Cancer Science
The official journal of the Japanese Cancer Association JCA

Methylation silencing of angiopoietin-like 4 in rat and human mammary carcinomas

Naoko Hattori, Eriko Okochi-Takada, Mizuho Kikuyama, Mika Wakabayashi, Satoshi Yamashita and Toshikazu Ushijima[1]

Division of Epigenomics, National Cancer Center Research Institute, Tokyo, Japan

(Received January 6, 2011/Revised March 30, 2011/Accepted April 1, 2011/Accepted manuscript online April 12, 2011/Article first published online May 12, 2011)

Aberrant DNA methylation is deeply involved in the development and progression of human breast cancers, but its inducers and molecular mechanisms are still unclear. To reveal such inducers and clarify the molecular mechanisms, animal models are indispensable. Here, to identify genes silenced by promoter DNA methylation in rat mammary carcinomas, we took a combined approach of methylated DNA immunoprecipitation (MeDIP)–CpG island (CGI) microarray analysis and expression microarray analysis after treatment with epigenetic drugs. MeDIP-CGI microarray revealed that among 5031 genes with promoter CGI, 465 were methylated in a carcinoma cell line induced by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), but not in normal mammary epithelial cells. By treatment of the cell line with 5-aza-2′-deoxycytidine and trichostatin A, 29 of the 465 genes were shown to be re-expressed. In primary mammary carcinomas, five (*Angptl4*, *Coro1a*, *RGD1304982*, *Tmem37* and *Ndn*) of the 29 genes were methylated in one or more of 25 samples. Quantitative expression analysis revealed that *Angptl4* had high expression in normal mammary glands, but low expression in primary carcinomas. Also in humans, *ANGPTL4* was unmethylated and expressed in normal mammary epithelial cells, but was methylated in 11 of 91 (12%) primary breast cancers. This is the first study to identify genes aberrantly methylated in rat mammary carcinomas, and *Angptl4* is a novel methylation-silenced gene both in rat and human mammary carcinomas. The combination of the MeDIP-CGI microarray analysis and expression microarray analysis after treatment with epigenetic drugs was effective in reducing the number of methylated genes that are not methylation silenced. (*Cancer Sci* 2011; 102: 1337–1343)

Aberrant epigenetic modifications, such as aberrant DNA methylation and histone modifications, are deeply involved in the development and progression of human cancers.[1–3] In human breast cancers, tumor-suppressor genes, such as *RASSF1A*, *BRCA1*, *CDKN2A* and *PTEN*, are silenced by aberrant methylation of promoter CpG islands (CGI).[4] Aberrant methylation could be detected not only in cancers but also in non-cancerous breast tissues, suggesting that an epigenetic field defect is formed in breast cancer patients.[5] Despite the deep involvement of aberrant DNA methylation, limited information is available on the factors that induce aberrant methylation during mammary carcinogenesis. Among the limited information, exposure to estrogen or a nonsteroidal estrogen, bisphenol A, was reported to change the methylation status of mammary epithelial progenitor cells aberrantly *in vitro*.[6,7] However, inducers and induction mechanisms of aberrant methylation *in vivo* are still almost unknown. To address these issues, animal models are indispensable.

Rat models are useful for the study of mammary carcinogenesis in terms of several features. Mammary carcinomas can be reproducibly induced by a wide range of a selection of chemicals, including 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP),[8,9] 7,12-dimethylbenz[*a*]anthracene (DMBA)[10] and *N*-nitroso-*N*-methylurea,[11] and also by radiation,[12] and their characteristics have been well established. The induced mammary carcinomas predominantly originated from mammary ducts similar to the majority of human breast cancers.[13] As an animal model, we can use animals with a homogeneous genetic background and make any intervention to clearly analyze the effects of specific factors, such as overexposure to estrogen and intake of a high fat diet, on a phenotype. However, to analyze inducers of aberrant methylation and its mechanisms, we need genes silenced by aberrant methylation in rat mammary carcinomas, which are not known yet.

In the present study, we aimed to identify methylation-silenced genes in rat primary mammary carcinomas. To this end, we applied two genome-wide methylation analyses, methylated DNA immunoprecipitation (MeDIP)–CGI microarray analysis and expression microarray analysis after treatment with epigenetic drugs.[14]

Materials and Methods

Rat primary tissue samples and carcinoma cell lines. PhIP-induced mammary adenocarcinomas were obtained from female (F344 × SD)F1 rats at the age of 56–69 weeks who were administrated 10 doses of PhIP (75 mg/kg) at the age of 6 weeks and a high-fat diet (23.5% corn oil).[15] DMBA-induced mammary adenocarcinomas were obtained from female (F344 × SD)F1 rats at the age of 25–32 weeks who were administrated a single dose of DMBA (50 mg/kg) at the age of 7 weeks.[16] Normal mammary glands were collected from 8-week-old untreated female (F344 × SD)F1 rats and 56–69-week-old female (F344 × SD)F1 rats without administration of PhIP by the gland isolation technique for mammary ducts.[17] Primary-cultured epithelial cells were maintained as rat mammary epithelial cells (RMEC).[17] Two carcinoma cell lines, PhIP7-4 and PhIP12-1, were established from two mammary carcinomas induced by PhIP, as previously reported.[18] Two carcinoma cell lines, DMBA334 and DMBA397, were established from the DMBA-induced mammary carcinomas described above.

Human breast cancer cell lines and tissue samples. MCF-7, T47-D, SK-BR-3, MDA-MB-231, MDA-MB-468, ZR-75-1, BT-474, Hs578T and mammary epithelial cell line (MCF10A) were purchased from the American Type Culture Collection (Rockville, MD, USA). Human mammary epithelial cells (HMEC) were purchased from Cambrex (East Rutherford, NJ, USA). Human breast cancers ($n = 91$) and adjacent non-cancerous tissues ($n = 21$), which were located at least 3 cm apart from cancers, were obtained from the surgical specimens of patients who underwent mastectomy. Human samples were obtained with informed consent and the analysis was approved by the institutional review boards.

[1]To whom correspondence should be addressed. E-mail: tushijim@ncc.go.jp

doi: 10.1111/j.1349-7006.2011.01955.x

5-Aza-2′-deoxycytidine and trichostatin A treatments. PhIP7-4 cells were seeded at a density of 2×10^5 cells/10 cm plate on day 0, exposed to freshly prepared 1 or 5 μM 5-aza-2′-deoxycytidine (5-aza-dC; Sigma, St Louis, MO, USA) for 24 h on days 1 and 2, treated with 100 or 300 nM trichostatin A (TSA; Sigma) for 24 h on day 3, and then harvested on day 4. BT-474 and MDA-MB-231 cells were seeded at a density of 5×10^5 cells/6 cm plate and 6×10^5 cells/10 cm plate, respectively, on day 0, and treated with 5-aza-dC (1 or 5 μM for BT474; 0.5 or 1 μM for MDA-MB-231) for 24 h on days 1 and 3, and harvested on day 4. The doses of 5-aza-dC (and TSA) were adjusted so that the growth of treated cells is suppressed to 40–80% of non-treated cells.

MeDIP-CGI microarray analysis. As described previously,[19,20] 5 μg of sonicated DNA was immunoprecipitated with 6 μg antibody against 5-methylcytidine (Diagnode, Liége, Belgium), and the precipitated DNA and input DNA were labeled with Cy5 and Cy3, respectively. The labeled probes were hybridized to a rat CGI oligonucleotide microarray (Agilent Technologies, Santa Clara, CA, USA) that contained 93 024 probes in or within 95 bp of 13 026 CGI with an average probe spacing of 100 bp. The microarray was scanned with an Agilent G2565BA microarray scanner (Agilent Technologies), and the scanned data were processed using Feature Extraction Ver.9.1 (Agilent Technologies) and Agilent G4477AA ChIP Analytics 1.3 software (Agilent Technologies). A signal of probes was converted into a ''M_e value'', which represented the methylation level as a value from 0–0.3 (unmethylated) to 0.6–1 (methylated).[19] For the rat CGI microarray, the formula for the M_e value was optimized as follows: M_e value = (signal log ratio $X[1 - P(X)] - 1.3)/4.0 + 0.5$.

