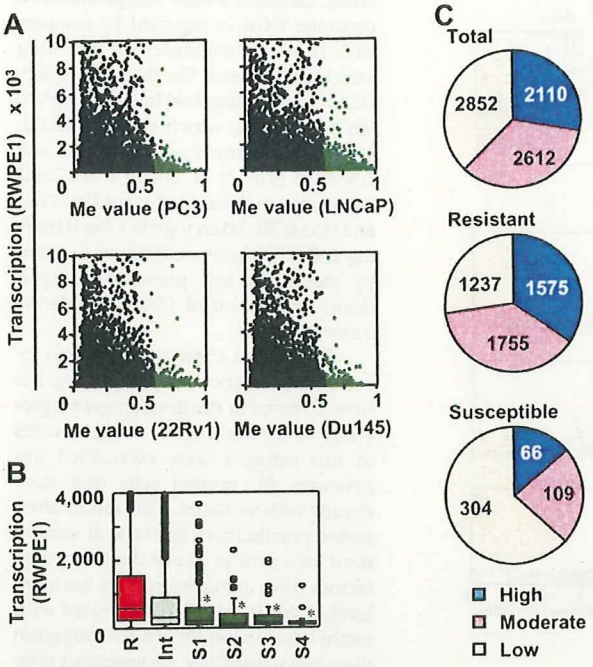

Figure 2. Low transcription levels of DNA methylation-susceptible genes in the normal prostatic cell line (RWPE1). (*A*) The association between DNA methylation levels (Me value of the NFRs) in each of the four prostate cancer cell lines (PC3, LNCaP, 22Rv1, and Du145) and transcription levels in RWPE1. (Green dots) Genes highly methylated in a cancer cell line. Genes highly methylated in a cancer cell line had low transcription levels in the normal cell line. (*B*) Transcription levels of resistant (R), intermediate (Int), and susceptible (S1–S4) genes in RWPE1. The boxes represent the 75th and 25th percentiles, and the line in the box represents the 50th percentile (the median). Whiskers represent the maximum data within (75th percentile + 1.5 × [75th percentile − 25th percentile]) and the minimum data within (25th percentile − 1.5 × [75th percentile − 25th percentile]). (Dots) The data not included between the whiskers. Transcription levels of Int, S1, S2, S3, and S4 were compared to that of R by the Mann-Whitney *U*-test (*$P < 1 \times 10^{-5}$). Susceptible genes had significantly lower expression levels than resistant genes. (*C*) The fraction of genes with high (blue) (signal intensity > 1000), moderate (pink) (250–1000), and low (yellow) (<250) transcription. Susceptible genes had a significantly larger fraction of genes with low transcription than the total genes.

The data obtained by the ChIP with microarray hybridization (ChIP-chip) analysis were validated by analyzing correlations between the signal ratio (immunoprecipitated DNA [IP]/whole cell extract [WCE]) obtained by ChIP-chip and those obtained by quantitative ChIP-PCR (Supplemental Fig. S7).

Using only genes with low transcription, we analyzed the association between the candidate instructive factors in the normal prostatic cell line and susceptibility to DNA methylation in prostate cancer cell lines. It was clear that H3Ac and H3K4me3 were elevated in resistant genes, and H3K27me3 was elevated in susceptible genes (Fig. 3A). In contrast, the H3K9me3 level was not different between resistant and susceptible genes. Notably, Pol II binding was remarkably higher in resistant genes (Fig. 3B). When further upstream regions and downstream regions were analyzed, resistant genes had elevated H3Ac and H3K4me3 mainly in their downstream regions, and susceptible genes had elevated H3K27me3 in their downstream regions and further upstream regions (Fig. 3C). Pol II binding was elevated mainly in the NFRs and then in downstream regions of resistant genes (Fig. 3C). In the mammary glands, exactly the same tendency was observed (Supplemental Fig. S8).

Next, within the normal prostatic cell line, the association between histone modifications and transcription levels was analyzed. Conforming to previous reports (Barski et al. 2007; Wang et al. 2008), genes with high and low transcription had elevated active and inactive histone modifications (Supplemental Fig. S9). Notably, among genes with low transcription, those without DNA methylation had elevated H3K27me3, confirming a previous report that H3K27me3 is involved in gene silencing independent of DNA methylation (Kondo et al. 2008). Within the normal mammary epithelial cells, the same tendency was observed.

Strongest association of Pol II binding with resistance to DNA methylation

The combination effect of H3K27me3 and one of the three active factors (H3Ac, H3K4me3, and Pol II binding) on DNA methylation susceptibility was then examined (Fig. 3D). All the three combinations were informative in distinguishing the resistant and susceptible genes, while Pol II binding gave the clearest discrimination. Multivariate logistic regression analysis was then performed to compare precisely the independent effects of H3Ac, H3K4me3, H3K9me3, H3K27me3, and Pol II binding on DNA methylation susceptibility. The genes with low transcription in the normal cell line (cells) were divided into quintiles according to the amounts of H3Ac, H3K4me3, H3K9me3, H3K27me3, and Pol II binding at the NFRs. Compared with the genes in the lowest quintile, multivariate-adjusted odds ratios (ORs) of genes in the other quintiles to become moderately or highly methylated in cancers (Int, and S1–S4 for the prostates; Int, and S1–S3 for the mammary glands) were calculated (Table 2). In the prostates, Pol II binding had the strongest independent association with resistance, and H3K27me3 had a strong and significant association with susceptibility. In the mammary glands, similar associations were observed. If the analysis was performed for the multivariate-adjusted odds ratio of genes to become highly methylated (S1–S4 for the prostates; and S1–S3 for the mammary glands), the association of Pol II binding became even clearer (Supplemental Table S3).

Finally, regardless of their transcription levels, all the genes were classified into genes with "active Pol II" (high/moderate transcription, high Pol II), those with "stalled Pol II" (low transcription, high Pol II), and those with "low Pol II" (low Pol II). The group of genes with low Pol II was further subdivided into those with and without H3K27me3. In the normal prostatic cell line, 47%, 13%, and 40% of genes had active, stalled, and low Pol II, respectively (Fig. 4A). Both genes with active Pol II and genes with stalled Pol II consisted mostly of resistant genes (Fig. 4B). In contrast, genes with low Pol II contained larger fractions of susceptible genes, and the presence of H3K27me3 remarkably increased the fraction. Similar results were obtained also in the mammary glands (Supplemental Fig. S10).

