Chapter 8
DNA Methylation Status in Chronic Liver Disease and Hepatocellular Carcinoma

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Abstract Alterations of DNA methylation are among the most consistent epigenetic changes observed in human cancers. In comparison with normal liver tissue, such alterations occur in a genome-wide manner in non-cancerous liver tissue showing chronic hepatitis or cirrhosis, which are widely considered to be precancerous conditions. DNA methylation alterations at the precancerous stage may rapidly generate more malignant cancers. Multiple tumor-related genes, such as the E-cadherin, HIC-1, p16, p15, TMS1/ASC, TIMP3, MGMT, RASSF1A 1, 14-3-3-σ, and SOCS-1 genes, are silenced by DNA hypermethylation in hepatocellular carcinomas (HCCs). Expression of DNA methyltransferase (DNMT) 1 is significantly higher in non-cancerous liver tissue showing chronic hepatitis or cirrhosis than in normal liver tissue. DNMT1 overexpression is also correlated with poorer tumor differentiation, portal vein involvement and intrahepatic metastasis of HCCs, and poorer patient outcome. On the other hand, overexpression of DNMT3b4, an inactive splice variant of DNMT3b, may lead to chromosomal instability through induction of DNA hypomethylation in pericentromeric satellite regions during hepatocarcinogenesis. Genome-wide DNA methylation profiling provides optimal indicators for carcinogenetic risk estimation in patients with chronic liver diseases, and for prognostication in patients with HCCs. With the intention of controlling hepatocarcinogenesis from the chronic liver disease stage, translational epigenetics have come of age.

Keywords Bacterial artificial chromosome array-based methylated CpG island amplification · DNA methylation · DNA methyltransferase · DNMT1 · DNMT3b · Precancerous condition · Prognostication · Risk estimation

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1 Introduction

DNA methylation, a covalent chemical modification resulting in addition of a methyl group at the carbon 5 position of the cytosine ring in CpG dinucleotides, is one of the most consistent epigenetic changes occurring in human cancers (Jones and Baylin 2002; Jones and Baylin 2007; Esteller 2008). DNA methyltransferases (DNMTs) transfer methyl groups from S-adenosylmethionine to cytosines. DNA methylation normally promotes a highly condensed heterochromatin structure associated with deacetylation of histones H3 and H4, loss of histone H3, lysine 4 (H3K4) methylation, and gain of H3K9 and H3K27 methylation. DNA methylation is a stable modification inherited throughout successive cell divisions, and is essential for X-chromosome inactivation, genome imprinting, silencing of transposons and other parasitic elements, and proper expression of genes (Cedar and Bergman 2009; Delcuve et al. 2009).

Human cancer cells show a drastic change in DNA methylation status, i.e., overall DNA hypomethylation and regional DNA hypermethylation. DNA hypomethylation induces chromosomal instability through decondensation of heterochromatin and enhancement of chromosomal recombination during carcinogenesis. DNA hypermethylation of CpG islands around the promoter regions silences tumor-supressor genes. Translational epigenetics have come of age (Issa and Kantarjian 2009), and empirical analysis of DNA methylation status in clinical tissue samples in connection with the clinicopathological diversity of human cancers is assuming increasing importance for the diagnosis, prevention, and therapy of cancers (Kanai and Hirohashi 2007; Kanai 2008, 2009).

2 DNA Methylation Alterations During Multistage Hepatocarcinogenesis

Although in the 1990s various genetic alterations were revealed using classical analytical techniques such as Southern blotting, especially in hepatocellular carcinomas (HCCs) that were poorly differentiated, large in size, and associated with metastasis, only a few of the molecular events occurring in the earlier stage of hepatocarcinogenesis were known. Since DNA methylation alterations may be correlated with chromosomal instability, we examined DNA methylation status on chromosome 16, which is known to be a hot spot for loss of heterozygosity (LOH) in HCCs, using Southern blotting with a DNA methylation-sensitive restriction enzyme. DNA methylation alterations at multiple loci on chromosome 16, in comparison with normal liver tissue samples obtained from patients without HCCs, were frequently revealed even in samples of non-cancerous liver tissue showing chronic hepatitis or liver cirrhosis, which are widely considered to be precancerous conditions, indicating that DNA methylation alterations are a very early event during multistage

hepatocarcinogenesis. This was one of the earliest reports of DNA methylation alterations at the precancerous stage (Kanai et al. 1996).