Definition of promoter CGI, and hierarchical clustering analysis. A CGI was defined as an assembly of probes within intervals <500 bp. A promoter CGI was defined as a CGI within a nucleosome-free region, which was defined as a region between a transcription start site (TSS) and its 200 bp upstream.[21] A TSS was determined using UCSC rn4 (Baylor Build 3.4, November 2004). According to these definitions, 5031 assemblies were defined as promoter CGI. The methylation level of a CGI was assessed by an average M_e value of the probes located within the CGI, and cut-off values of 0.6 and 0.3 were used for methylated and unmethylated CGI, respectively. Hierarchical clustering of promoter CGI by the Euclidean distance of their M_e value was performed using MultiExperimental Viewer v4.1 software (Dana-Farber Cancer Institute, Boston, MA, USA).

Oligonucleotide expression microarray analysis. Total RNA was isolated using ISOGEN (NIPPON GENE, Tokyo, Japan). Oligonucleotide microarray analysis was performed using a GeneChip Rat Genome 230 2.0 Array (Affymetrix, Santa Clara, CA, USA) with 54 000 probe sets, and 30 000 transcripts from 30 000 genes. From 7 μg of total RNA, double-stranded cDNA was synthesized and biotin-labeled cRNA was prepared using a BioArray High-Yield ENA transcript labeling kit (Enzo, Farmingdale, NY, USA). Twenty micrograms of labeled cRNA were fragmented and hybridized to the GeneChip oligonucleotide microarray. The scanned data were processed using GeneChip operating software and normalized so that the average of all genes on a GeneChip would be 500. A *P*-value for differential expression (change *P*-value) was calculated for each probe by an algorithm based on the Wilcoxon Signed-Rank test.

Methylation-specific PCR (MSP) and bisulfite sequencing. Sodium bisulfite treatment was performed as described previously using 1 μg of DNA digested with *Bam*HI (Toyobo, Tokyo, Japan),[22] and suspended in 40 μL of Tris-EDTA (TE) buffer. Fully methylated DNA was prepared by methylating genomic DNA using *Sss*I-methylase (New England Biolabs, Beverly, MA, USA). Fully unmethylated DNA was prepared by amplifying genomic DNA with ø29 DNA polymerase (GenomiPhi DNA Amplification kit; GE Healthcare UK, Buckinghamshire, UK).

Conventional MSP for screening purposes was conducted with primers specific to methylated DNA up to 39 cycles (Table S1), and samples with no PCR products amplified from methylated DNA were determined to be unmethylated samples.[22] Quantitative MSP (qMSP) was performed by real-time PCR using SYBR Green I and primers specific to methylated DNA at a locus and to a B2 repeat sequence, regardless of its methylation statuses for rat genes, or to unmethylated DNA for a human gene (Table S1). The number of DNA molecules was calculated as previously described.[23] The methylation level of a rat gene was calculated as the methylation percentage obtained as: ([number of DNA molecules methylated at a target CGI in a sample]/[number of B2 repeat in the sample])/([number of DNA molecules methylated at the target CGI in a *Sss*I-treated DNA]/[number of B2 repeat in the *Sss*I-treated DNA]) × 100; and that of a human gene as: (number of DNA molecules methylated)/([number of DNA molecules methylated] + [number of DNA molecules unmethylated]) × 100. The methylation level in a cancer sample was considered as aberrant when it was two or more times higher than the highest methylation level in normal samples.

For bisulfite sequencing, sodium bisulfite-treated DNA was amplified with primers common to methylated and unmethylated DNA sequences (Table S1). The PCR product was cloned into a pGEM-T Easy Vector (Promega, Madison, WI, USA), and 10 clones were sequenced using an ABI PRISM 310 sequencer (Applied Biosystems, Foster City, CA, USA).

Quantitative reverse transcription-PCR (qRT-PCR). DNase-treated total RNA (1 μg) was reverse-transcribed with a random hexamer (Invitrogen, Carlsbad, CA, USA) and Superscript III reverse transcriptase (Invitrogen). Quantitative PCR was carried out by real-time PCR using SYBR Green I and primers specific to genes. The primer sequences and PCR conditions are shown in Table S1. The amplification curve of a sample was compared with those of standard DNA samples with known copy numbers to obtain a copy number in the sample, as in qMSP. The number of target cDNA molecules was normalized to those of rat *Ppia* or human *GAPDH* cDNA molecules.[24]

Results

Isolation of genes methylation-silenced in a rat mammary carcinoma by integration of MeDIP-CGI microarray and expression microarray. To isolate CGI methylated in rat mammary carcinomas and identify a carcinoma with the largest number of aberrantly methylated CGI, we analyzed three primary carcinomas (PhIP7-4#1, DMBA334#1 and DMBA397#1), three carcinoma cell lines established from these primary samples, RMEC and a pool of four normal mammary glands by MeDIP-CGI microarray. Among the 5031 promoter CGI, 1745 were methylated in at least one carcinoma but not in any of the normal mammary glands. Hierarchical clustering analysis using the 1745 promoter CGI identified a group of promoter CGI methylated both in cell lines and primary carcinomas (Group A) and a group methylated mainly in carcinoma cell lines (Group B) (Fig. 1A). The number of methylated promoter CGI was the largest in the PhP7-4 cell line among the three cell lines, and 84% and 78% of the promoter CGI methylated in the DMBA334 and DMBA397 cell lines, respectively, overlapped with those in the PhIP7-4 cell line. Thus, we selected the PhIP7-4 cell line for further analysis, and isolated 465 promoter CGI methylated in this cell line but not in RMEC (Fig. 1B).

To select candidates for methylation-silenced genes, expression microarray analyses were conducted using the PhIP7-4 cell line before and after treatment with 5 μM 5-aza-dC and 100 nM TSA. The expression microarray data showed that 1705 genes

doi: 10.1111/j.1349-7006.2011.01955.x

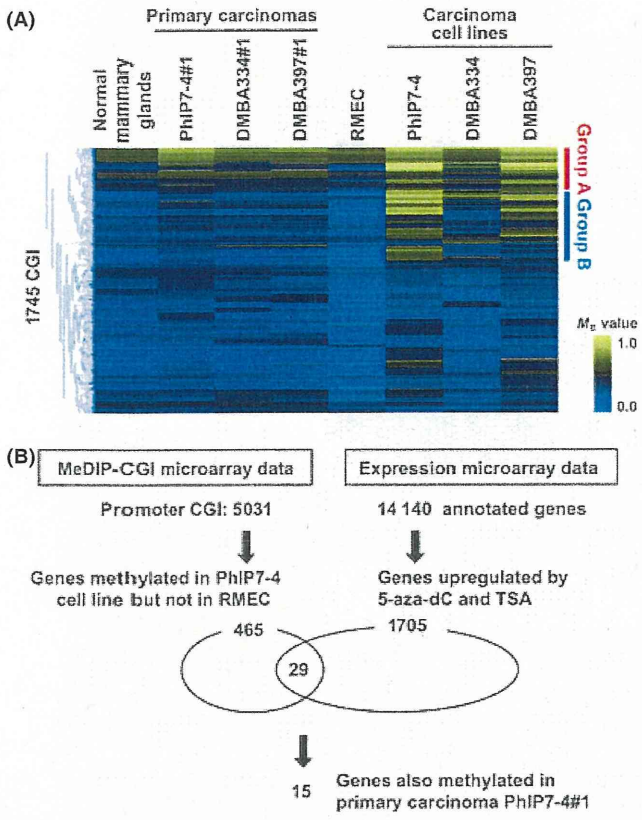

Fig. 1. Selection procedures of genes with methylated promoter CpG islands (CGI) in a rat mammary carcinoma cell line and its primary mammary carcinoma. (A) DNA methylation statuses of promoter CGI revealed by methylated DNA immunoprecipitation (MeDIP)–CGI microarray analysis. Hierarchical clustering of promoter CGI was performed using M_e values of 1745 promoter CGI methylated in at least one carcinoma and not methylated in normal mammary glands. The M_e values are represented by colors from blue (0.0) to yellow (1.0). (B) Selection procedure of genes with methylated promoter CGI in a rat mammary carcinoma cell line and then in its primary carcinoma. The number of methylated promoter CGI and that of genes upregulated by treatment of the PhIP7-4 cell line with 5-aza-2′-deoxycytidine (5-aza-dC) and trichostatin A (TSA) are shown. After integration of both sets of data, 29 candidate genes silenced in the PhIP7-4 cell line were obtained, and 15 genes were shown to be methylated also in its primary carcinoma (PhIP7-4#1). RMEC, rat mammary epithelial cells.