Discussion

In this study, we showed that Pol II binding in the NFRs in normal cell lines (cells) was closely associated with resistance to DNA methylation in cancer cell lines (cells) for the first time. The association between Pol II binding and resistance to DNA methylation was independent of transcriptional levels. It was also independent from the promoting effect of H3K27me3, and the combination of Pol II binding and H3K27me3 could explain a large part of the

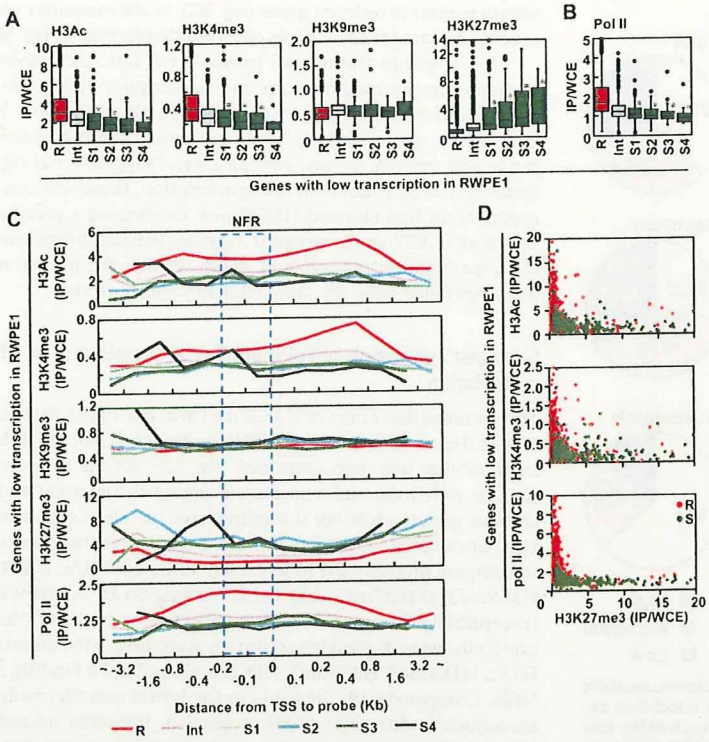

Figure 3. The association between the levels of candidate instructive factors in RWPE1 and DNA methylation susceptibility, among genes with low transcription in RWPE1. (*A*) Histone modification levels of genes with different susceptibilities to DNA methylation. For the box plot and statistical methods, refer to the legend to Figure 2B. Active histone modifications were elevated in resistant genes, and H3K27me3 was elevated in susceptible genes. (*B*) The association between Pol II binding and DNA methylation susceptibility. Pol II binding was associated with resistance even among genes with low transcription. (*C*) Levels of histone modifications and Pol II binding at various positions against the TSSs in RWPE1. Average levels of histone modifications and Pol II binding of CGIs continuous from their NFRs are shown. (Blue dotted rectangle) The NFRs. (*D*) The combination effect of one of the three active factors (H3Ac, H3K4me3, and Pol II binding) (*y*-axis) and H3K27me3 (*x*-axis) on resistance and susceptibility of genes with low transcription. (Red dots) DNA methylation-resistant genes; (green dots) DNA methylation-susceptible genes; they were separated by any of the three combinations.

instructive mechanisms for induction of DNA methylation. These data provided fundamental information on how the epigenetic fate of promoter CGIs is determined. The association between Pol II binding and resistance to DNA methylation can be potentially useful in the prediction of genes that will become silenced in cancer and other diseases.

Our multivariate analysis involving Pol II binding and histone modifications showed that the association between active histone modifications and resistance to DNA methylation was mostly overridden by that of Pol II binding, while the association between H3K27me3 and susceptibility to DNA methylation remained. It was reported that active histone modifications are involved in anchoring of the basal transcription factor TFIID (Vermeulen et al. 2007), which forms a transcription complex with Pol II. H3K4me3 is recognized by the PHD domain of TFIID, and acetylation of histone H3 lysine 9 and lysine 14 potentiates this interaction. It was therefore suggested that Pol II binding more directly works as a protection mechanism than active histone modifications, and that H3K27me3 has an independent mode of action.

Pol II forms a huge transcription complex of ~3 MDa with general transcription factors and other proteins (Boeger et al. 2005), and such a huge complex around promoter CGIs is expected to compete with DNA methyltransferases and their associated proteins. On the other hand, H3K27me3 is recognized by PRC2/3 (Hansen et al. 2008), which contains EZH2. Since EZH2 interacts with DNMT3A and DNMT3B (Vire et al. 2006), H3K27me3 is expected to signal binding of DNMT3A and DNMT3B. Taken together, Pol II binding and H3K27me3 are likely to function by preventing and promoting, respectively, recruitment of DNA methylation complexes.

Functional annotation analysis revealed that most of the susceptible genes were involved in the developmental processes of specific cells or tissues. Genes in this category were considered unnecessary for normal cells that have already differentiated. This raised alternative possibilities: The lack of current need for a gene is one of the instructive factors, or an unnecessary gene has a low level of Pol II, which is associated with methylation susceptibility. To distinguish these two possibilities, we examined overrepresentation of susceptible genes among genes with low Pol II levels after classification of genes by their function (Supplemental Table S4). As a result, in any categories of genes, susceptible genes were overrepresented among the genes with low Pol II levels, showing that the presence of Pol II was an independent factor for resistance to DNA methylation from functions of genes.

Specific genome structures are also known to be involved in the specificity of genes methylated, in addition to the instructive factors analyzed here. The presence of a repetitive sequence has been reported to be capable of functioning as a source of aberrant DNA methylation (Yates et al. 1999). In addition to methylation induction of individual genes, a cluster of genes can be methylated simultaneously in a cancer (Frigola et al. 2006). In this study, 64% and 50% of the susceptible genes in breast and prostate cancers, respectively, were unique to individual tumors. The susceptibility specific to a tissue is more likely to be due to Pol II binding and H3K27me3 rather, while susceptibility common to different tissues can be due to specific genome structures.

Genes moderately methylated were considered to be methylated in a fraction of cancer cells and thus to have been methylated after clonal expansion started. Genes highly methylated were considered to be present in all the cancer cells, and thus to have been methylated before clonal expansion. Therefore, DNA methylation susceptibility in normal cell line (cells) might be more precisely measured using genes highly methylated (Supplemental Table S3) than using genes highly and moderately methylated (Table 2).