Since the molecular weight of DNA fragments digested using a DNA methylation-sensitive restriction enzyme was higher in HCCs than in precancerous conditions, and the intensity of larger-sized bands appeared to be enhanced in the HCCs, the numbers of methylated CpG dinucleotides and cells showing DNA hypermethylation may increase progressively as precancerous conditions develop into HCCs. The incidence of DNA hypermethylation on chromosome 16 was significantly correlated with higher histological grade, portal vein involvement, and intrahepatic metastasis of HCCs. The presence of DNA methylation alterations in both precancerous conditions and progressed HCCs suggests that DNA methylation alterations in the precancerous stage may rapidly generate more malignant cancers (Kanai et al. 1996).

The E-cadherin tumor-suppressor gene is located on 16q22.1 near the hot spots for both DNA hypermethylation and LOH in HCCs. E-cadherin acts as a Ca^{2+}-dependent cell–cell adhesion molecule in the adherens junctions of epithelial cells (Hirohashi and Kanai 2003). Significant correlations between reduced expression of E-cadherin and poor prognosis have been reported in patients with cancers. We have demonstrated that the promoter region of the E-cadherin gene shows DNA methylation in human cancer cell lines lacking E-cadherin expression, and that E-cadherin expression is induced after treatment with the DNMT inhibitor 5-azacytidine in such cell lines (Yoshiura et al. 1995). At that time, only two genes, RB and VHL, were known to be tumor-suppressor genes silenced by DNA methylation. On the basis of our data, the E-cadherin gene became chronologically the third example of a tumor-suppressor gene silenced by DNA methylation.

DNA hypermethylation around the promoter region of the E-cadherin gene has been detected even in samples of non-cancerous liver tissue showing chronic hepatitis or cirrhosis. Heterogeneous E-cadherin expression in such non-cancerous liver tissue, which is associated with small focal areas of hepatocytes showing only slight E-cadherin immunoreactivity, might be due, at least partly, to DNA hypermethylation (Kanai et al. 1997). In HCCs, we found a significant correlation between DNA hypermethylation around the promoter region and reduced expression of E-cadherin (Kanai et al. 1997). This was the first demonstration of a significant correlation between DNA hypermethylation and reduced expression in a cohort of clinical tissue samples. DNA hypermethylation around the promoter region may participate in hepatocarcinogenesis through reduction of E-cadherin expression, resulting in loss of intercellular adhesiveness and destruction of tissue morphology.

The HIC (hypermethylated-in-cancer)-1 gene at the D17S5 locus (17q13.3) was the first tumor-suppressor gene to be identified in commonly methylated chromosomal loci in human cancers. We showed that DNA hypermethylation at the D17S5 locus was frequently detectable in non-cancerous liver tissue showing chronic hepatitis or cirrhosis (Kanai et al. 1999). The incidence and degree of DNA methylation at the D17S5 locus increased progressively as precancerous conditions developed

into HCCs. The expression level of HIC-1 mRNA in non-cancerous liver tissue showing chronic hepatitis or cirrhosis was significantly lower than that in normal liver tissue, and was further decreased in HCCs (Kanai et al. 1999).

The list of tumor-related genes whose expression levels are altered due to DNA hypo- or hypermethylation during hepatocarcinogenesis has recently been increasing. Silencing of cell cycle regulators such as p16 (Matsuda et al. 1999) and p15 (Wong et al. 2000), proapoptotic proteins such as TMS1/ASC (Kubo et al. 2004), matrix metalloproteinase inhibitor TIMP3 (Yu et al. 2002) and DNA repair protein MGMT (Matsukura et al. 2003), and multifunctional tumor-suppressor proteins such as RASSF1A (Schagdarsurengin et al. 2003) and 14-3-3-σ(Iwata et al. 2000), due to DNA hypermethylation has been reported in HCCs. DNA methylation of the cytokine mediator gene SOCS-1 (Okochi et al. 2003) has attracted attention because it may activate the JAK/STAT signaling pathway and mediate the molecular linkage between inflammation and hepatocarcinogenesis.

