI methylation and clinicopathological characteristics, including silencing of three tumor-suppressor genes (*CDKN2A*, *BRCA1* and *CDH1*), was also analyzed.

Materials and methods

Patients and tissue samples

Sixty-three breast cancer tissue specimens were obtained from patients who underwent mastectomy or breast-conserving surgery (stage I 22 cases; stage II 26 cases; stage III 15 cases and stage IV 0 case). Informed consent was obtained from all the patients, and analysis was approved by the institutional review boards. Cancer tissues were frozen after resection and stored at −80°C until extraction of genomic DNA. High-molecular weight DNA was extracted by the phenol–chloroform method. Histological types were evaluated according to the criteria of the Japanese Breast Cancer Society (22).

Bisulfite modification and quantitative methylation-specific polymerase chain reaction

Completely methylated DNA and completely unmethylated DNA were prepared by methylating genomic DNA with *Sss*I methylase (New England Biolabs, Beverly, MA) and amplifying genomic DNA with the GenomiPhi amplification system (GE Healthcare, Buckinghamshire, UK), respectively. Bisulfite modification was performed using 1 μg of *Bam*HI-digested genomic DNA as described previously (23). The modified DNA was suspended in 40 μl of Tris-EDTA buffer, and an aliquot of 1 μl was used for polymerase chain reaction (PCR) with a primer set specific to methylated or unmethylated sequences (supplementary Table 1 is available at *Carcinogenesis* Online). Using the completely methylated DNA and completely unmethylated DNA, an annealing temperature specific for each primer set was determined. Real-time PCR was performed using SYBR® Green I (BioWhittaker Molecular Applications, Rockland, ME) and an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The number of DNA molecules with methylated sequences and

Abbreviations: CGI, CpG island; CIMP, CpG island methylator phenotype; ESR, estrogen receptor; PCR, polymerase chain reaction; PGR, progesterone receptor.

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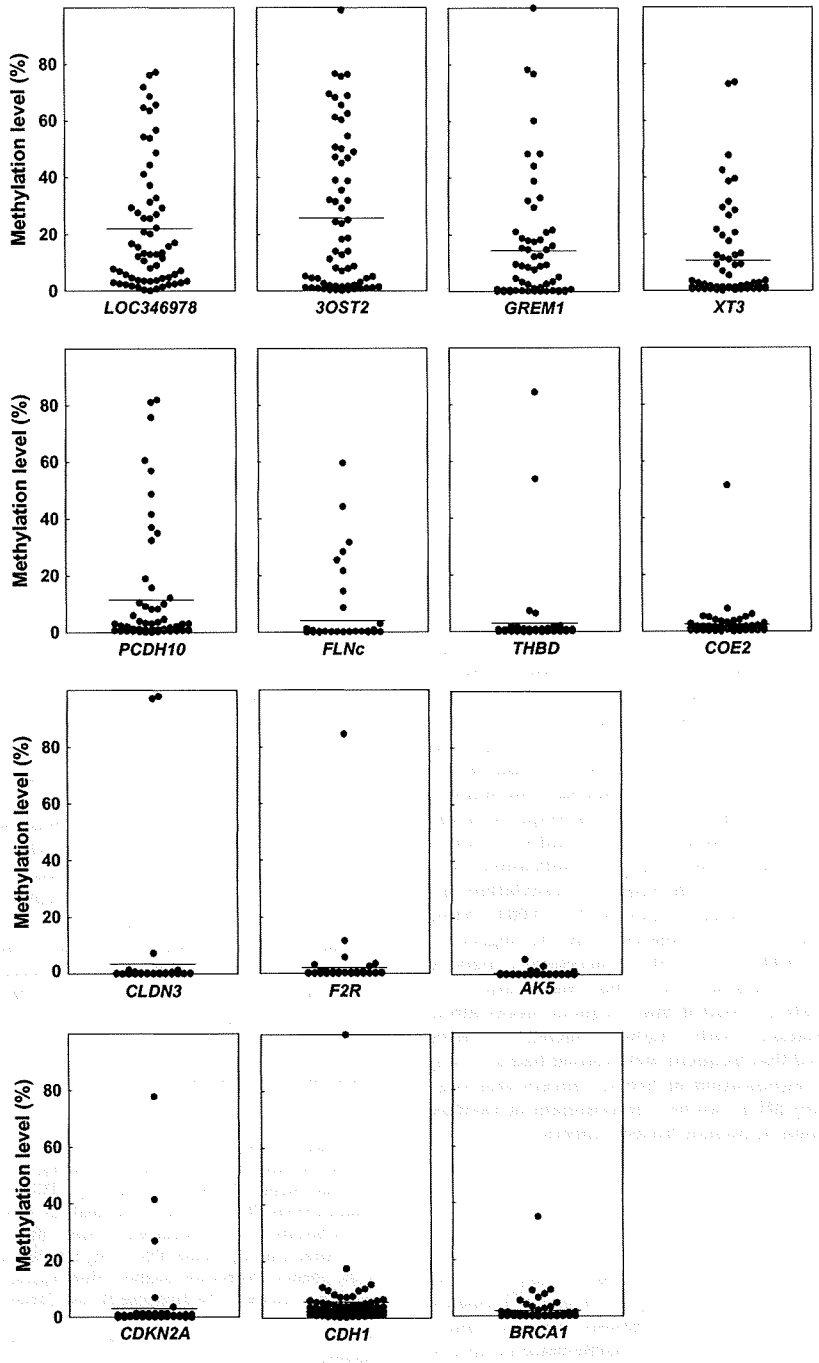

Fig. 1. Methylation levels in the 63 breast cancer samples. The numbers of DNA molecules methylated and unmethylated in promoter CGIs were obtained by quantitative methylation-specific PCR, and a methylation level was calculated as a fraction of methylated DNA molecules among the total DNA molecules. Some cancers had no methylation and the others had various levels of methylation. The methylation level in cancers was considered to represent the fraction of cancer cells in a sample and less occasionally the fraction of cells with methylation among cancer cells. We confirmed that we detected dense methylation of promoter CGIs by sequencing the quantitative methylation-specific PCR products obtained using primers specific to methylated DNA molecules (suppplementary Figure 3 is available at *Carcinogenesis* Online).

that with unmethylated sequences in a test sample were measured by comparing its amplification with those of standard samples that contained 10–10^6 DNA molecules. The standard samples were prepared by cloning PCR products of methylated and unmethylated sequences into the pGEM-T Easy vector (Promega, Madison, WI) or by purifying their PCR products using the Wizard SV Gel and PCR clean-up system (Promega). The 'methylation level' was calculated as the fraction of methylated DNA molecules among the total DNA molecules.