As materials, we used normal and cancer cell lines to perform efficient and precise ChIP experiments. It is known that cancer cell

Table 2. The association between the levels of candidate instructive factors and susceptibility to DNA methylation (Int and S)

	Lowest quintile	2nd quintile	3rd quintile	4th quintile	Highest quintile
Prostate					
H3Ac	1	0.78 (0.54–1.12)	0.86 (0.56–1.31)	0.86 (0.54–1.37)	0.91 (0.53–1.57)
H3K4me3	1	0.99 (0.70–1.41)	1.09 (0.74–1.61)	0.92 (0.61–1.38)	0.52 (0.34–0.82)
Pol II	1	0.83 (0.58–1.18)	0.78 (0.52–1.17)	0.40 (0.25–0.62)	0.22 (0.12–0.38)
H3K9me3	1	1.47 (1.00–2.15)	1.26 (0.85–1.86)	1.22 (0.82–1.80)	1.20 (0.81–1.78)
H3K27me3	1	1.41 (0.92–2.17)	2.88 (1.89–4.40)	5.95 (3.87–9.13)	11.20 (7.14–17.55)
Mammary gland					
H3Ac	1	0.95 (0.68–1.35)	0.63 (0.43–0.91)	0.44 (0.30–0.66)	0.42 (0.26–0.67)
H3K4me3	1	0.96 (0.68–1.34)	1.02 (0.71–1.47)	0.59 (0.40–0.87)	0.49 (0.31–0.75)
Pol II	1	1.22 (0.88–1.71)	1.29 (0.90–1.86)	1.14 (0.77–1.68)	0.67 (0.43–1.04)
H3K9me3	1	1.03 (0.76–1.41)	1.07 (0.78–1.47)	1.43 (1.03–1.99)	0.89 (0.64–1.25)
H3K27me3	1	1.61 (1.20–2.18)	2.44 (1.78–3.34)	3.96 (2.86–5.48)	6.44 (4.56–9.10)

Multivariate-adjusted odds ratio (OR) (95% confidence interval; 95% CI) to become methylated (Int, and S1–S4 for the prostates; and Int, and S1–S3 for the mammary glands) is shown for each group. The multivariate-adjusted OR (95% CI) was derived from analyses in which all other listed variables were included into the mode.

lines generally show a larger number of methylated genes than primary tumor cells when a single cancer cell line and a primary tumor sample are compared. However, when a large number of primary tumor samples are analyzed, most DNA methylation found in cancer cell lines is also observed in at least one of the primary tumor samples (Sato et al. 2003; Lodygin et al. 2005; Yamashita et al. 2006). Therefore, it is considered that DNA methylation susceptibility identified in cancer cell lines reflects that in the primary cancer cells as a whole.

In summary, Pol II binding and H3K27me3 in normal cell lines (cells) could predict the epigenetic fate of genes with promoter CGIs in cancer cell lines independently of transcription activity and are major components of instructive mechanisms of DNA methylation induction.

Methods

Cell culture

PC3, LNCaP, 22Rv1, Du145, MCF7, ZR-75-1, and MDA-MB468 (American Type Culture Collection) were maintained in RPMI1640. RWPE1 (American Type Culture Collection) was maintained in keratinocyte-SFM containing 5 ng/mL rEGF, 50 μg/mL bovine pituitary extract (Invitrogen). HMEC (Clonetics) was maintained in mammary epithelial cell serum-free growth medium containing 1% growth supplement (CELL Applications).

ChIP assay

About 1×10^7 cells were cross-linked with 1% formaldehyde for 10 min at room temperature, and washed with ice cold 1× PBS (−) twice. Cells were re-suspended in lysis buffer (50 mM Tris-HCl at pH 8.0, 1 mM EDTA, 1% [w/v] SDS), incubated for 10 min on ice, and then sonicated to shear DNA to an average length ranging from 200 to 1000 bp with a Bioruptor UCD-250 (Cosmo Bio). After DNA shearing, the lysate was centrifuged at 13,000 rpm for 10 min, and supernatant was recovered. The volume of supernatant containing 30 μg of sheared DNA was adjusted to 100 μL with lysis buffer, and then was diluted with 900 μL of dilution buffer (50 mM Tris-HCl at pH 8.0, 167 mM NaCl, 1.1% [w/v] Triton X-100, 0.11% [w/v] sodium deoxycholate [DOC]). Twenty microliters of sheared chromatin was recovered and was used as input DNA.

Diluted lysate was incubated with 2 μg of antibody against H3K4me3 (07-473; Millipore), H3K9me3 (07-442; Millipore), H3K27me3 (07-449; Millipore), H3Ac (06-599; Millipore), or Pol II (ab5095; Abcam), which was reported to be capable of detecting stalled Pol II (Muse et al. 2007) overnight at 4°C with rotation, and then immuno-complexes were collected with 25 μL of Dynabeads Protein A (Invitrogen Dynal AS). Collected beads were washed with 1× RIPA buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% [w/v] Triton X-100, 0.1% [w/v] SDS, 0.1% [w/v] DOC) containing 150 mM NaCl twice, 1× RIPA buffer containing 500 mM NaCl twice, LiCl wash buffer (10 mM Tris-HCl at pH 8.0, 0.25 M LiCl, 1 mM EDTA, 0.5% [w/v] NP-40, 0.5% [w/v] DOC), and 1× TE containing 50 mM NaCl. Beads were re-suspended with 1× TE, and the cross-links were reversed in the presence of 200 mM NaCl overnight at 65°C. DNA was recovered with RNase A and proteinase K treatment, followed by phenol extraction and ethanol precipitation, and dissolved in 100 μL of 1× TE. One microliter of DNA was used for quantitative ChIP-PCR to confirm the specificity of our

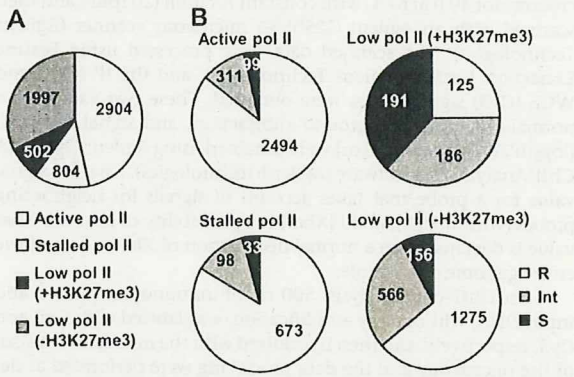

Figure 4. The association between Pol II binding and DNA methylation resistance in the total 6207 genes, regardless of transcription levels. (*A*) Classification of genes by Pol II status and H3K27me3 in the normal prostatic cell line. We were able to analyze transcription levels for 4567 of 5510 resistant, 1161 of 1330 intermediate, and 479 of 521 susceptible genes (total 6207 of 7361 genes) due to a difference in microarray platforms. Genes with high Pol II levels and high/moderate transcription levels were considered as those with "active Pol II." Genes with high Pol II levels but low transcription levels were considered as those with "stalled Pol II." Genes with low Pol II were further subdivided into those with and without H3K27me3. The numbers of genes with active, stalled, and low Pol II are shown. (*B*) The fractions of resistant, intermediate, and susceptible genes according to the Pol II and H3K27me3 statuses. Genes with either active or stalled Pol II had a larger fraction of resistant genes, and genes with low Pol II had a larger fraction of susceptible and intermediate genes.

ChIP technique (Supplemental Fig. S11) or to validate microarray results (Supplemental Fig. S7). Quantitative ChIP-PCR was performed using SYBR Green I (BioWhittaker Molecular Applications) and an iCycler Thermal Cycler (Bio-Rad Laboratories) as described previously (Nakajima et al. 2009). The primers used in quantitative ChIP-PCR are listed in Supplemental Table S5 (Kirmizis et al. 2004).