Microdissection techniques and PCR using microsatellite markers have been developed for detecting LOH in small numbers of cells from paraffin-embedded tissue samples. LOH has been reported even in microdissected specimens from tiny precancerous lesions in several organs. In order to re-examine whether aberrant DNA methylation precedes chromosomal instability during hepatocarcinogenesis, we examined 308 microdissected specimens obtained from lobules, pseudo-lobules, and regenerative nodules in non-cancerous liver tissue from patients with HCCs, and the HCCs themselves, for LOH and microsatellite instability (MSI) using 39 microsatellite markers. In addition, using methylation-specific PCR and combined bisulfite restriction enzyme analysis, we also studied the DNA methylation status of C-type CpG islands of the p16, THBS-1, and human hMLH1 genes, and MINT 1, 2, 12, 25, and 31 clones, which are known to be methylated in a cancer-specific, but not age-dependent manner (Kondo et al. 2000). The low incidence of microsatellite instability in HCCs was compatible with absence of silencing of the hMLH1 gene by DNA hypermethylation during hepatocarcinogenesis (Kondo et al. 1999, 2000). In non-cancerous liver tissue showing chronic hepatitis, LOH for at least one marker was found in 20% of informative microdissected specimens. In non-cancerous liver tissue showing cirrhosis, LOH for at least one marker was found in 15% of informative microdissected specimens. LOH was never detected in normal liver tissue from patients without HCCs or in non-cancerous liver tissue showing no remarkable histological findings from patients with HCCs. Although no degree of DNA methylation of any of the examined CpG islands was ever detected in normal liver tissue from patients without HCCs, DNA hypermethylation was found on at least one CpG island in 58% of microdissected specimens of non-cancerous liver tissue showing no remarkable histological features obtained from patients with HCCs in which LOH was never detected (Kondo et al. 2000). The incidence of DNA hypermethylation on CpG islands overwhelmed that of LOH at all stages of chronic hepatitis, liver cirrhosis, and HCC. Thus, aberrant DNA methylation is an earlier event preceding chromosomal instability during hepatocarcinogenesis, even when examined using PCR–LOH analysis and microdissection techniques.

3 Abnormalities of DNMTs During Hepatocarcinogenesis

3.1 Overexpression of DNMT1

With respect to the molecular backgrounds of DNA methylation alterations, we focused on abnormalities of DNMTs during hepatocarcinogenesis. The major DNMT, DNMT1, shows a preference for hemimethylated over unmethylated substrates in vitro, and targets replication foci by binding to proliferating cell nuclear antigen (PCNA) (Hermann et al. 2004). Thus, DNMT1 has been recognized as a "maintenance" DNMT that allows copying of the DNA methylation pattern on the parental strand to the newly synthesized daughter DNA strand. Mutational inactivation of the DNMT1 gene that can potentially cause genome-wide alterations of DNA methylation was never detected in HCCs (Kanai et al. 2003).

On the other hand, levels of DNMT1 mRNA expression are significantly higher in samples of non-cancerous liver tissue showing chronic hepatitis or cirrhosis than in normal liver tissue, and are even higher in HCCs (Sun et al. 1997; Saito et al. 2001). The incidence of DNMT1 overexpression in HCCs is significantly correlated with poorer tumor differentiation and portal vein involvement (Saito et al. 2003). Moreover, the recurrence-free and overall survival rates of patients with HCCs showing DNMT1 overexpression are significantly lower than those of patients with HCCs that do not (Saito et al. 2003).

3.2 Splicing Alteration of DNMT3b and DNA Hypomethylation in Pericentromeric Satellite Regions

Dnmt3b is required for DNA methylation of pericentromeric satellite regions in early mouse embryos (Okano et al. 1999). DNA hypomethylation in pericentromeric satellite regions is known to result in centromeric decondensation and enhanced chromosome recombination. Germline mutations of the DNMT3b gene have been reported in patients with immunodeficiency, centromeric instability, and facial anomalies (ICF) syndrome, a rare recessive autosomal disorder characterized by DNA hypomethylation of pericentromeric satellite regions (Hansen et al. 1999). In HCCs, DNA hypomethylation of these regions is correlated with copy number alterations on chromosome 1, where satellite regions are rich (Wong et al. 2001).