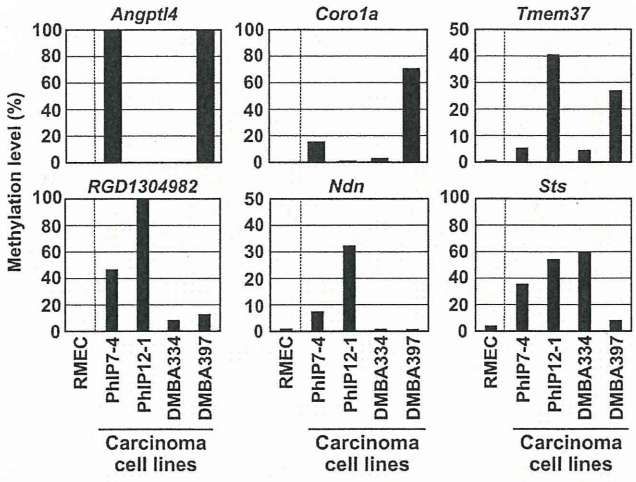

Fig. 2. Methylation levels of the six genes (*Angptl4*, *Coro1a*, *Tmem37*, *RGD1304982*, *Ndn* and *Sts*) in rat mammary carcinoma cell lines. Methylation levels of the seven genes selected by the data of methylated DNA immunoprecipitation–CpG islands (MeDIP-CGI) microarray data and expression microarray data were analyzed by quantitative methylation-specific PCR using rat mammary epithelial cells (RMEC) and the four mammary carcinoma cell lines (PhIP7-4, PhIP12-1, DMBA334 and DMBA397). Data of the six genes with high methylation levels in at least one carcinoma cell line are shown.

were upregulated twofold or more by the treatment, with *P*-values < 0.002 (Fig. 1B). By integrating the expression microarray data and the MeDIP-CGI microarray data, 29 genes were found to be methylated in the PhIP7-4 cell line and re-expressed by the epigenetic treatment. Based on the MeDIP-CGI microarray data, 14 genes methylated only in the cell line were excluded, and 15 genes methylated also in its primary tumor (PhIP7-4#1) were selected for further analysis (Fig. 1B).

Isolation of six genes methylated in rat mammary carcinoma cell lines. The data of MeDIP-microarray analysis was confirmed by conventional MSP of the PhIP7-4 cell line, and 11 of the 15 genes were methylated. Seven (*Angptl4*, *Coro1a*, *Tmem37*, *RGD1304982*, *Scin*, *Ndn* and *Sts*) of the 11 genes were not methylated in RMEC, while the remaining four were methylated (data not shown). The methylation levels of the seven genes were further quantified by qMSP in the RMEC and four carcinoma cell lines (the three cell lines used for the initial microarray analysis and PhIP12-1) (Fig. 2). Six genes (*Angptl4*, *Coro1a*, *Tmem37*, *RGD1304982*, *Ndn* and *Sts*) were highly methylated in one or more carcinoma cell lines (Fig. 2), supporting that aberrant methylation of these genes was present in epithelial cancer cells. In contrast, *Scin* had methylation levels <10% in all the carcinoma cell lines and the RMEC (data not shown).

Identification of five genes methylated in rat primary mammary carcinomas. Methylation levels of the six genes in primary carcinomas were quantified using 13 PhIP-induced primary mammary carcinomas, 12 DMBA-induced primary mammary carcinomas and seven normal mammary glands from age-matched control rats by qMSP (Fig. 3A). Two genes (*Angptl4* and *Coro1a*) were barely methylated in normal mammary glands, and were aberrantly methylated in three or more PhIP- and DMBA-induced carcinomas, showing carcinoma-specific aberrant methylation. *RGD1304982* was also barely methylated in normal mammary glands, and only one PhIP-induced carcinoma had aberrant methylation. Two genes (*Tmem37* and *Ndn*) had background methylation in normal mammary glands. *Tmem37* was aberrantly methylated in three of the 13 PhIP-induced and one of the 12 DMBA-induced carcinomas, and *Ndn* was aberrantly methylated only in two PhIP-induced carcinomas. The remaining one gene, *Sts*, was highly methylated in normal mammary glands, and carcinoma-specific methylation could not be confirmed.

The presence of dense methylation of the promoter CGI of *Angptl4* was analyzed by bisulfite sequencing of the PhIP7-4 cell line and a primary carcinoma (methylation level of 30% by qMSP) (Fig. 3B). Densely methylated DNA molecules were present in these two samples while they were absent in the RMEC and normal mammary glands (Fig. 3B).

Identification of *Angptl4* as a gene methylation silenced in rat mammary carcinomas with abundant expression in normal mammary glands. Expression in normal mammary glands was examined by qRT-PCR. *Angptl4* was highly expressed (Fig. 4A), but the other four genes (*Coro1a*, *Tmem37*, *RGD1304982* and *Ndn*) were not expressed (data not shown). *Angptl4* was not expressed in two carcinoma cell lines and

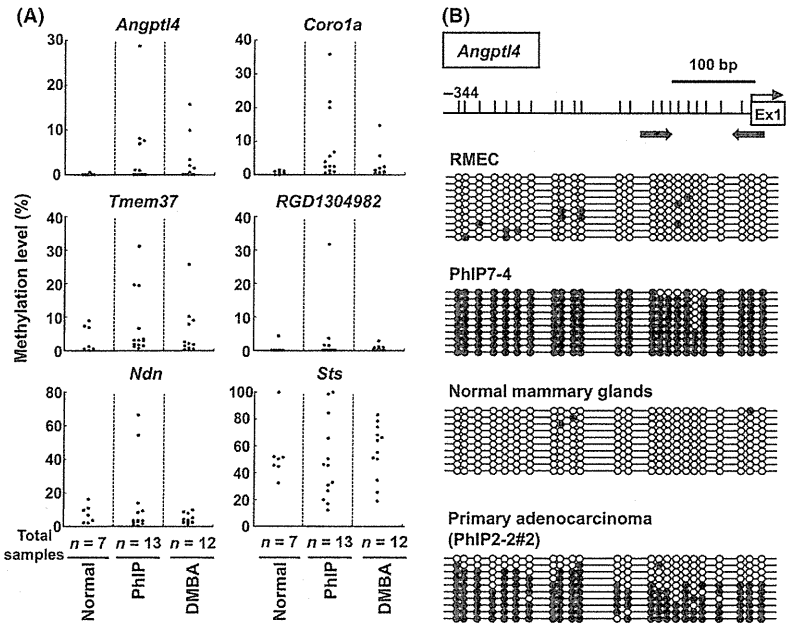

Fig. 3. Methylation of six genes in primary rat mammary carcinomas. (A) Methylation levels analyzed by quantitative methylation-specific PCR (qMSP) of seven normal mammary glands (Normal), and 13 PhIP-induced (PhIP) or 12 DMBA-induced (DMBA) primary mammary carcinomas. A carcinoma sample was considered to have aberrant methylation when its methylation level was two or more times higher than the highest methylation level in normal samples. (B) Presence of dense methylation in the PhIP7-4 cell line and a primary carcinoma, PhIP2-2#2. The methylation status of individual CpG sites (vertical lines, shown at the top) in the upstream region of *Angptl4* was determined by bisulfite sequencing. Closed circles, methylated CpG islands (CGI) sites; open circles, unmethylated CpG sites; closed arrows, location of the primers for MSP.

primary carcinomas, and unexpectedly also not in RMEC (Fig. 4A). Re-expression after the treatment of PhIP7-4 with 5-aza-dC and TSA was confirmed by qRT-PCR (Fig. 4B), supporting methylation silencing of *Angptl4*.

Methylation silencing of *ANGPTL4* in human breast cancer samples. Finally, we investigated methylation silencing of human *ANGPTL4*, which has a promoter CGI, as does rat *Angptl4*, in human breast cancers. *ANGPTL4* was expressed in two kinds of mammary epithelial cells (HMEC and MCF10A) that had unmethylated DNA (Fig. 5A). Among eight breast cancer cell lines, *ANGPTL4* was highly methylated in three cell lines (MDA-MB-231, MDA-MB-468 and BT-474), and had little expression in these cell lines (Fig. 5A). *ANGPTL4* was re-expressed by 5-aza-dC treatment of BT-474 and MDA-MB-231, showing that *ANGPTL4* was also silenced by methylation in human breast cancer cell lines (Fig. 5B).