Fluorescence in situ hybridization analysis of the HER2 amplification

Fluorescence *in situ* hybridization was performed using a PathVysion kit (Abbot Molecular, Des Plaines, IL) with our modification (24). The *HER2* locus

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and centromere of chromosome 17 (CEP17) were labeled by SpectrumOrange and SpectrumGreen fluorescence, respectively, and nuclei were counterstained with 4′, 6-diamidino-2-phenylindole. *HER2* and CEP17 signals were counted in 60 nuclei under a fluorescence microscope. Cancers with *HER2*:CEP17 ratio ≥2 were determined as *HER2* amplification positive.

Analysis of 3OST2 expression on cell growth

MCF7 Tet-Off cell line was purchased from Clontech Laboratories (Mountain View, CA). Full-length *3OST2* complementary DNA, cloned from human mammary epithelial cells, was inserted into the multiple cloning site of pTRE2hyg vector (Clontech Laboratories). The MCF7 Tet-Off cell line was transfected with the vector, and a stable clone was obtained by selection using hygromycin. Growth curves were analyzed by counting the cell numbers for the parental cell line, stable clones transfected with *3OST2*-expressing vector and with empty vector (without doxycycline). Overexpression of *3OST2* complementary DNA was confirmed by real-time reverse transcription–PCR analysis.

Sequencing analysis of quantitative methylation-specific PCR products

Quantitative methylation-specific PCR products of seven genes, *3OST2*, *FLNc*, *GREM1*, *THBD*, *PCDH10*, *XT3* and *LOC346978*, were cloned into pGEM-T Easy Vector (Promega). For each sample, ~10 clones were cycle sequenced using T7 primer, 5′-TAATACGACTCACTATAGGG-3′ and an Applied Biosystems 310 sequencer (Applied Biosystems, Foster City, CA).

Statistical analysis

Increasing or decreasing trends in no methylation, moderate methylation and then frequent methylation cancers were analyzed by the Mantel–Haenszel chi-square test. Differences between the frequent methylation *HER2*-positive cancers and moderate methylation (or no methylation) *HER2*-positive cancers were analyzed by the chi-square test. Correlation between the degree of *HER2* amplification and the number of methylated genes was analyzed using Pearson's correlation coefficient. All the analyses were performed using SPSS (SPSS, Chicago, IL).

Results

Quantitative methylation analysis of breast cancers

From 20 and 14 genes that were methylated in human breast and gastric cancers, respectively (18,19), we selected 11 genes (*LOC346978*, *3OST2*, *GREM1*, *XT3*, *PCDH10*, *FLNc*, *THBD*, *COE2*, *CLDN3*, *F2R* and *AK5*) and quantified their methylation levels in 63 breast cancers. These genes, except for *3OST2* and *CLDN3*, were not expressed in normal human mammary epithelial cells (18,19,25,26), and their silencing was unlikely to confer growth advantage to cells. Also, introduction of *3OST2* complementary DNA into MCF7 cells did not cause growth suppression (supplementary Figure 1 is available at *Carcinogenesis* Online), and its silencing was unlikely to confer growth advantage. Therefore, the majority of the 11 genes were considered to be suitable to detect the presence of a cellular environment that tends to induce methylation of promoter CGIs. We also analyzed methylation of three tumor suppressor genes (*CDKN2A*, *BRCA1* and *CDH1*) for clinicopathological analysis.

Quantitative methylation analyses of the 14 genes showed that some cancers had no methylation and the others had various levels

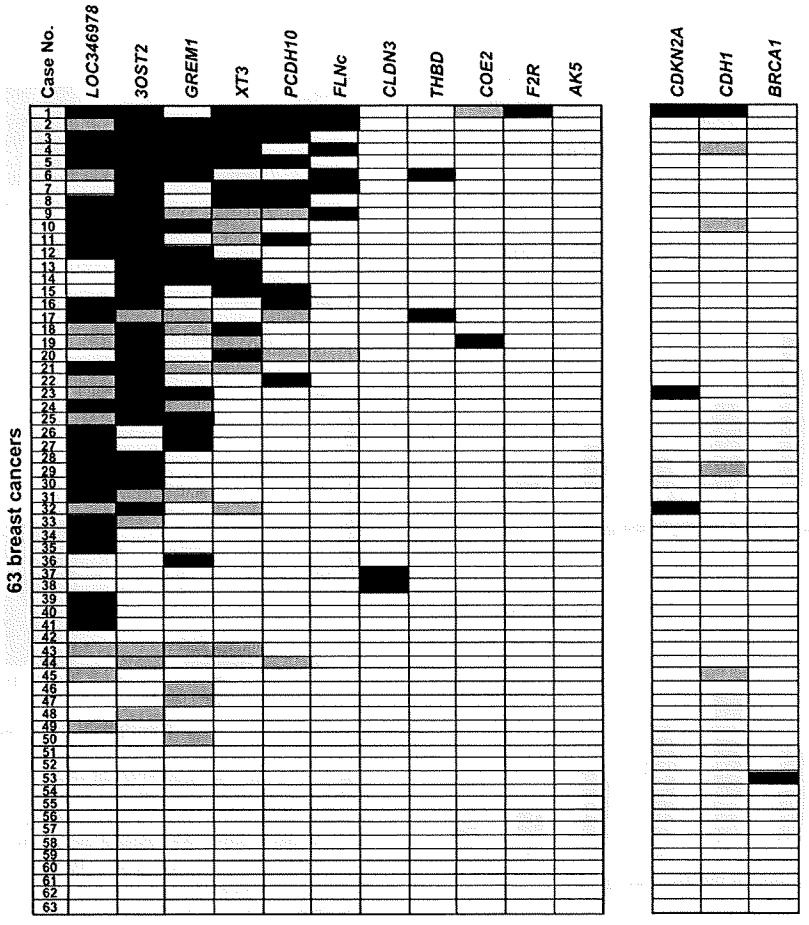

Fig. 2. Methylation profile of the 11 marker genes and three tumor suppressor genes in 63 breast cancers. Methylation in each sample was scored as positive or negative using two different cutoff values, and the 63 samples were aligned by the number of methylated CGIs. Methylation-positive samples using 10 and 20% as cutoff values are shown by gray and black boxes, respectively.