The major splice variant of DNMT3b in normal liver tissue samples is DNMT3b3, which possesses the conserved catalytic domains. DNMT3b4, on the other hand, lacks the conserved catalytic domains, although it retains the N-terminal domain required for targeting to heterochromatin sites. Samples of normal liver tissue show only a trace level of DNMT3b4 expression. The levels of DNMT3b4 mRNA expression and the ratio of DNMT3b4 mRNA to DNMT3b3 in samples of non-cancerous liver tissue obtained from patients with HCCs, and in HCCs themselves, were significantly correlated with the degree of DNA hypomethylation in

pericentromeric satellite regions (Saito et al. 2002). DNA demethylation on satellite 2 was observed in DNMT3b4-transfected human epithelial 293 cells (Saito et al. 2002). Since DNMT3b4 lacking DNMT activity competes with DNMT3b3 for targeting to pericentromeric satellite regions, DNMT3b4 overexpression may lead to chromosomal instability through induction of DNA hypomethylation in such regions.

Furthermore, the growth rate of DNMT3b4 transfectants was approximately double that of mock-transfectants soon after the introduction of DNMT3b4, when chromosomal instability may not yet have accumulated. STAT 1, which acts as an effector of interferon signaling, and the genes implicated in interferon signaling, were upregulated in DNMT3b4 transfectants relative to mock-transfectants (Kanai et al. 2004). It had been reported previously that inhibition of DNA methylation in cultured human cancer cells by 5-aza-2′-deoxycytidine induces a set of genes implicated in interferon signaling, primarily via overexpression of STAT1, 2, and 3 (Karpf et al. 1999). In cancer cells, DNMT3b may act to maintain the DNA methylation status of not only pericentromeric satellite regions, but also specific genes, probably in cooperation with DNMT1, and this may explain why inhibition of DNMT3b activity by induction of DNMT3b4 produced a similar result to the general inhibition of DNA methylation obtained with 5-aza-2′-deoxycytidine. Overexpression of DNMT3b4 plays a role in multistage carcinogenesis not only by inducing chromosomal instability, but also by affecting the expression of specific genes.

4 Altered Expression of Methyl-CpG Binding Proteins (MBDs)

MBDs, such as MeCP2, MBD1, MBD2, and MBD3, bind to methylated CpG dinucleotides, and their transcriptional repression domain recruits a transcriptional co-repressor complex containing histone deacetylases (Esteller 2008). Although many researchers have focused on cross-talk between DNA methylation and histone modification, abnormalities of MBDs in human cancers do not seem to have attracted much attention. The expression level of MeCP2 mRNA in HCCs with portal vein involvement is significantly lower than that in HCCs without such involvement, suggesting that reduced expression of MeCP2 may be associated with malignant progression of HCCs (Saito et al. 2001). Reduced expression of MBD2 mRNA has been observed in HCCs, suggesting that this may be associated with a particular step in human carcinogenesis (Saito et al. 2001). Unlike other MBDs recruiting histone deacetylase complexes, MBD4 is thought to act as a thymine DNA glycosylase, repairing G:T or G:U mismatches at CpG sites. The expression level of MBD4 mRNA in HCCs is significantly lower than that in the corresponding non-cancerous liver tissue and is significantly correlated with poorer tumor differentiation and portal vein involvement (Saito et al. 2001). Reduced MBD4 expression may result in frequent C-T transitions in tumor-suppressor genes.