Methylation of *ANGPTL4* was analyzed in 91 primary human breast cancers and non-cancerous breast tissues of 21 cancer patients by qMSP. Using a cut-off value of 10%,[25] methylation was detected in 11 of the 91 breast cancers (12%), while little methylation was detected in the non-cancerous tissues (Fig. 6). The methylation status in cancer was not associated with any clinicopathological characteristics, including age, clinical stage, tumor size, estrogen receptor status, progesterone receptor status, HER2 expression and recurrence (data not shown). These data showed that methylation silencing of *Angptl4* is commonly present in mammary carcinomas of both rats and humans.

Discussion

In the present study, genes with aberrant methylation of promoter CGI in rat primary mammary carcinomas were identified for the first time, and *Angptl4* was demonstrated as a novel methylation-silenced gene both in rat and human mammary carcinomas. The combination of the MeDIP-CGI microarray analysis and expression microarray analysis after epigenetic treatment was effective in reducing the number of methylated genes that were not methylation silenced.

ANGPTL4 is a secreted protein of the angiopoietin-like family, involved in lipid metabolism,[26] and upregulated by hypoxia.[27] It is known to be silenced in human gastric cancers[28] and in human melanoma cell lines.[29] The role of ANGPTL4 in mammary carcinomas remains controversial. Padua *et al.*,[30] reported that overexpression of ANGPTL4 mediated lung metastasis of estrogen receptor-negative breast cancer cells. In contrast, Foreman *et al.* and Girroir *et al.*[31,32] reported that *Angptl4* was upregulated by PPARβ/∂ activation, and inhibited growth of human and mouse mammary carcinoma cell lines. Here we found that *Angptl4* was expressed in rat mammary glands and human mammary epithelial cells, and methylation silenced in rat mammary carcinomas and human breast cancers. Together with Foreman *et al.* and Girroir *et al.* findings, *Angptl4* was suggested to be a tumor-suppressor gene.

The other four genes (*Coro1a*, *Tmem37*, *RGD1304982* and *Ndn*) were likely to have been methylated as a consequence of rat mammary carcinogenesis because they were not expressed in normal mammary glands. However, several interesting features have been reported about *Ndn* and *RGD1304982*. Human *NDN* is reported to suppress the growth of osteosarcoma cells, have anti-angiogenic effects both *in vitro* and *in vivo*, and interact with tumor suppressor p53.[33,34] A putative quinone oxidoreductase-like protein 2, encoded by *RGD1304982*, has homology to human NAD(P)H:quinone oxidoreductase 1 (*NQO1*) and NRH:quinone oxidoreductase 2 (*NQO2*), which are known to stabilize p53.[35] The possibility remains that *Ndn* and *RGD1304982* expression is induced in response to cellular stresses in normal mammary glands, and that these two genes function as tumor suppressors.

doi: 10.1111/j.1349-7006.2011.01955.x

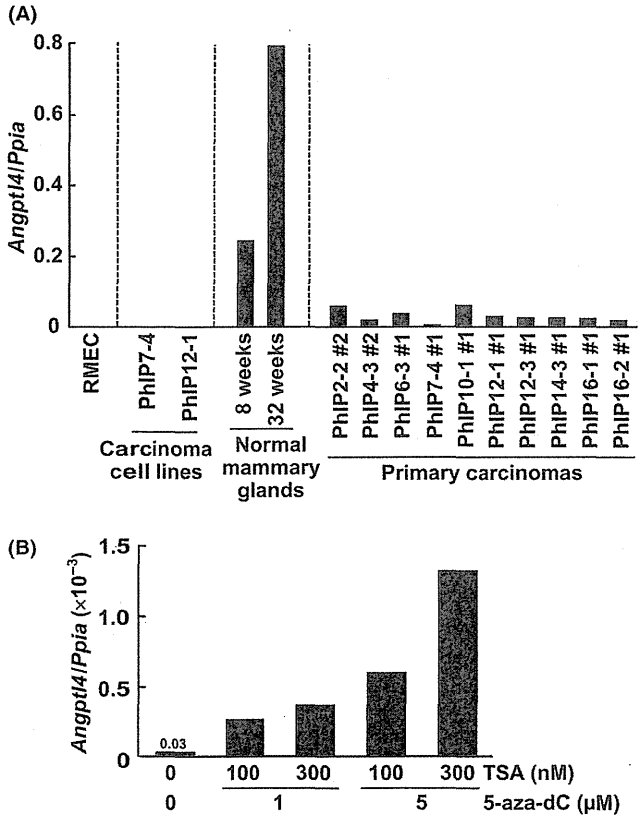

Fig. 4. Expression analysis of rat *Angptl4*. (A) *Angptl4* expression in rat mammary epithelial cells (RMEC), normal mammary glands, carcinoma cell lines (PhIP7-4 and PhIP12-1) and 10 PhIP-induced primary carcinomas analyzed by qRT-PCR. *Angptl4* expression was lost in the cell lines, and decreased in all the carcinomas. (B) Re-expression of *Angptl4* after epigenetic treatment of PhIP7-4 cells with 5-aza-2′-deoxycytidine (5-aza-dC) and trichostatin A (TSA). *Angptl4* re-expression was confirmed.

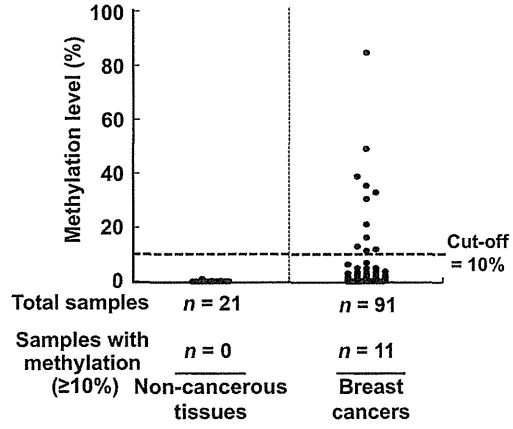

Fig. 6. Methylation levels of human *ANGPTL4* obtained by quantitative methylation-specific PCR (qMSP) of primary human tissue samples. Methylation was barely observed in non-cancerous tissues, but high methylation levels were observed in breast cancer tissues. Using a threshold of 10%, 11 cancers showed aberrant methylation.

Two (*Tmem37* and *Ndn*) of the five genes aberrantly methylated in mammary carcinomas were slightly methylated in normal-appearing mammary glands of old rats (56–69 weeks old) (Fig. 3A), which did not contain cancerous tissues. This aberrant methylation was not observed in mammary glands of young rats (8 weeks old) (data not shown), suggesting that these two genes are methylated in an age-dependent manner. It is known that age-dependent methylation can be accelerated by inflammation in the colon,[36] and there is a possibility that these genes can be used as efficient markers to assess the effects of possible inducers of aberrant methylation during mammary carcinogenesis.

Four rat primary carcinomas and two human breast cancer cell lines (T-47D and Hs578T) did not express *ANGPTL4*, although their DNA was unmethylated (Figs 4A, 5A). It is frequently observed that a gene methylation silenced in some cancer cell lines is not expressed in other cell lines by mechanisms other than CGI methylation, such as loss of transcription factors or signal dysregulation.[37] In the case of *ANGPTL4*, its expression is known to be stimulated by TGFß signaling,[30] which is disrupted in various kinds of cancers. *Angptl4* was not expressed even in RMEC (Fig. 4A), suggesting that under *in vitro* culture conditions factors required for *ANGPTL4* expression are lacking.

Methodologically, we integrated the MeDIP-CGI microarray data and those by expression microarray analysis after epigenetic treatment to identify methylation-silenced genes. The integration effectively reduced the number of candidate genes from

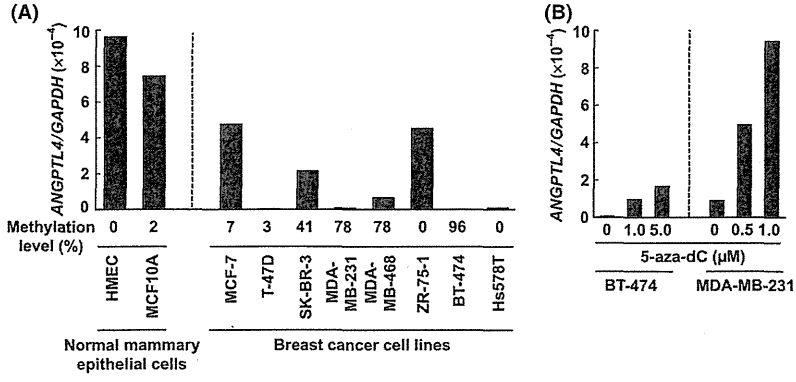

Fig. 5. Methylation silencing of human *ANGPTL4*. (A) Expression levels of human *ANGPTL4* in normal mammary epithelial cells and cancer cell lines by qRT-PCR, along with its methylation levels by qMSP. *ANGPTL4* expression was very low in the three cell lines (MDA-MB-231, MDA-MB-468 and BT-474) with high methylation levels. (B) Re-expression of *ANGPTL4* after treatment of breast cancer cell lines (BT-474 and MDA-MB-231) with 5-aza-2′-deoxycytidine (5-aza-dC).