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of methylation (Figure 1). Such distribution of methylation levels was typically observed for *FLNc*, *THBD*, *CLDN3*, *F2R* and *CDKN2A*. The presence of such distribution confirmed previous findings that cancer samples could essentially be classified into two groups: cancers with methylation of a specific gene and those without (11,19,27). Counting cancer cells in the tissue section samples showed that two samples with least cancer cells contained cancer cells with fractions of 19.8 ± 5.2% and 22.9 ± 0.3% (mean ± SD). Based on these data, we adopted two cutoff values 10 and 20% to score each cancer sample as positive or negative. When overall distribution of methylation was examined, similar patterns of cancers with methylation were observed using the two cutoff values (Figure 2). Using either value, the number of methylated genes in a cancer did not obey bimodal distribution and looked quite similar (Figure 3). Therefore, we adopted a cutoff value of 20% to score individual cancers as positive or negative for methylation.

Then, the 63 cancers were classified by the frequency of CGI methylation. To avoid biases due to a cutoff number of methylated genes, we classified the cancers into three groups, those with no methylation, moderate methylation and frequent methylation, using two different cutoff numbers for frequent methylation. Using a cutoff number of three methylated genes or more, 16, 26 and 21 cases were classified into cancers with frequent methylation, moderate methylation and no methylation, respectively. Using a cutoff number of four methylated genes or more, 8, 34 and 21 cases were classified into those with frequent methylation, moderate methylation and no methylation, respectively.

Association between frequent CGI methylation and the HER2 amplification

The presence of *HER2* amplification was analyzed by fluorescence *in situ* hybridization, and 24 of 63 (38%) cancers had *HER2* amplification (supplementary Figure 2 is available at *Carcinogenesis* Online). The extent of amplification ranged from 2.0- to 16.8-fold. Using a cutoff number of three for frequent methylation, the fractions of cancers with *HER2* amplification were 11/16, 11/26 and 2/21 in cancers with frequent methylation, moderate methylation and no methylation, respectively (Figure 4A). Using a cutoff number of four, it was 6/8, 16/34 and 2/21, respectively (Figure 4B).

When correlation between the degree of CGI methylation and fraction of cancers with *HER2* amplification was examined by trend analysis, a highly significant increasing trend was observed from cancers with no methylation, to those with moderate methylation and then to those with frequent methylation ($P < 0.001$ for both of cutoff numbers). When cancers with frequent methylation and those with no methylation were compared, the former had a significantly higher fraction ($P = 0.003$ and 0.001 for cutoff numbers of four and three, respectively). Also, the degree of *HER2* amplification showed a correlation with the number of methylated genes (correlation coefficient = 0.411, $P = 0.002$) (Figure 5 and supplementary Table 2 is available at *Carcinogenesis* Online). This demonstrated that frequent CGI methylation had an association with *HER2* amplification.

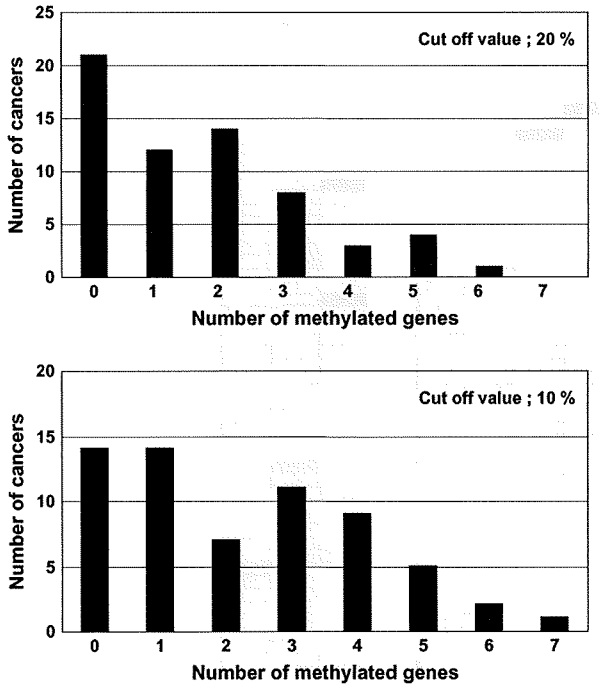

Fig. 3. Distribution of the number of methylated genes in a cancer. Similar distribution patterns were observed using two different cutoff values. The distribution was not bimodal, and involvement of multiple mechanisms in frequent methylation in breast cancers was suggested.