5 Genome-Wide DNA Methylation Analysis

5.1 DNA Methylation Alterations During Multistage Hepatocarcinogenesis Occur in a Genome-Wide Manner

Recently, we have employed bacterial artificial chromosome (BAC) array-based methylated CpG island amplification (BAMCA) (Inazawa et al. 2004) for DNA methylation analysis on a genomic-wide scale. Many researchers in this field use promoter arrays to identify genes that are methylated in cancer cells. However, the promoter regions of specific genes are not the only target of DNA methylation alterations in human cancers. Genomic regions in which DNA hypomethylation affects chromosomal instability may not be contained in promoter arrays. Moreover, aberrant DNA methylation of large chromosome regions, which are regulated in a coordinated manner in human cancers due to a process of long-range epigenetic silencing, has recently attracted attention (Frigola et al. 2006). Therefore, we used a BAC array that may be suitable for overviewing the DNA methylation status of individual large regions among all chromosomes. In fact, using BAMCA, we have shown successfully that particular DNA methylation profiles in the kidney at the precancerous stage are inherited by clear cell renal cell carcinomas developing in individual patients, and that these determine the aggressiveness of the tumors and patient outcome (Arai et al. 2009a). Diagnostic indicators for urothelial carcinomas have also been established using BAMCA (Nishiyama et al. 2009).

In samples of non-cancerous liver tissue obtained from patients with HCCs, many BAC clones showed DNA hypo- or hypermethylation (Panel N of Fig. 8.1a) in comparison with normal liver tissue from patients without HCCs (Panel C of Fig. 8.1a). Patients showing DNA hypo- or hypermethylation on more BAC clones in their non-cancerous liver tissue samples frequently developed metachronous or recurrent HCCs after hepatectomy, whereas patients showing DNA hypo- or hypermethylation on fewer BAC clones in their non-cancerous liver tissue samples rarely did so, suggesting that DNA methylation alterations at the precancerous stage may not occur randomly but are prone to development of more malignant HCCs potentially through the induction of chromosomal instability and silencing of tumor-suppressor genes (Arai et al. 2009b). In HCCs themselves, more BAC clones showed DNA hypo- or hypermethylation, the degree of which was further increased (Panel T of Fig. 8.1a) in comparison with non-cancerous liver tissue obtained from the same patients (Arai et al. 2009b).

There were no significant differences in the number of BAC clones showing DNA hypo- or hypermethylation in samples of normal liver tissue from male and female patients without HCCs, and in non-cancerous and cancerous liver tissue from male and female patients with HCCs, respectively. Wilcoxon test identified BAC clones in which DNA methylation status differed significantly between hepatitis B virus (HBV)- and hepatitis C virus (HCV)-positive patients with HCCs in both cancerous