MeDIP

Five micrograms of genomic DNA was sheared by sonication using a VP-5s homogenizer (TAITEC) to a length of ~300 bp (Supplemental Fig. S12). Generally, there are nine to 53 CpG sites in 300-bp regions of promoter CGI (Nakajima et al. 2009), and this number of CpG sites is sufficient for efficient immunoprecipitation by MeDIP (Keshet et al. 2006). After heat denaturation for 10 min at 95°C, DNA was incubated with 5 μg of antibody against 5-methyl cytidine (Diagnode) in 1× IP buffer (10 mM Na-phosphate at pH 7.0, 140 mM NaCl, 0.05% [w/v] Triton X-100) overnight at 4°C with rotation. Immuno-complexes were collected with 70 μL of Dynabeads Protein A, washed with 1× IP buffer four times, and were recovered by Proteinase K treatment, followed by phenol extraction and ethanol precipitation. DNA was dissolved in 26 μL of 1× TE.

CGI oligonucleotide microarray analysis

Genome-wide analysis of DNA methylation, histone modifications, and Pol II binding was carried out using a human CGI oligonucleotide microarray (Agilent technologies) that contained 237,220 probes in or within 95 bp of CGI covering 27,800 CGIs, with an average probe spacing of 100 bp.

For MeDIP-CGI microarray analysis, immunoprecipitated DNAs from 4.33 μg of sonicated DNA and 0.96 μg of input DNA, without any amplification, were labeled with Cy5 and Cy3, respectively, using an Agilent Genomic DNA Labeling kit PLUS (Agilent technologies). Labeled DNA was hybridized to the microarray for 40 h at 67°C with constant rotation (20 rpm), and then scanned with an Agilent G2565BA microarray scanner (Agilent Technologies). The scanned data were processed using Feature Extraction Ver.9.1 (Agilent Technologies), and the IP (Cy5) and WCE (Cy3) signal values were obtained. These two values were normalized using background subtraction, and signal log ratio [$\log_2$(IP/WCE)] and *P*[Xbar] were obtained using Agilent G4477AA ChIP Analytics 1.3 software (Agilent Technologies). Xbar is a signal value for a probe that takes account of signals for neighboring probes (within 1 kb), and *P*[Xbar] is a probability of how the Xbar value is deviated from a normal distribution of Xbar values of the entire genome of a sample.

For ChIP-chip analysis, 500 ng of immunoprecipitated and input DNA, without any amplification, was labeled with Cy5 and Cy3, respectively, and then hybridized with the microarray. A scan of the microarray and the data processing were performed as described above. The levels of each histone modification or Pol II binding were assessed by the signal ratio (IP/WCE). Genes were classified into those with high and low levels of each histone modification or Pol II binding when they had signal intensities higher and lower, respectively, than the average signal intensity of total probes. The microarray data (MeDIP-CGI microarray and ChIP-chip analyses) were submitted to the GEO database under accession no. GSE15154.

Calculation of Me value

The Me value of each probe was calculated as Me value = [signal log ratio × (1 − *P*[Xbar]) − 1.3]/2.6 + 0.5. The Me value was developed to give a value between 0 and 1 that linearly correlates with the amount of methylated DNA molecules at a specific locus and is not influenced by the genome-overall methylation levels. The Me value of a single probe is known to correlate well with an average DNA methylation level of CpG sites within 200 bp from the probe (Yamashita et al. 2009).

Definition of genomic regions

The position of each probe against a TSS was determined using UCSC hg18 (NCBI Build 36.1, March 2006). A CGI was defined as an assembly of probes with intervals <500 bp. CGIs were classified into four categories, promoter 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 from genes). A CGI spanning both a promoter region and gene body was split into a promoter CGI and a gene body CGI. A putative NFR was defined as a region between a TSS, determined by UCSC hg18 (NCBI Build 36.1, March 2006), and its 200 bp upstream. Since TSSs are inherently variable for some genes (Suzuki et al. 2001), and the size of NFRs are different according to studies (Yuan et al. 2005; Gal-Yam et al. 2006), the locations are approximate, but expected to be correct as a whole. According to these definitions, 34,697 assemblies of probes were defined as CGIs, and 9624 assemblies were defined as NFRs. Genes with multiple NFRs because of their multiple TSSs were analyzed as different genes. DNA methylation status and histone modifications/Pol II binding in each CGI (or NFR) were assessed by an average Me value and signal ratio, respectively, of the probes located within each CGI (or NFR). A single CGI (or NFR) contains 6.8 (2.0) probes on average.

Gene expression analysis by oligonucleotide microarray

Expression microarray analysis was performed by a GeneChip Human Genome U133 Plus 2.0 expression microarray (Affymetrix) that contained 54,000 probe sets from 39,000 genes. From 8 μg of total RNA, the first-strand cDNA was synthesized with SuperScript III reverse transcriptase (Invitrogen) and a T7-(dT) 24 primer (Amersham Bioscience). Double-stranded cDNA was then synthesized, and biotin-labeled cRNA was synthesized using a BioArray HighYield RNA transcript labeling kit (Enzo). Twenty micrograms of labeled cRNA was fragmented and hybridized to the GeneChip oligonucleotide microarray. The microarray was stained and scanned according to the protocol from Affymetrix. The scanned data were processed using GeneChip operating software (ver. 1.4). The signal intensity of each probe was normalized so that the average signal intensity of all the probes on a microarray would be 500. Average signal intensity of all the probes for a gene was used as its transcription level. Genes were classified into those with high (>1000), moderate (250–1000), and low (<250) transcription according to their signal intensities.

Multivariate analysis and other statistical tests

To evaluate the independent contribution of each predictor variable (H3Ac, H3K4me3, Pol II binding, H3K9me3, or H3K27me3 level) in relation to the other four predictor variables on DNA methylation susceptibility (an outcome variable), multivariate logistic regression analysis was performed. Susceptible genes were defined as (1) those moderately and highly methylated in cancer cell lines (Int, and S1–S4 for the prostates; Int, and S1–S3 for the mammary glands), or (2) those highly methylated in cancer cell lines (S1–S4 for the prostates; and S1–S3 for the mammary glands). The predictor variables were classified into quintiles according to

H3Ac, H3K4me3, Pol II binding, H3K9me3, or H3K27me3 levels of the NFRs to create dummy variables. This was done because a log linear relationship was unclear between the raw value (signal ratio of each gene) and DNA methylation susceptibility. Multivariate-adjusted ORs and 95% confidence intervals (CIs) of genes in each quintile for DNA methylation susceptibility were calculated, including all predictor variables simultaneously in the model using SAS software, ver. 9.1 (SAS Institute Inc, *SAS/STAT 9.1 User's Guide*, SAS Institute Inc., Cary, NC). Using the lowest quintile as a reference, we calculated multivariate-adjusted ORs of genes in each quintile, which reflect DNA methylation susceptibility relative to the reference while controlling for the simultaneous effect of all the other predictor variables included in the model.