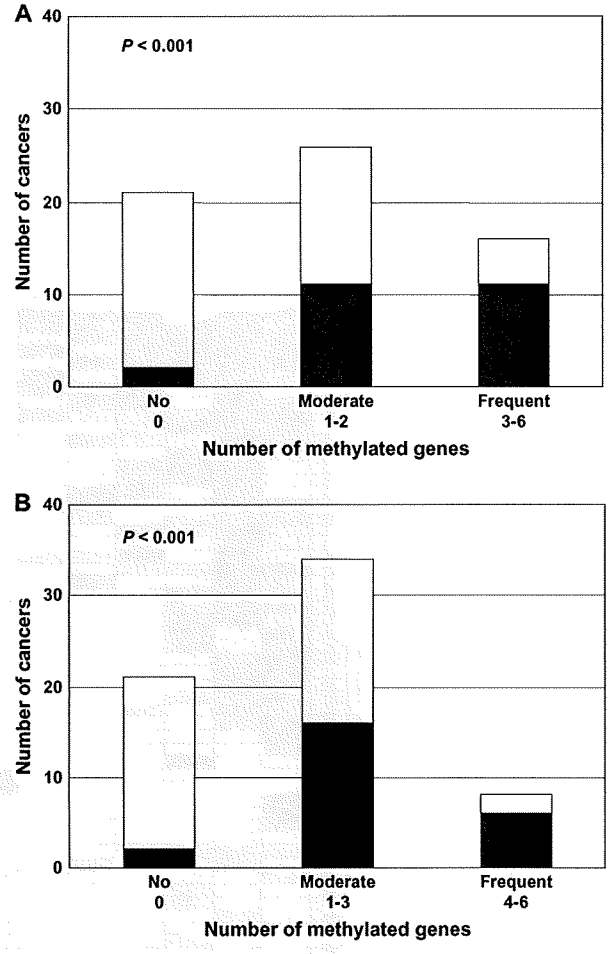

Fig. 4. The correlation between the degree of frequent methylation and *HER2* amplification. Two different cutoff numbers were used to define frequent methylation. These analyses adopted a cutoff value of 20% for methylation-positive. (**A**) Frequent methylation was defined as cancers with methylation of three or more genes. (**B**) Frequent methylation was defined as cancers with methylation of four or more genes. Whichever cutoff number was used, a clear increasing trend of *HER2*-positive cancers in no methylation, moderate methylation and then frequent methylation groups was observed ($P < 0.001$ for both of the two cutoff numbers). Closed and open boxes represent cancers with and without *HER2* amplification, respectively.

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Association between frequent CGI methylation and other clinicopathological features, including methylation of tumor suppressor genes

The correlation between frequent CGI methylation and methylation of three tumor suppressor genes, *CDKN2A*, *CDH1* and *BRCA1*, was analyzed (Table I). However, none of the three genes showed any correlation (P = 0.557, 0.157 and 0.232, respectively). Regarding other clinicopathological characteristics, the degree of frequent CGI methylation correlated with higher nuclear grades (P = 0.001). The degree of frequent CGI methylation tended to show correlations with advanced pathological stage (P = 0.068) and post-menopausal status (P = 0.044). However, no association was observed with lymph node metastasis and negative expression of estrogen receptor (ESR) or progesterone receptor (PGR).

Discussion

The present study demonstrated for the first time that frequent CGI methylation in breast cancers had a highly significant association with *HER2* amplification. Regarding DNA methylation and HER2 overexpression, Fiegl *et al.* (28) previously found that methylation levels of four genes (*CDH13*, *PGR*, *HSD17B4* and *MYOD1*) and one gene (*BRCA1*), which were selected from 35 genes, correlated with HER2 expression positively and inversely, respectively (P = 0.01–0.04). Methylation levels of individual genes in cancers are affected by the content of cancer cells, and, also, the correlation observed in the study was considered to be due to interaction between a function of an individual gene and HER2 overexpression. In contrast, here, we focused on the abnormality in epigenetic regulation in cancers. To estimate its degree, we used marker genes that were unlikely to confer growth advantages even if methylated, scored their methylation as positive or negative and integrated the information from the 11 marker genes into the frequency of methylation in a cancer sample. The cancers were classified into three groups, namely those with frequent methylation, moderate methylation and no methylation. As a result, a very strong association between frequent methylation and *HER2* amplification ($P < 0.001$) was demonstrated. Also, the degree of frequent methylation showed a clear correlation with the degree of *HER2* amplification. *BRCA1* methylation did not correlate with the degree of frequent methylation or *HER2* amplification (P = 0.806).

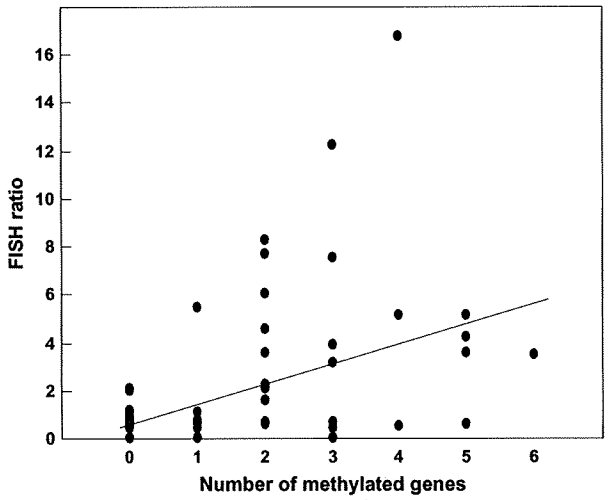

Fig. 5. The correlation between the number of methylated genes and degree of *HER2* amplification. The degree of *HER2* amplification showed a strong correlation with the number of methylated genes (correlation coefficient = 0.411, P = 0.002).

Table I. Association between frequent CGI methylation and clinicopathological features, including methylation of tumor suppressor genes

	No methylation	Moderate methylation	Frequent methylation	*P* value
CDKN2A methylation (+/−)	2/19	0/26	1/15	0.557
BRCA1 methylation (+/−)	1/20	0/26	0/16	0.232
CDH1 methylation (+/−)	0/21	0/26	1/15	0.157
Menopausal (pre/post)	12/9	9/17	4/12	0.044
Stage (I/II/III)	9/11/1	9/8/9	4/7/5	0.068
Lymph node metastasis (positive/negative)	6/15	13/13	7/9	0.308
ESR (positive/negative)	15/6	14/12	11/5	0.779
PGR (positive/negative)	17/4	14/12	9/7	0.100
Nuclear grade (1/2/3)	4/10/7	2/7/17	0/2/14	0.001

Frequent methylation was defined as breast cancers with methylation of three or more genes. Increasing or decreasing trends were tested by Mantel–Haenszel chi-square.

The association between frequent methylation and *HER2* amplification has clinical implications. It is known that *HER2* amplification status can show a discrepancy between primary and metastatic sites in a small fraction of patients (29). There is a possibility that HER2-negative breast cancers at initial diagnosis change into HER2 positive at their recurrence and that the presence of frequent methylation at the initial diagnosis can be used to predict such cases. Since accurate detection of HER2-positive cancers is very important to implement appropriate treatment, including trastuzumab (17), future studies to predict the *HER2* amplification status using frequent methylation and to clarify the mechanism of the association are warranted. Also, the effect of frequent methylation on long-term survival is important. So far, only 5 of 63 cases suffered from recurrence (one frequent methylation, three moderate methylation and one no methylation cases), and the effect cannot be statistically analyzed. Since the association between *HER2* amplification and poor survival (without trastuzumab) is well established, the effect of frequent methylation on long-term survival seems worth being analyzed in the future.