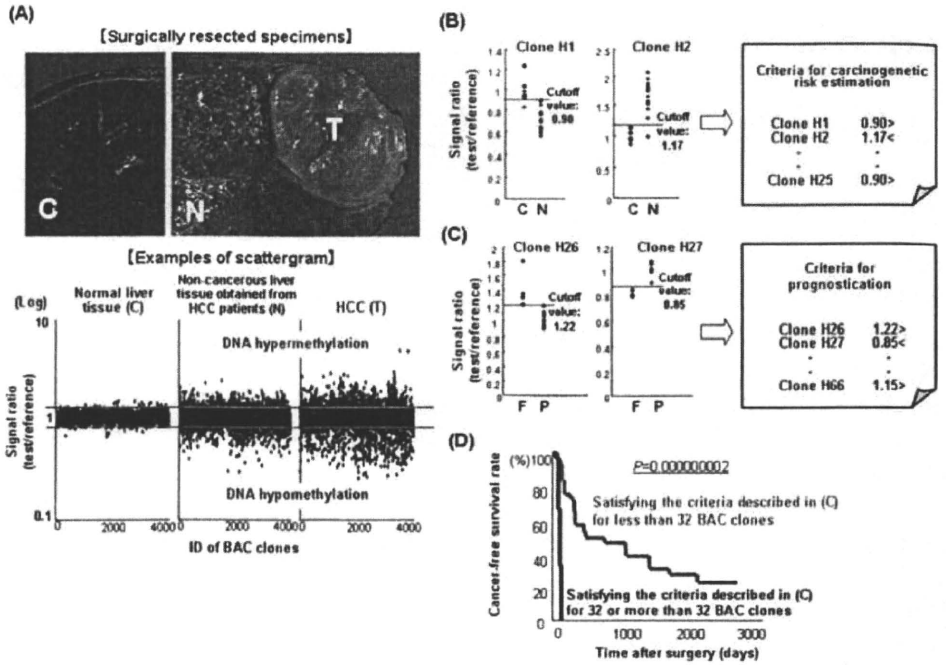

Fig. 8.1 Carcinogenetic risk estimation and prognostication based on genome-wide DNA methylation profiling. (**a**) Results of bacterial artificial chromosome (BAC) array-based methylated CpG island amplification (BAMCA). In non-cancerous liver tissue obtained from patients with HCCs (N), many BAC clones showed DNA hypo- or hypermethylation in comparison with normal liver tissue from patients without HCCs (C). In HCCs themselves (T), more BAC clones showed DNA hypo- or hypermethylation, and the degree of DNA hypo- or hypermethylation was further increased. (**b**) Cutoff values for each of the 25 BAC clones selected using a bioinformatics approach were set to discriminate non-cancerous liver tissue obtained from patients with HCCs (N) from normal liver tissue (C), and the criteria for carcinogenetic risk estimation were established using the 25 BAC clones. (**c**) Cutoff values for each of the 41 BAC clones selected using a bioinformatics approach were set to discriminate the poorer outcome group (P) from the favorable-outcome group (F), and the criteria for prognostication were established using the 41 BAC clones. (**d**) The cancer-free survival rate of patients with HCCs in the validation cohort satisfying the criteria for 32 or more BAC clones was significantly lower than that of patients with HCCs satisfying the criteria for less than 32 BAC clones

and non-cancerous liver tissue. The DNA methylation status of such BAC clones may reflect the HBV- and HCV-specific molecular mechanisms inducing DNA methylation alterations.

5.2 Carcinogenetic Risk Estimation Based on DNA Methylation Profiles

The effectiveness of surgical resection for HCC is limited, unless the disease is diagnosed early at the asymptomatic stage. Therefore, surveillance at the precancerous stage will become a priority. To reveal the baseline liver histology, microscopic

examination of liver biopsy specimens is performed in patients with HBV or HCV infection prior to interferon therapy. Therefore, carcinogenetic risk estimation using such liver biopsy specimens would be advantageous for close follow-up of patients who are at high-risk of HCC development. Since even subtle alterations of DNA methylation profiles at the precancerous stage are stably preserved on DNA double strands by covalent bonds, they may be better indicators for risk estimation than mRNA and protein expression profiles, which can be easily affected by the microenvironment of precursor cells.

To estimate the degree of carcinogenetic risk based on DNA methylation profiles, we omitted potentially insignificant BAC clones associated only with inflammation and/or fibrosis and focused on BAC clones for which DNA methylation status was inherited by HCCs from the precancerous stage, i.e., BAC clones in Groups I, II, III, and IV. Group I: BAC clones in which the average signal ratio of non-cancerous liver tissue obtained from patients with HCCs was higher than that of normal liver tissue and the average signal ratio of HCCs was even higher than that of non-cancerous liver tissue obtained from patients with HCCs. Group II: BAC clones in which the average signal ratio of non-cancerous liver tissue obtained from patients with HCCs was higher than that of normal liver tissue, and the average signal ratio of HCCs did not differ from that of non-cancerous liver tissue obtained from patients with HCCs. Group III: BAC clones in which the average signal ratio of non-cancerous liver tissue obtained from patients with HCCs was lower than that of normal liver tissue, and the average signal ratio of HCCs was even lower than that of non-cancerous liver tissue obtained from patients with HCCs. Group IV: BAC clones in which the average signal ratio of non-cancerous liver tissue obtained from patients with HCCs was lower than that of normal liver tissue, and the average signal ratio of HCCs did not differ from that of non-cancerous liver tissue obtained from patients with HCCs. From the BAC clones of Groups I, II, III, and IV, in which the DNA methylation status was inherited by HCCs from non-cancerous liver tissue, the top 25 BAC clones for which DNA methylation status was able to discriminate non-cancerous liver tissue from patients with HCCs in the learning cohort from normal liver tissue with sufficient sensitivity and specificity were identified using a bioinformatics approach (Arai et al. 2009b). By 2D hierarchical clustering analysis using these 25 BAC clones, normal liver tissue and non-cancerous liver tissue obtained from patients with HCCs in the learning cohort were successfully subclassified into different subclasses without any error. We set the cutoff values for each of the 25 BAC clones to discriminate non-cancerous liver tissue obtained from patients with HCCs in the learning cohort from normal liver tissue, and established the criteria for carcinogenetic risk estimation using the 25 BAC clones (Fig. 8.1b). Based on these criteria, both the sensitivity and specificity for diagnosis of non-cancerous liver tissue samples obtained from patients with HCCs in the learning cohort, as being at high-risk of carcinogenesis, were 100% (Arai et al. 2009b). Our criteria enabled diagnosis of additional non-cancerous liver tissue samples obtained from patients with HCCs in the validation cohort as being at high-risk of carcinogenesis with a sensitivity and specificity of 96% (Arai et al. 2009b).