The fractions of genes with low transcription were compared between different groups of genes by the χ^2-test. The transcription, histone modification, and Pol II binding levels were compared between two groups of genes by the Mann-Whitney's *U*-test.

Functional annotation analysis

Functional annotation analysis was performed by DAVID bioinformatics resources (Dennis et al. 2003; Huang et al. 2009). The enrichment of genes in a biological process (a Gene Ontology criterion) was analyzed by comparing a fraction of genes with an ontology among the resistant (or susceptible) genes with that among all the genes.

Acknowledgments

We thank Hiroyuki Sasaki for his critical reading of this manuscript. H.T. is a recipient of Research Resident Fellowships from the Foundation for Promotion of Cancer Research. This study was supported by Grants-in-Aid for the Third-Term Comprehensive Cancer Control Strategy from the Ministry of Health, Labour and Welfare, Japan; for the Priority-area Research from the Ministry of Education, Science, Culture, and Sport, Japan; and a grant from Uehara Life Science Foundation.

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Received March 1, 2009; accepted in revised form July 30, 2009.

Cancer Letters 285 (2009) 182–189

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

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

Kosuke Hosoya [a,c], Satoshi Yamashita [a], Takayuki Ando [a], Takeshi Nakajima [b], Fumio Itoh [c], Toshikazu Ushijima [a,*]

[a] Carcinogenesis Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan
[b] Endoscopy Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan
[c] Division of Gastroenterology and Hepatology, Department of Internal Medicine, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki 216-8511, Japan

ARTICLE INFO

Article history:
Received 3 April 2009
Received in revised form 14 May 2009
Accepted 15 May 2009

Keywords:
Epigenetics
DNA methylation
Gastric cancer
Helicobacter pylori
Passenger

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.

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],

* Corresponding author. Tel.: +81 3 3547 5240; fax: +81 3 5565 1753.
E-mail address: tushijim@ncc.go.jp (T. Ushijima).

0304-3835/$ - see front matter © 2009 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2009.05.016

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

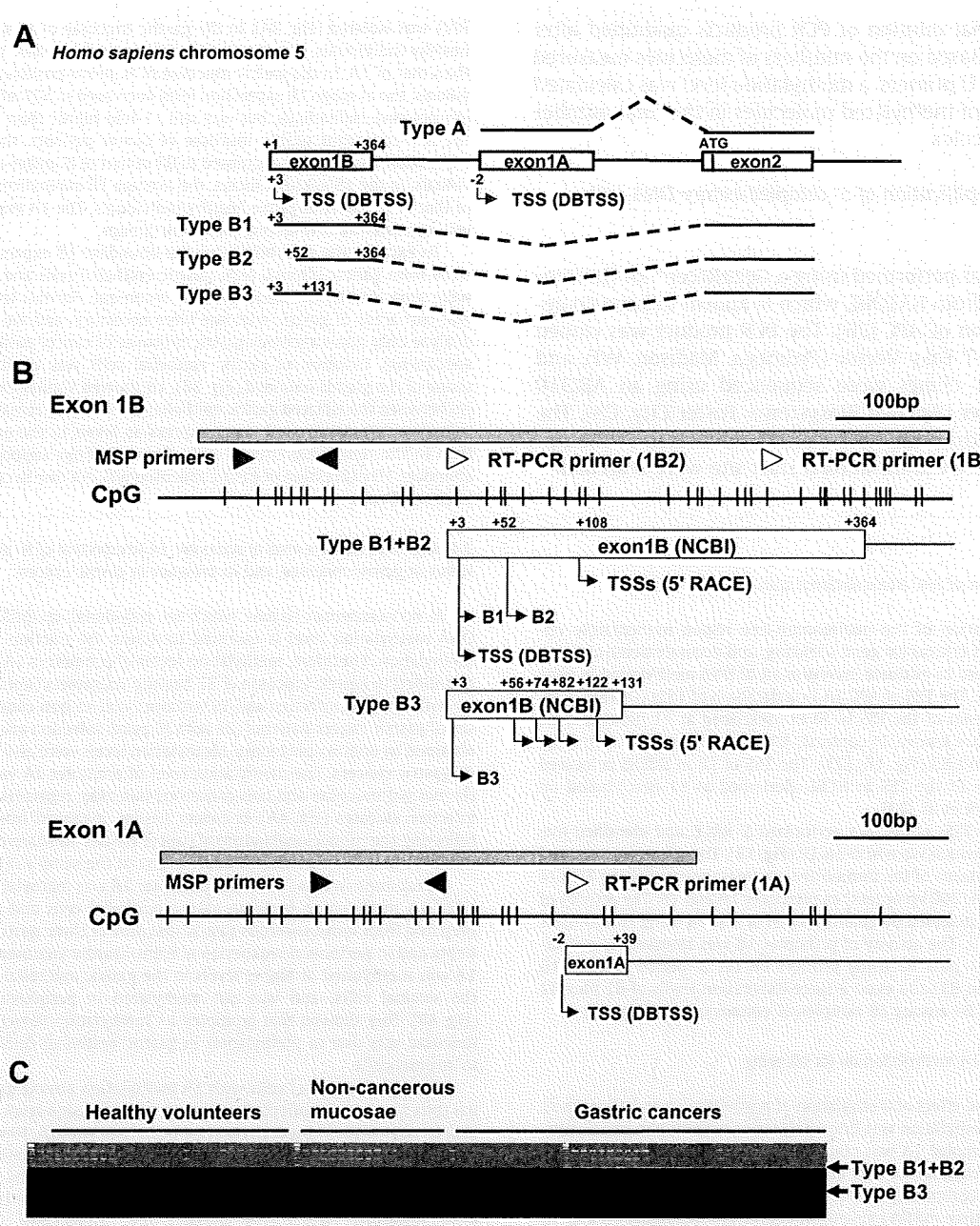

Fig. 1. Genomic and mRNA structures of *APC* 1A and 1B. (A) Splicing forms of 1A and 1B. One form of 1A and three variants of 1B are known to be produced from individual promoters. (B) CpG maps of promoters 1A and 1B and their TSSs. The TSS of *APC* 1A in a database of TSSs (DBTSS) is located 2 bp downstream 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