Some breast cancers with *HER2* amplification belonged to the moderate methylation or no methylation groups although the majority of cancers with *HER2* amplification belonged to the frequent methylation group. This was in contrast with the case of neuroblastomas, where all the neuroblastomas with *MYCN* amplification had frequent methylation, CIMP, with only one exception (12,13). Therefore, the relationship between frequent methylation and *HER2* amplification in breast cancers seems more complex than the relationship between CIMP and *MYCN* amplification in neuroblastomas. Not only frequent methylation could lead to *HER2* amplification through chromosomal instability (9), which was our initial expectation, but also *HER2* amplification could lead to frequent methylation or they might have common inducers.

The degree of frequent methylation also correlated with higher nuclear grades. It also tended to show association with advanced stages and post-menopausal status. It has been reported that *CDH1* methylation was associated with negative ESR and PGR expressions (P = 0.06 and 0.09, respectively) and that frequent methylation of seven tumor suppressor genes was associated with poor differentiation (30). It has also been reported that PGR expression was negatively associated with *ESR1*, *TGFBR2*, *PPTGS2* and *CDH13* methylation (P = 0.01–0.04) (31) and that ESR and PGR expressions were positively and negatively associated with *HIN-1/RASSF1A* and *RIL/CDH13* methylation, respectively (32). Taken together, the frequent methylation in breast cancers was weakly associated with advanced stages, negative PGR and ESR expressions and poor differentiation (higher nuclear grades). Nevertheless, the correlation between frequent methylation and *HER2* amplification was much stronger than these associations in our study. It was considered that quantitative analysis of marker genes was advantageous to clarify the strong association.

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The cutoff value of methylation levels to score cancer samples as positive or negative for methylation was determined based upon the fraction of cancer cells in two samples with their smallest contents (20%). To count methylation in a fraction of cancer cells, we also tested a cutoff value, 10%, but quite similar results were obtained (Figure 3). Regarding the cutoff number for frequent methylation, we tried three and four but observed a highly significant association using both numbers (Figure 4). This excluded a possibility that a false-positive association between frequent methylation and *HER2* amplification was observed due to arbitrary cutoff values or numbers. Also, we confirmed that we detected dense methylation of promoter CGIs by our quantitative methylation-specific PCR analysis by sequencing the PCR products. Almost all the CpG sites in the products were densely methylated (supplementary Figure 3 is available at *Carcinogenesis* Online). Finally, we confirmed that the methylation detected in cancer tissues originated from cancer cells. Methylation levels of nine genes that showed high methylation levels ($\geq$10%) in some cancer samples were measured in 11 pairs of non-cancerous breast and cancer tissues (supplementary Figure 4 is available at *Carcinogenesis* Online). The methylation levels of all the genes were elevated only in cancer tissues, and the methylation we detected was considered to originate from cancer cells.

In summary, frequent methylation in breast cancers had a strong association with *HER2* amplification.

Supplementary material

Supplementary Tables 1 and 2 and Figures 1–4 can be found at http://carcin.oxfordjournals.org/

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Development of a Novel Output Value for Quantitative Assessment in Methylated DNA Immunoprecipitation-CpG Island Microarray Analysis

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Abstract

In DNA methylation microarray analysis, quantitative assessment of intermediate methylation levels in samples with various global methylation levels is still difficult. Here, specifically for methylated DNA immunoprecipitation-CpG island (CGI) microarray analysis, we developed a new output value. The signal log ratio reflected the global methylation levels, but had only moderate linear correlation ($r = 0.72$) with the fraction of DNA molecules immunoprecipitated. By multiplying the signal log ratio using a coefficient obtained from the probability value that took account of signals in neighbouring probes, its linearity was markedly improved ($r = 0.94$). The new output value, Me value, reflected the global methylation level, had a strong correlation also with the fraction of methylated CpG sites obtained by bisulphite sequencing ($r = 0.88$), and had an accuracy of 71.8 and 83.8% in detecting completely methylated and unmethylated CGIs. Analysis of gastric cancer cell lines using the Me value showed that methylation of CGIs in promoters and gene bodies was associated with low and high, respectively, gene expression. The degree of demethylation of promoter CGIs after 5-aza-2′-deoxycytidine treatment had no association with that of induction of gene expression. The Me value was considered to be useful for analysis of intermediate methylation levels of CGIs.

Key words: epigenetics; CpG island microarray; 5-aza-2′-deoxycytidine; methylation silencing; gastric cancer

1. Introduction

DNA methylation plays a critical role during mammalian development and differentiation. Methylation of a CpG island (CGI) in a gene promoter region has been known to repress transcription of its downstream gene.[1] At the same time, DNA methylation statuses of CGIs are faithfully inherited upon cell replication,[2] and are considered to work as a stable switch of gene transcription.[1] Once a promoter CGI is aberrantly methylated, it leads to permanent aberrant silencing of its downstream gene. Aberrant DNA methylation is deeply involved in human cancers,[3] and is also likely to be involved in other human-acquired disorders.[4]

There is a great interest in genome-wide analysis of DNA methylation, and new technologies involving microarrays and next-generation sequencers are being developed,[5] replacing traditional techniques.[6] In comparison with techniques using next-generation sequencers, microarray techniques are cost-effective and do not need complex bioinformatics. Their hybridization probes can be prepared by bisulphite modification of unmethylated cytosines, use of methylation-sensitive restriction enzymes, and affinity purification. The affinity purification can be performed by an antibody against 5-methylcytidine or by methylated DNA binding domains (MBDs). It has an advantage over the use of restriction enzymes, since genomic regions analysed by affinity purification

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are not limited to restriction sites of methylation-sensitive enzymes.