465 (by the MeDIP-CGI microarray analysis) and 1705 (by the expression microarray analysis) after epigenetic treatment) to 29 genes. The MeDIP-CGI microarray can isolate methylated CGI, but these are not always located in genomic regions important for gene silencing.[19] The expression microarray analysis after epigenetic treatment, also known as chemical genomic screening[14,38] or pharmacological unmasking,[39] is a simple and cost-effective method in identifying methylation-silenced genes using cell lines.[14,29,38] However, genes isolated by this method are known to contain many genes that were induced by the actions of 5-aza-dC other than DNA demethylation, such as activation of the p53 pathway.[40] The approach taken here is a more effective way to identify methylation-silenced genes by reducing the number of methylated genes that are not methylation silenced.

A rat CGI microarray was custom designed in the present study. Its use successfully led to identification of a novel methylation-silenced gene not only in rat but also in human mammary carcinomas. Rats are widely used in the fields of biomedical research, such as cancers, toxicology, physiology and cardiovascular diseases, and a large amount of data using rat models has been accumulated.[41,42] The rat CGI microarray can be used for various epigenetic research in rats.

In conclusion, this is the first study that identified genes aberrantly methylated in rat mammary carcinomas. *Angptl4* is a novel methylation-silenced gene both in rat and human mammary carcinomas.

Acknowledgments

N.H. is the recipient of a Research Resident Fellowship from the Foundation for Promotion of Cancer Research. This study was supported by a Grant-in-Aid for the Third-Term Comprehensive Cancer Control Strategy from the Ministry of Health, Labour and Welfare, Japan, and by the A3 Foresight Program from the Japan Society for the Promotion of Science.

Disclosure Statement

The authors have no conflict of interest.

References

1 Esteller M. Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat Rev Genet* 2007; **8**: 286–98.
2 Jones PA, Baylin SB. The epigenomics of cancer. *Cell* 2007; **128**: 683–92.
3 Linhart HG, Lin H, Yamada Y *et al.* Dnmt3b promotes tumorigenesis *in vivo* by gene-specific de novo methylation and transcriptional silencing. *Genes Dev* 2007; **21**: 3110–22.
4 Hinshelwood RA, Clark SJ. Breast cancer epigenetics: normal human mammary epithelial cells as a model system. *J Mol Med* 2008; **86**: 1315–28.
5 Yan PS, Venkataramu C, Ibrahim A *et al.* Mapping geographic zones of cancer risk with epigenetic biomarkers in normal breast tissue. *Clin Cancer Res* 2006; **12**: 6626–36.
6 Cheng AS, Culhane AC, Chan MW *et al.* Epithelial progeny of estrogen-exposed breast progenitor cells display a cancer-like methylome. *Cancer Res* 2008; **68**: 1786–96.
7 Weng YI, Hsu PY, Liyanarachchi S *et al.* Epigenetic influences of low-dose bisphenol A in primary human breast epithelial cells. *Toxicol Appl Pharmacol* 2010; **248**: 111–21.
8 Ghoshal A, Preisegger KH, Takayama S *et al.* Induction of mammary tumors in female Sprague–Dawley rats by the food-derived carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine and effect of dietary fat. *Carcinogenesis* 1994; **15**: 2429–33.
9 Snyderwine EG, Thorgeirsson UP, Venugopal M *et al.* Mammary gland carcinogenicity of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine in Sprague-Dawley rats on high- and low-fat diets. *Nutr Cancer* 1998; **31**: 160–7.
10 Russo J, Tay LK, Ciocca DR *et al.* Molecular and cellular basis of the mammary gland susceptibility to carcinogenesis. *Environ Health Perspect* 1983; **49**: 185–99.
11 Sukumar S, McKenzie K, Chen Y. Animal models for breast cancer. *Mutat Res* 1995; **333**: 37–44.
12 Imaoka T, Nishimura M, Iizuka D *et al.* Radiation-induced mammary carcinogenesis in rodent models: what's different from chemical carcinogenesis? *J Radiat Res (Tokyo)* 2009; **50**: 281–93.
13 Russo J, Russo IH. Atlas and histologic classification of tumors of the rat mammary gland. *J Mammary Gland Biol Neoplasia* 2000; **5**: 187–200.
14 Suzuki H, Gabrielson E, Chen W *et al.* A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer. *Nat Genet* 2002; **31**: 141–9.
15 Okochi E, Watanabe N, Sugimura T *et al.* Single nucleotide instability: a wide involvement in human and rat mammary carcinogenesis? *Mutat Res* 2002; **507**: 101–11.
16 Kuramoto T, Morimura K, Yamashita S *et al.* Etiology-specific gene expression profiles in rat mammary carcinomas. *Cancer Res* 2002; **62**: 3592–7.
17 Okochi E, Watanabe N, Shimada Y *et al.* Preferential induction of guanine deletion at 5′-GGGA-3′ in rat mammary glands by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine. *Carcinogenesis* 1999; **20**: 1933–8.
18 Watanabe N, Okochi E, Hirayama Y *et al.* Single nucleotide instability without microsatellite instability in rat mammary carcinomas. *Cancer Res* 2001; **61**: 2632–40.
19 Yamashita S, Hosoya K, Gyobu K *et al.* Development of a novel output value for quantitative assessment in methylated DNA immunoprecipitation-CpG island microarray analysis. *DNA Res* 2009; **16**: 275–86.
20 Takeshima H, Yamashita S, Shimazu T *et al.* The presence of RNA polymerase II, active or stalled, predicts epigenetic fate of promoter CpG islands. *Genome Res* 2009; **19**: 1974–82.
21 Yuan GC, Liu YJ, Dion MF *et al.* Genome-scale identification of nucleosome positions in *S. cerevisiae*. *Science* 2005; **309**: 626–30.
22 Yamashita S, Takahashi S, McDonell N *et al.* Methylation silencing of transforming growth factor-beta receptor type II in rat prostate cancers. *Cancer Res* 2008; **68**: 2112–21.
23 Niwa T, Tsukamoto T, Toyoda T *et al.* Inflammatory processes triggered by *Helicobacter pylori* infection cause aberrant DNA methylation in gastric epithelial cells. *Cancer Res* 2010; **70**: 1430–40.
24 Weisinger G, Gavish M, Mazurika C *et al.* Transcription of actin, cyclophilin and glyceraldehyde phosphate dehydrogenase genes: tissue- and treatment-specificity. *Biochim Biophys Acta* 1999; **1446**: 225–32.
25 Terada K, Okochi-Takada E, Akashi-Tanaka S *et al.* Association between frequent CpG island methylation and *HER2* amplification in human breast cancers. *Carcinogenesis* 2009; **30**: 466–71.
26 Mandard S, Zandbergen F, van Straten E *et al.* The fasting-induced adipose factor/angiopoietin-like protein 4 is physically associated with lipoproteins and governs plasma lipid levels and adiposity. *J Biol Chem* 2006; **281**: 934–44.
27 Le Jan S, Amy C, Cazes A *et al.* Angiopoietin-like 4 is a proangiogenic factor produced during ischemia and in conventional renal cell carcinoma. *Am J Pathol* 2003; **162**: 1521–8.
28 Kaneda A, Kaminishi M, Yanagihara K *et al.* Identification of silencing of nine genes in human gastric cancers. *Cancer Res* 2002; **62**: 6645–50.
29 Nobeyama Y, Okochi-Takada E, Furuta J *et al.* Silencing of tissue factor pathway inhibitor-2 gene in malignant melanomas. *Int J Cancer* 2007; **121**: 301–7.
30 Padua D, Zhang XH, Wang Q *et al.* TGFbeta primes breast tumors for lung metastasis seeding through angiopoietin-like 4. *Cell* 2008; **133**: 66–77.
31 Foreman JE, Sharma AK, Amin S *et al.* Ligand activation of peroxisome proliferator-activated receptor-beta/delta (PPARbeta/delta) inhibits cell growth in a mouse mammary gland cancer cell line. *Cancer Lett* 2009; **288**: 219–25.
32 Girroir EE, Hollingshead HE, Billin AN *et al.* Peroxisome proliferator-activated receptor-beta/delta (PPARbeta/delta) ligands inhibit growth of UACC903 and MCF7 human cancer cell lines. *Toxicology* 2008; **243**: 236–43.
33 Shibui T, Higo Y, Tsutsui TW *et al.* Changes in expression of imprinted genes following treatment of human cancer cell lines with non-mutagenic or mutagenic carcinogens. *Int J Oncol* 2008; **33**: 351–60.
34 Taniura H, Kobayashi M, Yoshikawa K. Functional domains of necdin for protein-protein interaction, nuclear matrix targeting, and cell growth suppression. *J Cell Biochem* 2005; **94**: 804–15.
35 Gong X, Kole L, Iskander K *et al.* NRH:quinone oxidoreductase 2 and NAD(P)H:quinone oxidoreductase 1 protect tumor suppressor p53 against 20s proteasomal degradation leading to stabilization and activation of p53. *Cancer Res* 2007; **67**: 5380–8.
36 Issa JP, Ahuja N, Toyota M *et al.* Accelerated age-related CpG island methylation in ulcerative colitis. *Cancer Res* 2001; **61**: 3573–7.
37 Fatemi M, Pao MM, Jeong S *et al.* Footprinting of mammalian promoters: use of a CpG DNA methyltransferase revealing nucleosome positions at a single molecule level. *Nucleic Acids Res* 2005; **33**: e176.