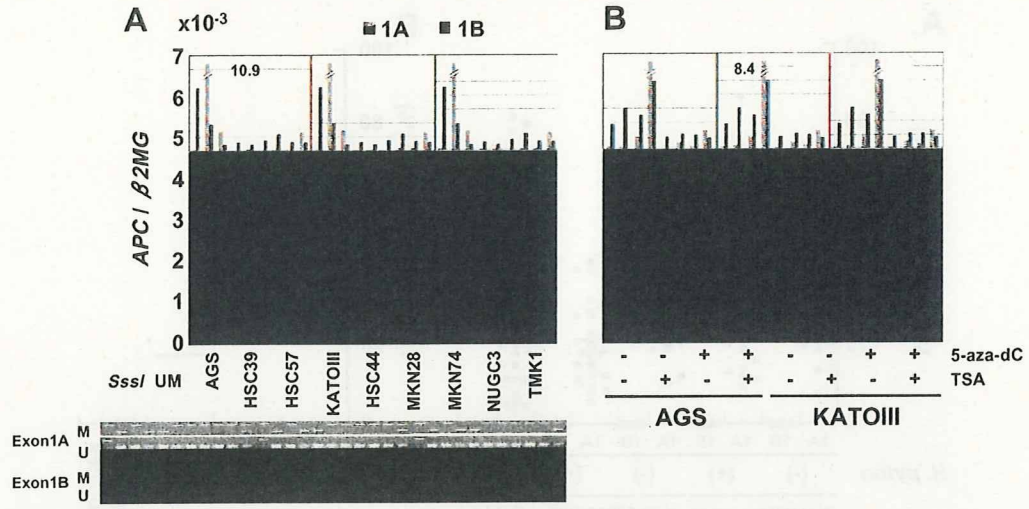

Fig. 2. Methylation-silencing of 1A in human gastric cancer cell lines. (A) qRT-PCR of *APC* 1A and 1B, and MSP of promoters 1A and 1B in nine cell lines. The mRNA quantity of each gene was normalized to that of β2-microgloburin (β2MG). It was found that 1A was consistently repressed in the six cell 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.

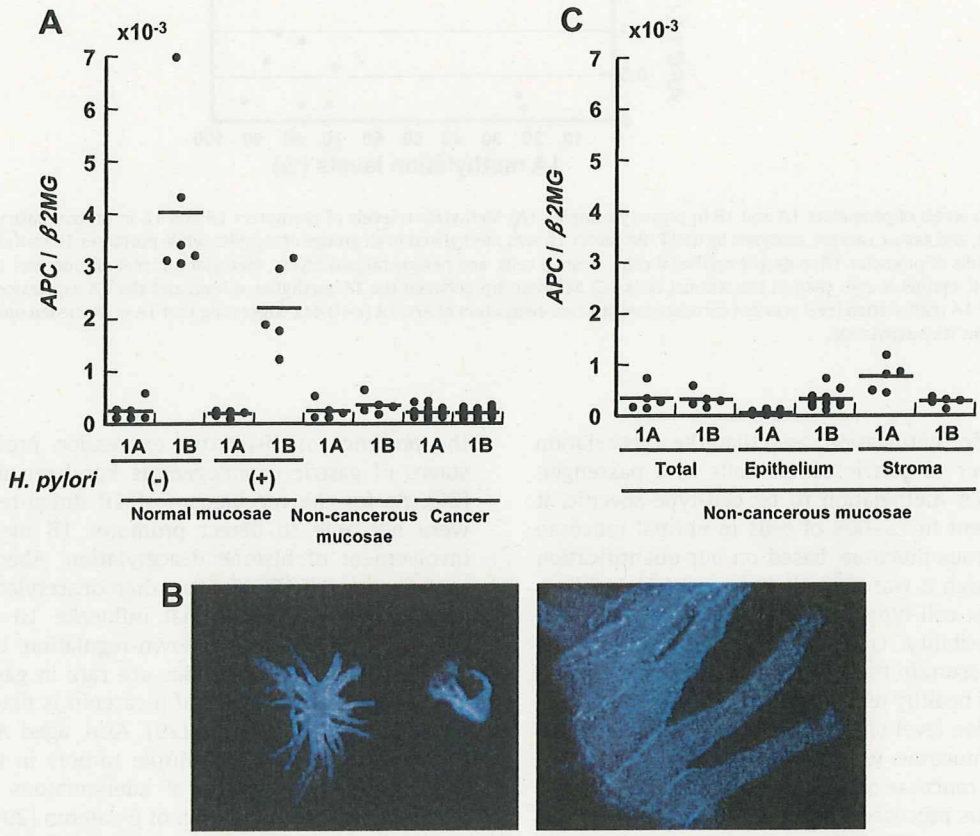

Fig. 3. Expression levels of *APC* 1A and 1B in primary samples. (A) Expression levels of 1A and 1B in normal gastric mucosae, non-cancerous mucosae, and gastric cancers. In *H. pylori*-negative gastric mucosae of healthy volunteers, the average 1B expression level was 15-fold higher than that of 1A. 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.

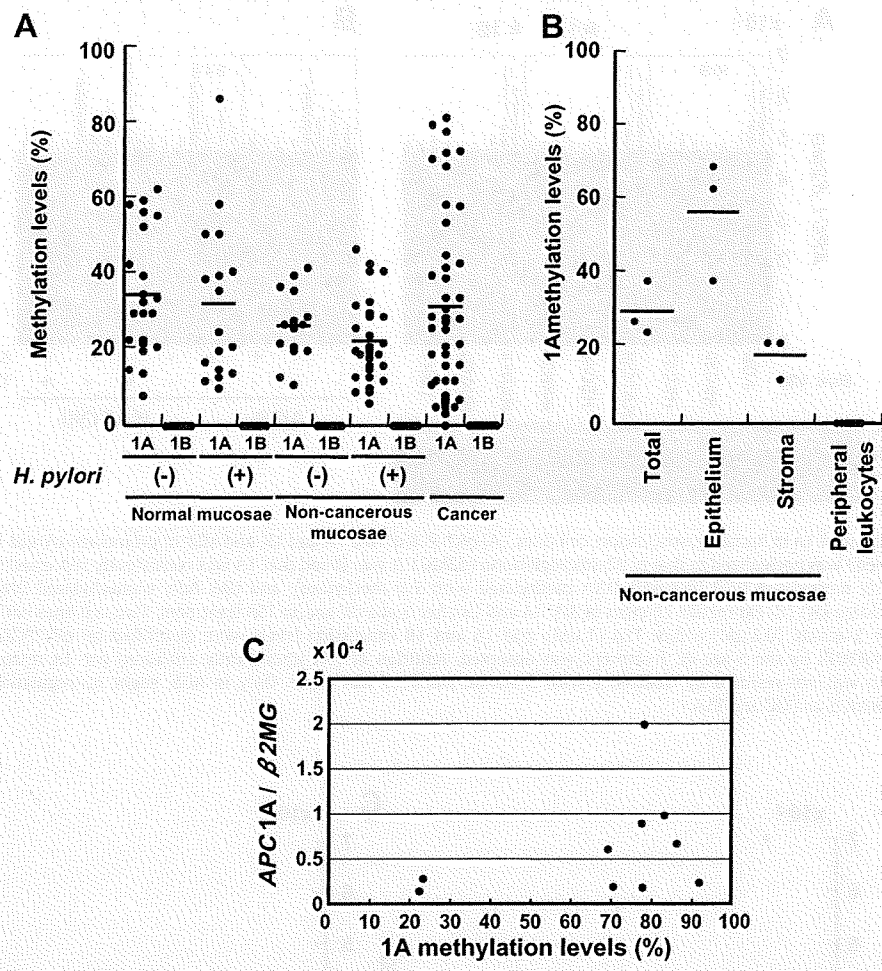

Fig. 4. Methylation levels of promoters 1A and 1B in primary samples. (A) Methylation levels of promoters 1A and 1B in normal gastric mucosae, non-cancerous mucosae, and gastric cancers, analyzed by qMSP. Promoter 1A was methylated in all groups of samples while promoter 1B was never methylated. (B) 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.