Methylated DNA immunoprecipitation (MeDIP)-microarray analysis has been used to obtain a high-resolution whole-genome DNA methylation profile of various genomes.[7–17] However, quantitative assessment of intermediate methylation levels has been hampered by the difficulty in appropriate normalization. Methylation levels have a unique distribution pattern that is essentially different from gene transcription and is likely to be bimodal.[17,18] Also, global methylation levels in different samples are highly variable, and there are few reference genes that have consistent methylation levels across various samples. To overcome these issues, two methods (Batman and MEDME) were recently developed.[14,16]

Batman (Bayesian tool for methylation analysis) transforms a signal log ratio of an individual probe to a value of methylation level taking account of the methylation levels of nearby CpG sites using standard Bayesian techniques. It is capable of processing data obtained by microarray and by next-generation sequencers. The method was validated by bisulphite sequencing of sperm samples,[14] and its validity in samples with different global methylation levels remains to be established. MEDME (modelling experimental data with MeDIP enrichment) weighs signal log ratios of individual probes using a logistic model and signals obtained by neighbouring probes and by using completely methylated DNA samples.[16] Both Batman and MEDME had good correlation with methylation levels obtained by bisulphite sequencing ($R^2 = 0.82$ and 0.75, respectively). Also, both are capable of processing data from both CpG-rich and -poor regions, and this made their conversion algorithm complex as a trade-off.

In this study, we developed a novel output value, the 'Me value', that can be calculated from raw output values, and confirmed that the value had a linear correlation with methylation levels of genomic regions using samples with various global methylation levels. The Me value was used to clarify how methylation of CGIs in various positions against transcription start sites (TSSs) is associated with gene expression, and how demethylation of CGIs is associated with re-expression of genes.

2. Materials and methods

2.1. Cell lines and tissue samples

AGS and KATOIII gastric cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and the Japanese Collection of Research Bioresources (Tokyo, Japan), respectively. HSC39 and HSC57 gastric cancer cell lines were gifted by Dr K. Yanagihara, National Cancer Center Research Institute, Tokyo, Japan. Treatment with a demethylating agent, 5-aza-2'-deoxycytidine (5-aza-dC, Sigma, St Louis, MO, USA), was performed as in our previous study (AGS, 3 days, 1 μM).[19] A normal gastric tissue sample was prepared by pooling endoscopic biopsy specimens from three healthy volunteers with informed consents. High-molecular-weight DNA was extracted by the phenol/chloroform method with RNase A treatment.

2.2. MeDIP and quantification of the number of immunoprecipitated DNA molecules

Five micrograms of genomic DNA were sonicated by a VP-5s homogenizer (TAITEC, Saitama, Japan) to fragment lengths between 200 and 800 bp. The mode of fragment length was about 300 bp. After heat denaturation at 95°C for 10 min, DNA was incubated with 5 μg antibody against 5-methylcytidine (Diagnode, Liége, Belgium) in 1× IP buffer [10 mM Na-phosphate, pH 7.0, 140 mM NaCl, 0.05% (w/v) Triton X-100] at 4°C overnight. Immune complexes were collected with Dynabeads Protein A (Invitrogen Dynal AS, Oslo, Norway), washed with 1× IP buffer four times, treated with Proteinase K, and purified by phenol and chloroform extraction and isopropanol precipitation.

To assess the fraction of immunoprecipitated (IP) DNA molecules among that of the total DNA (whole cell extract DNA, WCE) molecules, the number of IP and WCE molecules was quantified by real-time PCR using SYBR® Green I (BioWhittaker Molecular Applications, Rockland, ME, USA) and an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) as described previously.[20] All primers used in this study are listed in Supplementary Table S1.

2.3. CGI microarray analysis

Methylation microarray analysis was carried out using a human CGI oligonucleotide microarray (Agilent Technologies, Santa Clara, CA, USA) that contained 237 220 probes in or within 95 bp either side of a CGI and covered 27 800 CGIs with an average probe spacing of 100 bp. IP from 4.5 μg of sonicated DNA and 1.0 μg of WCE, without any amplification, were labelled with Cy5 and Cy3, respectively, using an Agilent Genomic DNA Labeling Kit PLUS (Agilent Technologies). Labelled DNA was hybridized to the microarray at 67°C for 40 h with constant rotation (20 rpm), and then scanned with an Agilent G2565BA microarray scanner (Agilent Technologies).

From the scanned data, signal values of the IP and WCE were obtained using Feature Extraction Ver.9.1 (Agilent Technologies). These two signal values were

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normalized using background subtraction, and signal ratio (IP/WCE), signal log ratio [$\log_2$(IP/WCE)], $P[X]$, and $P[\bar{X}]$ were obtained using Agilent G4477AA ChIP Analytics 1.3 software (Agilent Technologies). The $P[X]$ and $P[\bar{X}]$ values, which are used in chromatin immunoprecipitation (ChIP)-on-chip analysis to obtain a binding call,[21–25] were defined as the probability how the X ($\bar{X}$) value deviates from Gaussian distribution of X ($\bar{X}$) values of the entire genome of a sample. Here, the X value for a probe was obtained as the difference between the IP and the WCE signals after adjusting the symmetry of its distribution. The $\bar{X}$ value for a probe was calculated as an average X, taking account of signals for neighbouring probes (within 1 kb of the probe). In addition, to calculate signal log ratio in experiments specifically referred to, the two signal values were also normalized by the Median and the Lowess normalization methods. The microarray results were submitted to the GEO database (GSE15291).

2.4. *Calculation of the Me value*

The Me value of each probe (site Me value) was calculated as Me value = [signal log ratio $\times$ (1 − $P[\bar{X}]$) − k]/I + 0.5. The $P[\bar{X}]$ value and signal log ratio normalized using background subtraction were used for this formula. The [signal log ratio $\times$ (1 − $P[\bar{X}]$)] value mostly ranged from 0 to 2.6 in this study, and in general, the distribution depends on the microarray platform. Accordingly, the constant I was fixed at 2.6 in this study, so that the Me value would be within a range between 0 and 1. Me values larger than 1 and those smaller than 0, which were occasionally produced after calculation, were corrected to 1 and 0, respectively. The constant k was calculated as [the signal log ratio of CGIs that had a 50% fraction of DNA molecules IP (1.7 in this study) − 0.4], which equalled to 1.3 in this study. The signal log ratio of CGIs with 50% methylation depends on the microarray platform, labelling method, and mixture rate of IP and WCE, but does not need to be changed once established to suit a protocol.