doi: 10.1111/j.1349-7006.2011.01955.x

38 Yamashita S, Tsujino Y, Moriguchi K *et al.* Chemical genomic screening for methylation-silenced genes in gastric cancer cell lines using 5-aza-2′-deoxycytidine treatment and oligonucleotide microarray. *Cancer Sci* 2006; **97**: 64–71.
39 Yamashita K, Upadhyay S, Osada M *et al.* Pharmacologic unmasking of epigenetically silenced tumor suppressor genes in esophageal squamous cell carcinoma. *Cancer Cell* 2002; **2**: 485–95.
40 Wang H, Zhao Y, Li L *et al.* An ATM- and Rad3-related (ATR) signaling pathway and a phosphorylation-acetylation cascade are involved in activation of p53/p21Waf1/Cip1 in response to 5-aza-2′-deoxycytidine treatment. *J Biol Chem* 2008; **283**: 2564–74.
41 Jacob HJ. Functional genomics and rat models. *Genome Res* 1999; **9**: 1013–6.
42 Toyokuni S. Iron and carcinogenesis: from Fenton reaction to target genes. *Redox Rep* 2002; **7**: 189–97.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primers for MSP, RT-PCR and bisulfite sequencing.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

Carcinogenesis vol.32 no.1 pp.35–41, 2011
doi:10.1093/carcin/bgq219
Advance Access publication October 27, 2010

Insufficient role of cell proliferation in aberrant DNA methylation induction and involvement of specific types of inflammation

Keun Hur, Tohru Niwa, Takeshi Toyoda[1,2], Tetsuya Tsukamoto[1,3], Masae Tatematsu[1], Han-Kwang Yang[4] and Toshikazu Ushijima*

Carcinogenesis Division, National Cancer Center Research Institute, 1-1 Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan, [1]Division of Oncological Pathology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa, Nagoya 464-8681, Japan, [2]Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan, [3]Department of Pathology, Fujita Health University School of Medicine, 1-98 Dengakugakubo, Kutsukake-cho, Toyoake, Aichi 470-1192, Japan and [4]Department of Surgery and Cancer Research Institute, Seoul National University, College of Medicine, 28 Yeongeon-dong, Jongno-gu, Seoul 110-744, Korea

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

Chronic inflammation is deeply involved in induction of aberrant DNA methylation, but it is unclear whether any type of persistent inflammation can induce methylation and how induction of cell proliferation is involved. In this study, Mongolian gerbils were treated with five kinds of inflammation inducers [*Helicobacter pylori* with cytotoxin-associated gene A (CagA), *H.pylori* without CagA, *Helicobacter felis*, 50% ethanol (EtOH) and saturated sodium chloride (NaCl) solution]. Two control groups were treated with a mutagenic carcinogen that induces little inflammation (20 p.p.m. of *N*-methyl-*N*-nitrosourea) and without any treatment. After 20 weeks, chronic inflammation with lymphocyte and macrophage infiltration was prominent in the three *Helicobacter* groups, whereas neutrophil infiltration was mainly observed in the EtOH and NaCl groups. Methylation levels of eight CpG islands significantly increased only in the three *Helicobacter* groups. By Ki-67 staining, cell proliferation was most strongly induced in the NaCl group, demonstrating that induction of cell proliferation is not sufficient for methylation induction. Among the inflammation-related genes, *Il1b*, *Nos2* and *Tnf* showed increased expression specifically in the three *Helicobacter* groups. In human gastric mucosae infected by *H.pylori*, *NOS2* and *TNF* were also increased. These data showed that inflammation due to infection of the three *Helicobacter* strains has a strong potential to induce methylation, regardless of their CagA statuses, and increased cell proliferation was not sufficient for methylation induction. It was suggested that specific types of inflammation characterized by expression of specific inflammation-related genes, along with increased cell proliferation, are necessary for methylation induction.

Introduction

Aberrant DNA methylation of promoter CpG islands (CGIs) is deeply involved in human carcinogenesis (1,2). As inducers of aberrant DNA methylation, aging and chronic inflammation have been suggested because methylation was present in colonic tissues of the aged (3) and patients with long-standing ulcerative colitis (4–6), in the liver with chronic hepatitis (7) and in gastric tissues with *Helicobacter pylori* (*H.pylori*)-induced gastritis (8,9). Especially in the stomach, accumulation levels of aberrant methylation correlate with risk of gastric cancers (8,10–12). Chronic inflammation is characterized by transition of inflammatory cell types from polymorphonuclear cells (mainly neutrophils) to mononuclear cells (lymphocytes and macrophages) and persistent cell proliferation (13). However, it is still unclear whether chronic inflammation with infiltration of mononuclear cells and expression of specific genes or simply persistent inflammation is important for methylation induction and how cell proliferation is involved in it.

As an animal model for methylation induction, we recently demonstrated that inflammation triggered by *H.pylori* infection induces aberrant methylation in the stomach of Mongolian gerbils (*Meriones unguiculatus*) (14). In the gerbil stomach, *H.pylori* with a bacterial virulence factor, cytotoxin-associated gene A (CagA), which is associated with a high risk of human gastric cancers (15), can induce more severe inflammation than that without (16). *Helicobacter felis*, which does not possess CagA (17), can induce chronic gastritis without direct damage of epithelial cells (18,19). High concentrations of ethanol (EtOH) and sodium chloride (NaCl) can induce gastric erosion associated with inflammation (20–22). Their repeated administration can induce persistent inflammation with cell proliferation without transition of inflammatory cell types. In contrast, little inflammation is induced by *N*-methyl-*N*-nitrosourea (MNU), a mutagenic gastric carcinogen (23).

Regarding inflammation-related genes, high expression of *IFNG*, *IL1B*, *TNF*, *NOS2* and *COX2* has been reported in human gastritis induced by *H.pylori* infection (24,25). Also in gerbils, high expression of *Ifng*, *Il1b*, *Cox2* and *Nos2* has been observed (26,27). Our previous time-course study after *H.pylori* infection and eradication in gerbils showed that expression levels of *Cxcl2*, *Il1b*, *Nos2* and *Tnf* were correlated with methylation levels in gastric epithelial cells (GECs) (14). In humans, a polymorphism of *IL1B* is associated with gastric cancer risk (28) and with methylation of multiple genes in gastric cancers (29).

In this study, using five inducers of inflammation (*H.pylori* with CagA, *H.pylori* without CagA, *H.felis*, EtOH and NaCl) and a carcinogen control (MNU), we aimed to clarify the roles of transition of inflammatory cell types, induction of cell proliferation and specific inflammation-related genes in methylation induction.