Acknowledgement

This study was supported by Grants-in-Aid for the Third-term Comprehensive Cancer Control Strategy and for Pioneering Basic Research from the Ministry of Health, Labour and Welfare, Japan.

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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Carcinogenesis vol.30 no.3 pp.466–471, 2009
doi:10.1093/carcin/bgp021
Advance Access publication January 23, 2009

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

Kotoe Terada[1,2], Eriko Okochi-Takada[1], Sadako Akashi-Tanaka[3], Kazuaki Miyamoto[4], Kiyomi Taniyama[4], Hitoshi Tsuda[5], Kiyoshi Asada[1], Michio Kaminishi[2] and Toshikazu Ushijima[1,*]

[1]Carcinogenesis Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan, [2]Department of Surgical Metabolic Care and Endocrine Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo 113-8655, Japan, [3]Department of Surgery, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan, [4]Institute for Clinical Research, National Hospital Organization Kure Medical Center/ Chugoku Cancer Center, Hiroshima 737-0023, Japan and [5]Pathology Laboratory, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

*To whom correspondence should be addressed. Tel: +81 3 3547 5240; Fax: +81 3 5565 1753; Email: tushijim@ncc.go.jp

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.

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

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

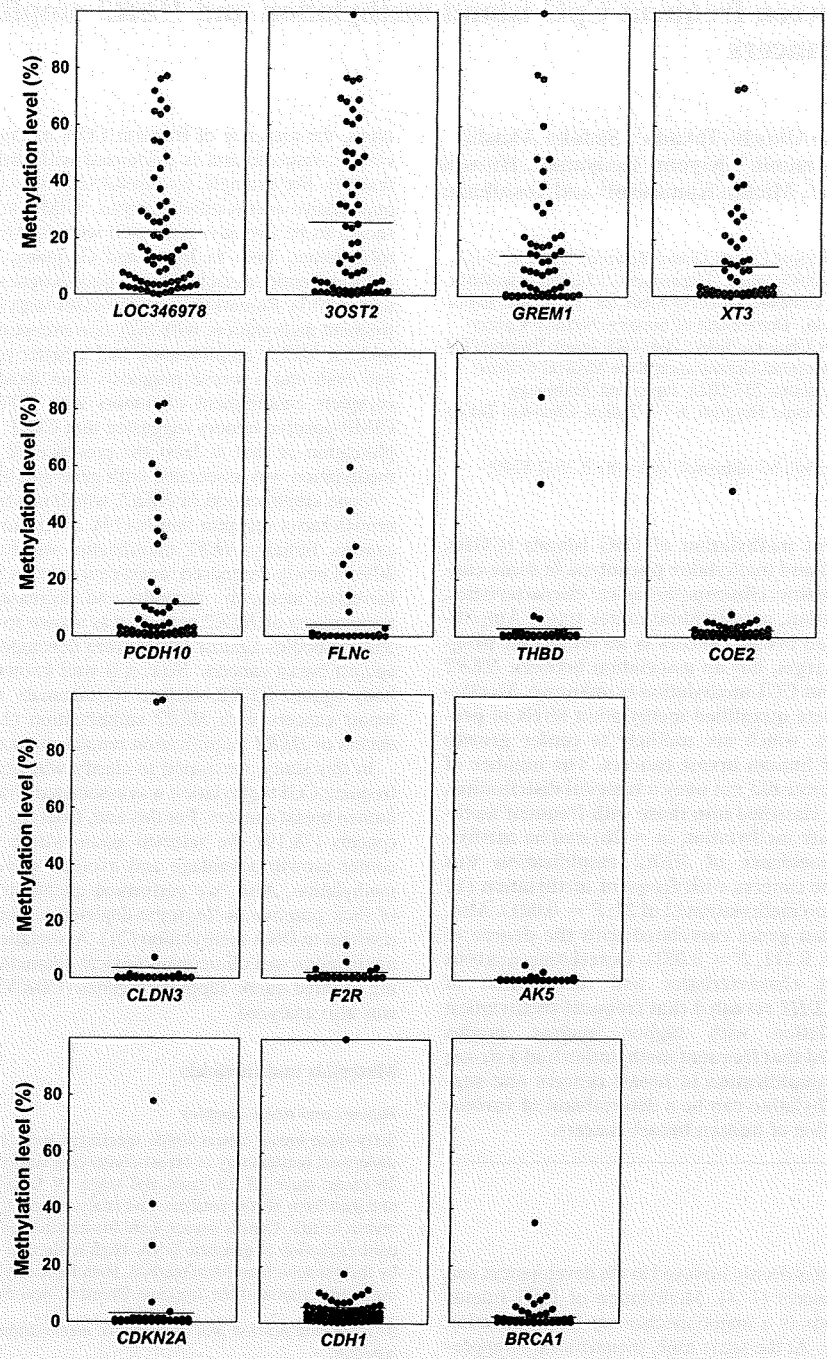

Fig. 1. Methylation levels in the 63 breast cancer samples. The numbers of DNA molecules methylated and unmethylated in promoter CGIs were obtained by quantitative methylation-specific PCR, and a methylation level was calculated as a fraction of methylated DNA molecules among the total DNA molecules. 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