The Me value was calculated only for probes with high reliability. To select such probes, first, probes that yielded extremely high signal intensities (5-fold higher than average) for the WCE (Cy-3) were excluded. Since the signals obtained for the WCE should be the same theoretically for all the probes, extremely high signals were considered to be due to cross-hybridization. Then, continuity of signal log ratios of neighbouring probes was enforced. If the value of a probe was higher than those of neighbouring probes on both sides, it was corrected to their average because the value was likely to be an error. In addition, efficiency in labelling and hybridization in each microarray analysis was monitored by the signal log ratio and the fraction of DNA molecules IP by MeDIP at 10 probe loci. The data processing for the Me value was performed by Excel 2007 (Microsoft, Redmond, WA, USA), and the templates are available upon request.

2.5. *Definitions of genomic regions*

The position of each probe against a TSS was determined using UCSC hg18 (NCBI Build 36.1, March 2006). A single CGI was defined as an assembly of probes in the CGI microarray with intervals <500 bp. CGIs were classified into four categories: upstream CGIs (within 10 kb upstream of the TSS), divergent CGIs (within 10 kb upstream of the TSSs of two genes that are transcribed in opposite directions), gene body CGIs, and downstream CGIs (within 10 kb downstream of genes). A CGI spanning both an upstream region and a gene body was split into an upstream CGI and a gene body CGI. A putative promoter region (promoter) was defined as a region between a TSS, determined by UCSC hg18 (NCBI Build 36.1, March 2006) and its 200 bp upstream. According to these definitions, 34 697 assemblies of probes were defined as CGIs, and 9624 assemblies were defined as promoters. Genes with multiple promoters were analysed as different genes because of their multiple TSSs. CGIs that could not be classified by these criteria (4164 CGIs) were omitted from the following analysis. An average number of probes that covered a single CGI (or a single promoter) was 6.8 (2.0), and the distribution of the numbers is shown in Supplementary Fig. S1.

2.6. *Expression microarray analysis*

Microarray analysis of gene expression was performed using GeneChip (Affymetrix) as described previously,[19,26] and the signal intensities were normalized, so that the average intensity of all the genes on a microarray would be 500. The average signal intensity of all the probes for a gene was used as its expression level. Genes with signal intensities of 1000 or more and of 250 or less were defined as those with high and low expression, respectively.

2.7. *Bisulphite treatment, methylation-specific PCR, and bisulphite sequencing*

Bisulphite modification was performed using *Bam*HI-digested genomic DNA as descried previously.[26] Methylation-specific PCR (MSP) was performed using bisulphite-treated DNA as descried previously.[26] Bisulphite sequencing was performed after cloning the PCR product (10 clones or more for each sample). We used the data of methylation status previously analysed.[19,26–30]

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2.8. *Determination of methylation levels of a genomic region and CGI (or promoter) using the microarray data*

A methylation level of a genomic region analysed by quantitative PCR of IP DNA molecules was assessed by an output value of a probe within the PCR product and closest to the forward primer, or a probe closest to the PCR product when no probes were present within the PCR product. A methylation level of a region analysed by bisulphite sequencing (200 bp) was assessed by an output value of a probe in the centre of the region. This was possible because bisulphite sequencing was performed for a region larger than 100 bp upstream and downstream of a probe, and methylation statuses of CpG sites within the 200 bp region were scored. A methylation level of a CGI (or promoter) was assessed by an average of site Me values of the probes located within the CGI (or promoter), which was defined as the CGI (or promoter) Me value.

3. Results and discussion

3.1. *Assessment of current output values for methylation levels*

We first examined whether or not distribution of the available output values reflected the global methylation levels. The output values analysed were: (i) the signal log ratio, which is most frequently used in microarray analysis, (ii) the $P[X]$ value, and (iii) $P[\bar{X}]$ value, which is often used in ChIP-on-chip analysis. Their distribution was analysed in two samples, a cell line (AGS) with frequent CGI methylation[26] and the same cell line after treatment with 5-aza-dC. Our previous study showed that AGS after 5-aza-dC treatment has demethylation of at least 421 promoter CGIs,[19] and its global methylation level was expected to shift towards unmethylated ranges. Among the three values, the signal log ratio showed such a shift (Fig. 1A). On the other hand, the $P[X]$ and $P[\bar{X}]$ values did not show such a shift (Fig. 1B and C).

Next, a linear correlation with methylation level, represented here by the fraction of DNA molecules IP by the anti-5-methylcytidine antibody, was examined for individual output values using 31 genomic regions located within various CGIs. In addition to the three output values, (iv) the signal ratio (background subtraction normalization), (v) the signal log ratio with Median normalization, and (vi) the signal log ratio with the Lowess normalization were analysed. Although $P[\bar{X}]$ gave a correlation coefficient of -0.91 (r, Pearson's), the absolute values of correlation coefficients using other output values were smaller than 0.72 (Supplementary Fig. S2). Median and Lowess normalization did not improve the linear correlation.

3.2. *Development of a novel output value 'Me value'*

To improve the linearity of the signal log ratio, which reflected the global methylation levels, $P[\bar{X}]$, which had a strong linear correlation, was used as a coefficient to multiply it. Because $P[\bar{X}]$ showed an inverse correlation, $(1 - P[\bar{X}])$ was used as a coefficient to multiply the signal log ratio '$=(1 - P[\bar{X}]) \times$ signal log ratio'. This value showed a higher correlation coefficient (0.93) than the other output values (Supplementary Fig. S2). This value was scaled to a value with a minimum value of 0 and a maximum value of 1 using the constants in Section 2, and the scaled output value was designated as the 'Me value'. The Me value had the largest correlation coefficient (0.94) among all of the output values analysed. The distribution of the Me value reflected the global methylation levels by showing a shift towards smaller values after demethylation (Fig. 1D).