Materials and methods

Preparation of Helicobacter strains

Helicobacter pylori with CagA (ATCC 43504, also known as NCTC 11637) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). *Helicobacter pylori* without CagA, SS1, was kindly provided by Professor Takashi Joh at Nagoya City University (30). *Helicobacter felis* (ATCC 49179) was also obtained from ATCC. Each strain was inoculated in Brucella broth (Becton Dickson, Cockeysville, MD) with 7% vol/vol heat-inactivated fetal bovine serum and incubated at 37°C under microaerobic conditions using an AnaeroPack Campylo (Mitsubishi Gas Chemical, Tokyo, Japan) for 24 h. For the culture of *H.felis*, 0.1% wt/vol of BactoAgar (Becton Dickson) was supplemented. Before harvesting bacteria, their mobility and shape were confirmed under phase contrast microscopy.

Animal experiments and sample preparation

Five-week-old male Mongolian gerbils (MGS/Sea; Kyudo, Tosu, Japan) were randomly assigned to seven groups of eight animals each. Gerbils in groups for *Helicobacter* treatment were inoculated with ~10^8 CFU/gerbil of *H.pylori* ATCC 43504 (ATCC group), *H.pylori* SS1 (SS1 group) or *H.felis* (HF group) and were kept without further treatment. Gerbils in groups of EtOH and NaCl treatment were administered with 5 ml/kg body wt of 50% EtOH group and saturated NaCl group, respectively, by gavage twice a week from 5 to 25 weeks of age. Gerbils in the group of MNU treatment (MNU group) were administered with 20 p.p.m. of MNU (Sigma–Aldrich, St Louis, MO) in drinking water from 5 to 25 weeks of age. A control group was kept without any treatment.

Abbreviations: CagA, cytotoxin-associated gene A; CGI, CpG island; Dnmt, DNA methyltransferase; EtOH, ethanol; GEC, gastric epithelial cell; MNU, *N*-methyl-*N*-nitrosourea; NaCl, sodium chloride; PCR, polymerase chain reaction; qRT–PCR, quantitative reverse transcriptase–polymerase chain reaction.

At age 25 weeks, all the animals were killed, and their stomachs were resected. From the posterior wall of the pyloric region, GECs were isolated by the gland isolation technique (31) for DNA and RNA extraction. The anterior wall of the pyloric region was further cut into two pieces: one for RNA extraction from the mucosal and submucosal layers and the other for histological analysis. DNA and RNA were extracted as described previously (14). As controls in immunohistochemistry of DNA methyltransferases (Dnmts), adult male mice (C57BL/6N, 11 weeks of age; CLEA Japan, Tokyo, Japan) were purchased and stomachs were resected. The animal experiment protocols were approved by the Committee for Ethics in Animal Experimentation.

Histological analysis

After fixation with 10% neutral formalin, tissues were embedded in paraffin and sections at 3 μm thickness were prepared. For histological analysis, hematoxylin and eosin staining was performed by a routine method. The degrees of infiltration of mononuclear and polymorphonuclear cells, intestinal metaplasia and heterotopic proliferative glands were graded on a four-point scale (0–3; 0, no or faint; 1, mild; 2, moderate and 3, marked) as described previously (32). For immunohistochemical analysis, a rabbit anti-human Ki-67 (Clone SP6; Thermo Fisher Scientific, Fremont, CA) antibody was purchased. Rabbit anti-mouse Dnmt1 (33), Dnmt3a (34) and Dnmt3b (34) antibodies were kindly provided by Professor Shoji Tajima at Osaka University. Rehydrated sections were incubated in HistoVT one (Nacalai Tesque, Kyoto, Japan) at 80°C for 40 min to unmask the antigen. After blocking with 0.5% bovine serum albumin in phosphate-buffered saline, sections were incubated with each primary antibody overnight, and the immune complex was visualized by a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Microscopic images were captured using the BZ-9000 microscope system (Keyence, Osaka, Japan). To analyze the number of the positive cells, more than five gastric glands in at least three different optic fields were counted, and the labeling index was calculated as a percentage of the positive cells relative to the total counted cells.

Human clinical samples

Human gastric mucosae were obtained by endoscopic biopsy from 7 *H.pylori*-negative (4 men and 3 women; average age 70, ranging from 44 to 83) and 18 *H.pylori*-positive (8 men and 10 women; average age 64, ranging from 46 to 81) persons with informed consents and approval of Institutional Review Boards. Their *H.pylori* infection statuses were determined by the serum anti-*H.pylori* IgG test (SBS, Kanazawa, Japan). Endoscopic superficial gastritis was observed in six of the seven *H.pylori*-negative persons and atrophic gastritis was observed in 14 of the 18 *H.pylori*-positive cases. RNA was extracted with ISOGEN (Wako, Osaka, Japan).

Gene expression analysis

The number of complementary DNA molecules was quantified by quantitative reverse transcriptase–polymerase chain reaction (qRT–PCR) as described previously (14). The number of complementary DNA molecules obtained by gene-specific primers (supplementary Table 1 is available at *Carcinogenesis* Online) was normalized to *Gapdh* (*GAPDH*) expression.

Methylation analysis

Methylation levels of gerbil CGIs (HE6, HG2, SA9, SC3, SD2, SE3, SF12 and SH6) were analyzed by quantitative methylation-specific polymerase chain reaction (PCR) and were expressed as a percentage of methylated reference as described previously (14). Bisulfite sequencing was conducted after cloning of PCR products after bisulfite modification as described previously (14).

Statistic analysis

To evaluate significant difference between two independent groups of sample data, the Mann–Whitney *U*-test was employed.

Results

Characterization of five kinds of inflammation triggered by the inducers

Gerbils were treated with five kinds of inflammation inducers (*H.pylori* ATCC 43504, *H.pylori* SS1, *H.felis*, EtOH and saturated NaCl solution) and also with MNU (Figure 1A). By histological examination of the pyloric area, the ATCC group had marked infiltration of mononuclear and polymorphonuclear cells into mucosae and submucosae and glands with intestinal metaplasia and heterotopic proliferative glands were occasionally observed (Figure 1B and Table I). The SS1 and HF groups showed milder infiltration of polymorphonuclear and mononuclear cells, less heterotopic proliferative glands and no intestinal metaplasia. The EtOH group showed infiltration of almost only polymorphonuclear cells. The NaCl group showed no or little infiltration of inflammatory cells but had thickened lamina propria. The MNU group showed no histological inflammatory changes but also had thickened lamina propria.

The kinds of infiltrating inflammatory cells were also assessed by qRT–PCR analysis [*Cd3g* (T cell), *Emr1* (macrophage), *Ela2* (neutrophil) and *Ms4a1* (B cell)] of gastric tissues containing both mucosal and submucosal layers (Figure 1C). In the ATCC, SS1 and HF groups, expression of all the four inflammatory cell markers was markedly elevated and met the typical features of chronic inflammation, such as infiltration of mononuclear cells. The macrophage and neutrophil markers were very high in the ATCC group. In the EtOH and NaCl groups, the neutrophil marker was in the same range as in the three *Helicobacter* groups, the macrophage marker was half, and the T- and B-cell markers were almost absent, showing that the inflammation in these groups was persistent acute inflammation. In the MNU group, none of the four markers were significantly elevated. These expression data were in accordance with the histological data, except for the polymorphonuclear infiltration in the NaCl group.

Induction of DNA methylation by the three Helicobacter strains but not by EtOH and NaCl

To assess methylation in GECs (not in infiltrating leukocytes), we used eight of the 10 CGIs known to be methylated in gerbil GECs as markers because these eight CGIs (HE6, HG2, SA9, SC3, SD2, SE3, SF12 and SH6) have been shown not to be methylated in peripheral blood cells (14). First, methylation levels of these CGIs were measured by quantitative methylation-specific PCR in GECs isolated by the gland isolation technique in each group (Figure 2A). The ATCC group had high methylation levels (significant in all the eight CGIs). The SS1 and HF groups also had high methylation levels (significant in six CGIs; HE6, HG2, SA9, SD2, SF12 and SH6) but lower than the ATCC group. The EtOH, NaCl and MNU groups had no increases of methylation in any CGIs.