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

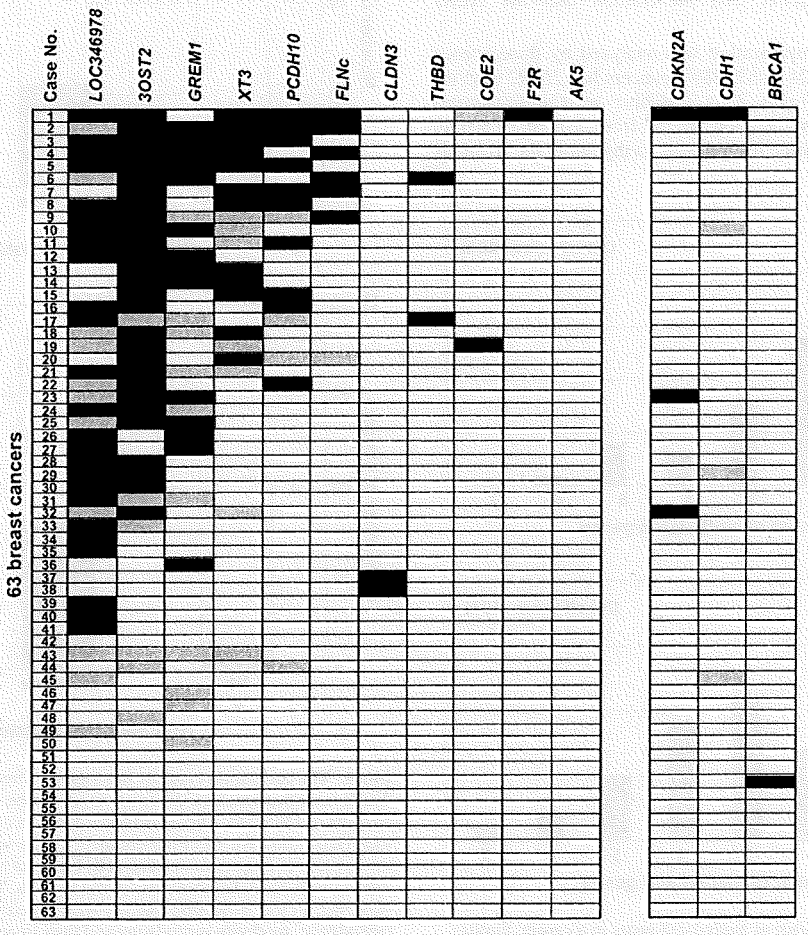

Fig. 2. Methylation profile of the 11 marker genes and three tumor suppressor genes in 63 breast cancers. Methylation in each sample was scored as positive or negative using two different cutoff values, and the 63 samples were aligned by the number of methylated CGIs. Methylation-positive samples using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

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.

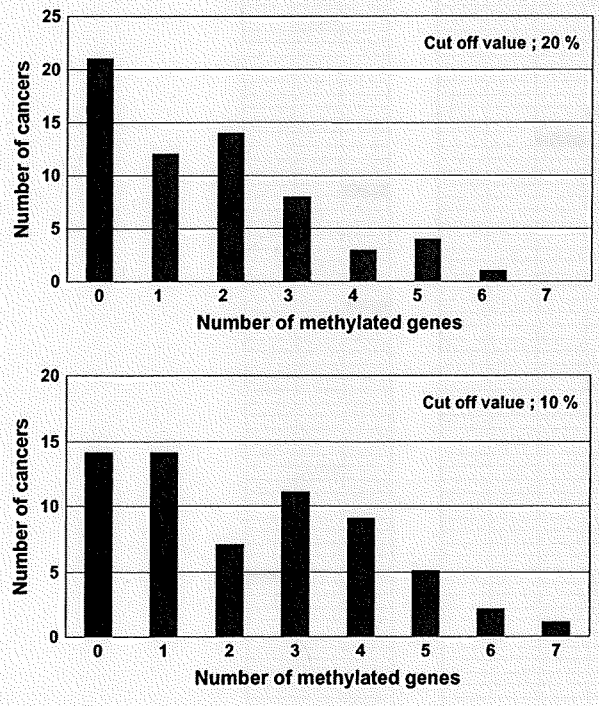

Fig. 3. Distribution of the number of methylated genes in a cancer. Similar distribution patterns were observed using two different cutoff values. The distribution was not bimodal, and involvement of multiple mechanisms in frequent methylation in breast cancers was suggested.

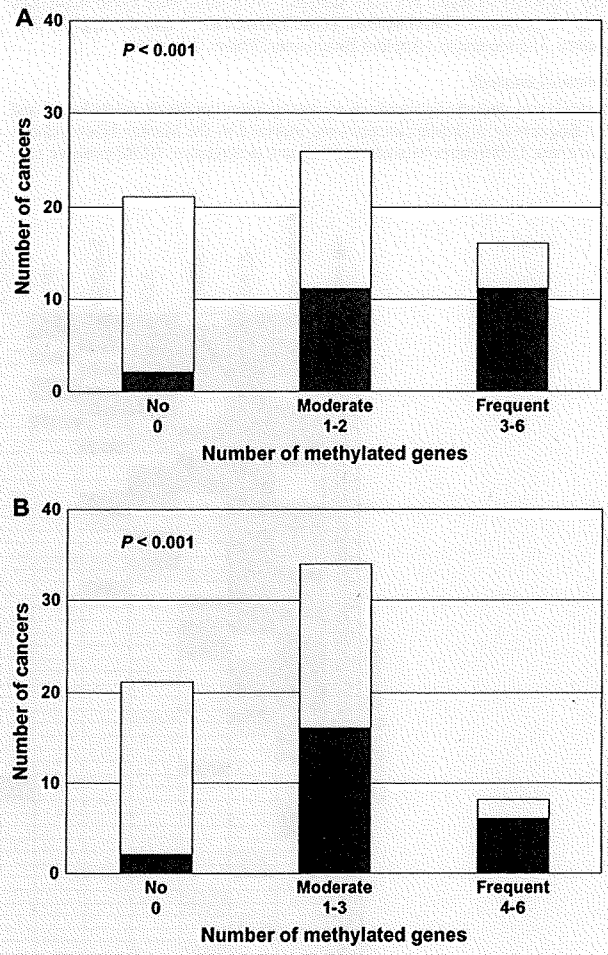

Fig. 4. The correlation between the degree of frequent methylation and *HER2* amplification. Two different cutoff numbers were used to define frequent methylation. These analyses adopted a cutoff value of 20% for methylation-positive. (**A**) Frequent methylation was defined as cancers with methylation 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.

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).

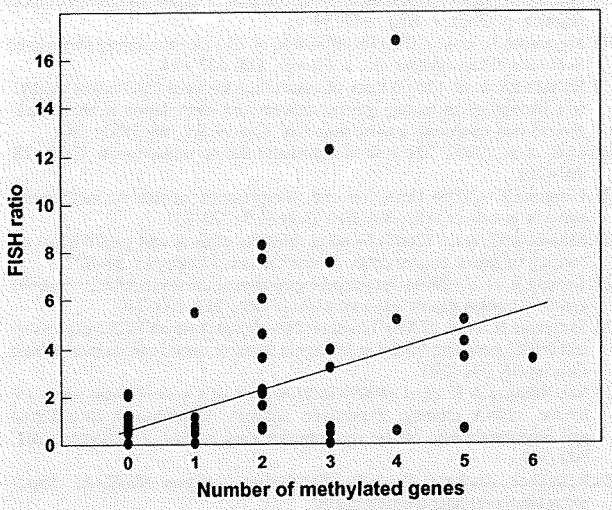

Fig. 5. The correlation between the number of methylated genes and degree of *HER2* amplification. The degree of *HER2* amplification showed a strong correlation with the number of methylated genes (correlation coefficient = 0.411, P = 0.002).

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.

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/

Funding

Third-term Comprehensive Cancer Control Strategy and Pioneering Basic Research from the Ministry of Health, Labor and Welfare, Japan.

Acknowledgements

The authors are grateful to Dr S.Yamamoto for his advice in statistical analyses.

Conflict of Interest Statement: None declared.

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Received August 20, 2008; revised December 16, 2008; accepted January 11, 2009