The number of BAC clones satisfying the criteria in non-cancerous liver tissue samples showing chronic hepatitis obtained from patients with HCCs was not significantly different from that in non-cancerous liver tissue samples showing cirrhosis obtained from patients with HCCs. In addition, the average number of BAC clones satisfying the criteria was significantly lower in samples of liver tissue obtained from patients who were infected with HBV or HCV, but who had never developed HCCs, than that in non-cancerous liver tissue samples obtained from patients with HCCs. Our criteria not only discriminated non-cancerous liver tissue obtained from patients with HCCs from normal liver tissue, but may also be applicable for classifying liver tissue obtained from patients who are followed up because of HBV or HCV infection, chronic hepatitis, or cirrhosis into that which may generate HCCs and that which will not (Arai et al. 2009b). We intend to validate the reliability of such risk estimation prospectively using liver biopsy specimens obtained prior to interferon therapy from a large cohort of patients.

5.3 Prognostication of Patients with HCCs Based on DNA Methylation Profiles

To establish criteria for prognostication of patients with HCCs, in the learning cohort, HCC samples obtained from patients who had survived more than 4 years after hepatectomy and HCC samples obtained from patients who had suffered recurrence within 6 months and died within a year after hepatectomy were defined as a favorable-outcome group and a poor-outcome group, respectively. Wilcoxon test revealed that the signal ratios of 41 BAC clones differed significantly between the two groups (Arai et al. 2009b). By 2D hierarchical clustering analysis using the 41 BAC clones, HCCs in two groups were subclassified into different subclasses without any error. We set cutoff values for each of the 41 BAC clones to discriminate the poor-outcome group in the learning cohort from the favorable-outcome group, and established criteria for prognostication using the 41 BAC clones (Fig. 8.1c). Multivariate analysis revealed that satisfying the criteria for 32 or more BAC clones was a predictor of recurrence, and was independent of parameters that are already known to have prognostic impact, such as histological differentiation, portal vein tumor thrombi, intrahepatic metastasis, and multicentricity (Arai et al. 2009b).

To confirm these criteria, additional HCC samples were analyzed by BAMCA as a validation study. The cancer-free (Fig. 8.1d) and overall survival rates of patients with HCCs satisfying the criteria for 32 or more BAC clones were significantly lower than those of patients with HCCs satisfying the criteria for less than 32 BAC clones. Such prognostication using liver biopsy specimens obtained before transarterial embolization and radiofrequency ablation may be advantageous even for patients who undergo such therapies. The reliability of such prognostication needs to be validated again prospectively using surgically resected specimens or biopsy specimens.

6 Perspective

DNA methylation alterations associated with DNA methyltransferase abnormalities, such as overexpression of DNMT1 and splicing alterations of DNMT3b, may participate in multistage hepatocaricnogenesis from the precancerous stage to the malignant progression stage. DNA methylation alterations at the precancerous stage may rapidly generate more malignant HCCs. Genome-wide DNA methylation profiling can provide optimal indicators for carcinogenetic risk estimation and prognostication using surgically resected specimens or liver biopsy specimens. Elucidation of the molecular backgrounds of DNA methylation alterations in chronic liver disease may provide clues for epigenetic prevention and therapy of HCCs.