To confirm the presence of densely methylated DNA molecules, bisulfite sequencing of HE6 was performed in one gerbil in each group (Figure 2B). Gerbils in the ATCC, SS1 and HF groups had densely methylated DNA molecule(s), and their fractions (3, 1–2, 1 of 24, respectively) were in accordance with the methylation level obtained by quantitative methylation-specific PCR. Gerbils in the EtOH, NaCl and MNU groups had no densely methylated molecules. These data showed that aberrant methylation of these CGIs was induced only by inflammation triggered by the three *Helicobacter* strains, most potently by *H.pylori* ATCC 43504-induced inflammation but not by EtOH- or NaCl-induced inflammation.

Insufficient role of cell proliferation in methylation induction

Cell proliferation was analyzed by immunohistochemistry of Ki-67 in gastric mucosae (Figure 3A) and counting the Ki-67 labeling indices (Figure 3B). All the treatment groups showed significant increases in Ki-67 labeling indices. The three *Helicobacter*-infected groups and the NaCl-treated group showed very high Ki-67 labeling indices. The NaCl-treated group, especially which did not show increased methylation levels, showed the highest Ki-67 labeling index. This result showed that induction of cell proliferation is not sufficient to induce DNA methylation.

Inflammation-related genes associated with methylation induction

To dissect inflammation components responsible for methylation induction, qRT–PCR analysis of 10 inflammation-related genes [*Cox2*, *Cxcl2* (*MIP-2*), *Ifng*, *Il1b*, *Il2*, *Il4*, *Il6*, *Il7*, *Nos2* (*iNos*) and *Tnf* (Tnf-α)] was performed using RNA collected from gastric tissues that contained both GECs and inflammatory cells (Figure 4A). In the three *Helicobacter*-infected groups, *Il1b*, *Nos2* and *Tnf* were significantly upregulated. *Ifng*, *Il2*, *Il4* and *Il6* were significantly upregulated in the

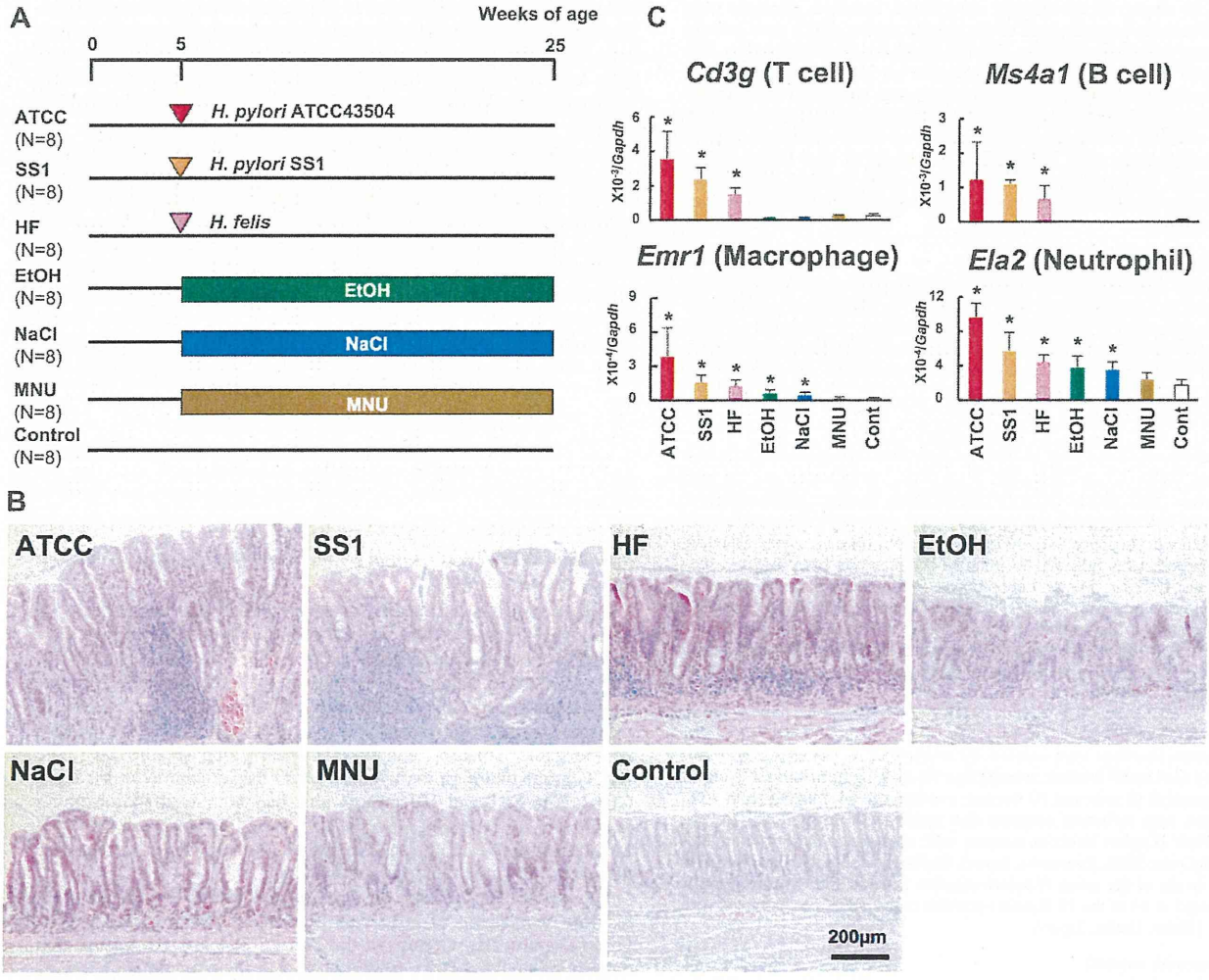

Fig. 1. Treatment of Mongolian gerbils by five inflammation inducers and MNU. (**A**) Experimental design. (**B**) Histology of gastric mucosa after treatment for 20 weeks. Transition of inflammatory cells was observed in the three *Helicobacter* groups. (**C**) Expression levels of inflammatory cell markers. Infiltration of T and B cells was prominent in the three *Helicobacter* groups. Values are shown as mean + SD. $^*P < 0.05$ compared with the control group.

Table I. Histological changes induced by the five inflammation inducers and MNU

Group	Infiltration of mononuclear cells	Infiltration of polymorphonuclear cells	Intestinal metaplasia	Heterotopic proliferative glands
ATCC	2.8 ± 0.5*	2.3 ± 0.7*	0.9 ± 0.6*	1.4 ± 0.9*
SS1	1.6 ± 0.5*	1.1 ± 0.7*	0.0 ± 0.0	0.3 ± 0.5
HF	1.6 ± 0.8*	0.7 ± 0.5*	0.0 ± 0.0	0.4 ± 0.8
EtOH	0.0 ± 0.0	0.9 ± 0.3*	0.0 ± 0.0	0.1 ± 0.3
NaCl	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
MNU	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Values are shown as mean ± SD.
$^*P < 0.01$ compared with control group.

SS1, HF, EtOH and NaCl groups but not in the ATCC group. Expression levels of these genes tended to be higher in the EtOH and NaCl groups than in the SS1 and HF groups. The MNU group did not show any significant changes compared with the control group. These results suggested that upregulation of *Il1b*, *Nos2* and *Tnf* was associated with methylation induction.

Expression of Dnmts

Dnmts are the final effectors that methylate DNA (35). To analyze the relation between expression of Dnmts and aberrant methylation induction, we conducted immunohistochemistry of Dnmts. Antibodies against mouse Dnmt1, Dnmt3a and Dnmt3b were tested in gerbils, and those against Dnmt1 and Dnmt3a were confirmed to have high sensitivity and specificity (supplementary Figure 1 is available at *Carcinogenesis* Online).

Dnmt1 protein was localized in the nuclei of GECs around the proliferative zone of gastric glands (supplementary Figures 1 and 2 are available at *Carcinogenesis* Online). In the ATCC, SS1, HF and NaCl groups, the number of GECs expressing Dnmt1 protein was markedly increased and the highest labeling index was observed in the NaCl group (Figure 4B). The profile of Dnmt1 expression was the same as that of Ki-67 (Figure 3B), indicating that Dnmt1 expression was elevated in association with increased cell proliferation. Dnmt3a protein was localized in the nuclei of most GECs except in some cells in the bottom of the glands. Although GECs expressing Dnmt3a protein significantly decreased in the ATCC, EtOH and MNU groups, the degree of decrease was small (Figure 4B and supplementary Figures 1 and 3 are available at *Carcinogenesis* Online). These results showed that the fractions of GECs expressing Dnmt1 and Dnmt3a in gastric glands were not associated with methylation induction.