The Me value was generated taking advantage of the signal log ratio and $P[\bar{X}]$. The signal log ratio was not quantitative but reflected the global methylation levels. On the other hand, $P[\bar{X}]$ had a high linear correlation with the fraction of DNA molecules IP by the anti-5-methylcytidine antibody. The $P[\bar{X}]$ value is obtained as a probability value that takes account of neighbouring probes, and reflects the methylation level of a small local region. Since the vast majority of CpG sites within a CGI are (un)methylated when the CGI is (un)methylated,[31] the $P[\bar{X}]$ value was considered to have an advantage in faithful reflection of the local methylation status.

3.3. *High accuracy of MeDIP-CGI microarray with Me value*

In addition to the linear correlation between the Me value and the fraction of DNA molecules IP, a linear correlation between the Me value and the fraction of methylated CpG sites was analysed. Fractions of methylated CpG sites of 11 genomic regions (each 200 bp) with a variety of methylation levels were obtained by bisulphite sequencing in four different cell lines with different global methylation levels[26] (44 values in total). The Me value was obtained for a probe (site Me value) in the centre of the genomic region analysed. A strong correlation ($r = 0.88$, Fig. 2) was observed. The correlation was stronger than any of those obtained by the other output values. When the analysis was limited to genomic regions with intermediate Me values, the correlation coefficients were 0.75 (Me value: 0.1–0.9); 0.53 (0.2–0.8); 0.47 (0.3–0.7); and 0.19 (0.4–0.6). These data further supported that a site Me value

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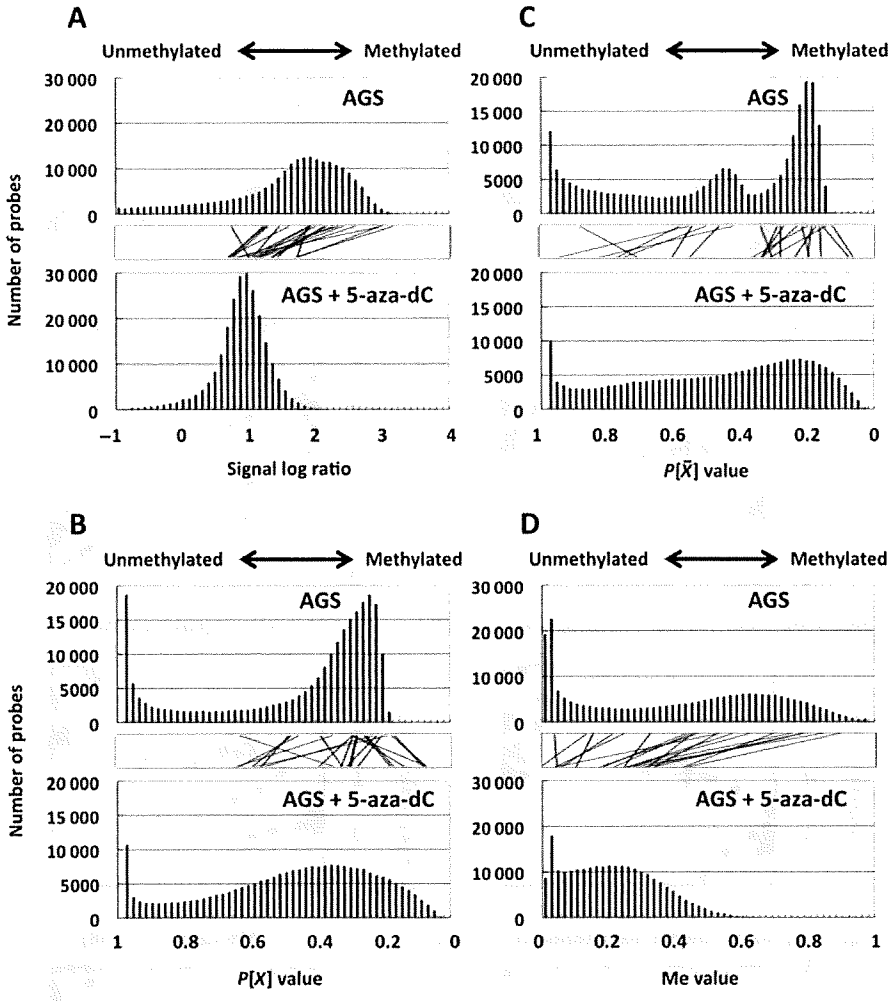

Figure 1. Distribution of methylation levels assessed by the available output values and the Me value in a gastric cancer cell line, AGS, and the same cell line after 5-aza-dC treatment. The analysis was performed for the signal log ratio with background subtraction normalization (A), the $P[X]$ value (B), the $P[\bar{X}]$ value (C), and the Me value (D). Lines between the two bar graphs indicate values of individual probes (randomly selected representative 20 values). Distributions of the signal log ratio and the Me value reflected the global methylation level, as shown by their shift towards smaller values in cells with 5-aza-dC treatment. In contrast, those of the $P[X]$ and $P[\bar{X}]$ values did not show the shift.

reflects a methylation level of a small genomic region (200 bp), even if it is within an intermediate range, and that the Me value is useful for the analysis of various biological samples, such as tissue samples.

Cancer tissues sometimes show mixtures of methylated and unmethylated CpG sites on the same DNA molecule (mosaic pattern). Even in this case, multiple CpG sites are usually located within the average size of shearing DNA (300 bp) because there are 9–53 CpG sites in 300 bp regions of promoter CGIs.[20] If two or more CpG sites are methylated, such DNA molecules are reported to be efficiently IP.[9] Therefore, it is expected that, for most CGIs, the Me value will work even in samples with mosaic pattern methylation, such as cancer tissues.

Next, detection of completely methylated and unmethylated statuses of CGIs was attempted. Using output values other than the Me value, this has been achieved by optimizing cut-off values depending upon samples because their global methylation levels were highly variable. Since the Me value well reflected the global methylation levels, we tried to use cut-off CGI Me values common to different samples. Methylation statuses of 113 CGIs in four cell lines with different global methylation levels[26] (452 values in total) were scored using various cut-off CGI Me values. A high specificity with little compromise of sensitivity was achieved with the cut-off values of 0.6 and 0.4 for highly methylated and unmethylated CGIs, respectively (Supplementary Fig. S3). Accuracies

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