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

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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 $\sim 10^8$ 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 1). 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

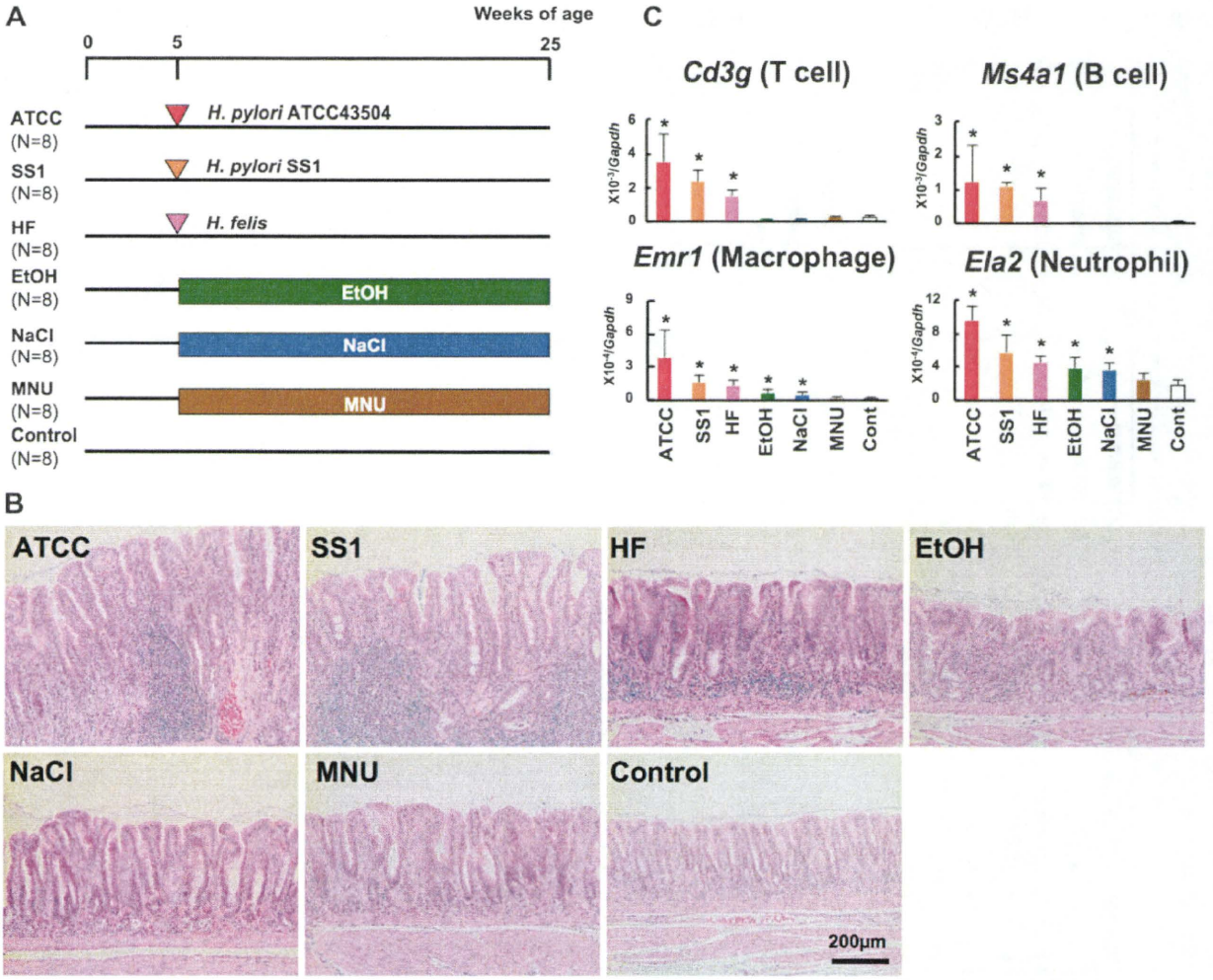

Fig. 1. Treatment of Mongolian gerbils by five inflammation inducers and MNU. (**A**) Experimental design. (**B**) Histology of gastric mucosa after treatment for 20 weeks. Transition of inflammatory cells was observed in the three *Helicobacter* groups. (**C**) Expression levels of inflammatory cell 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.

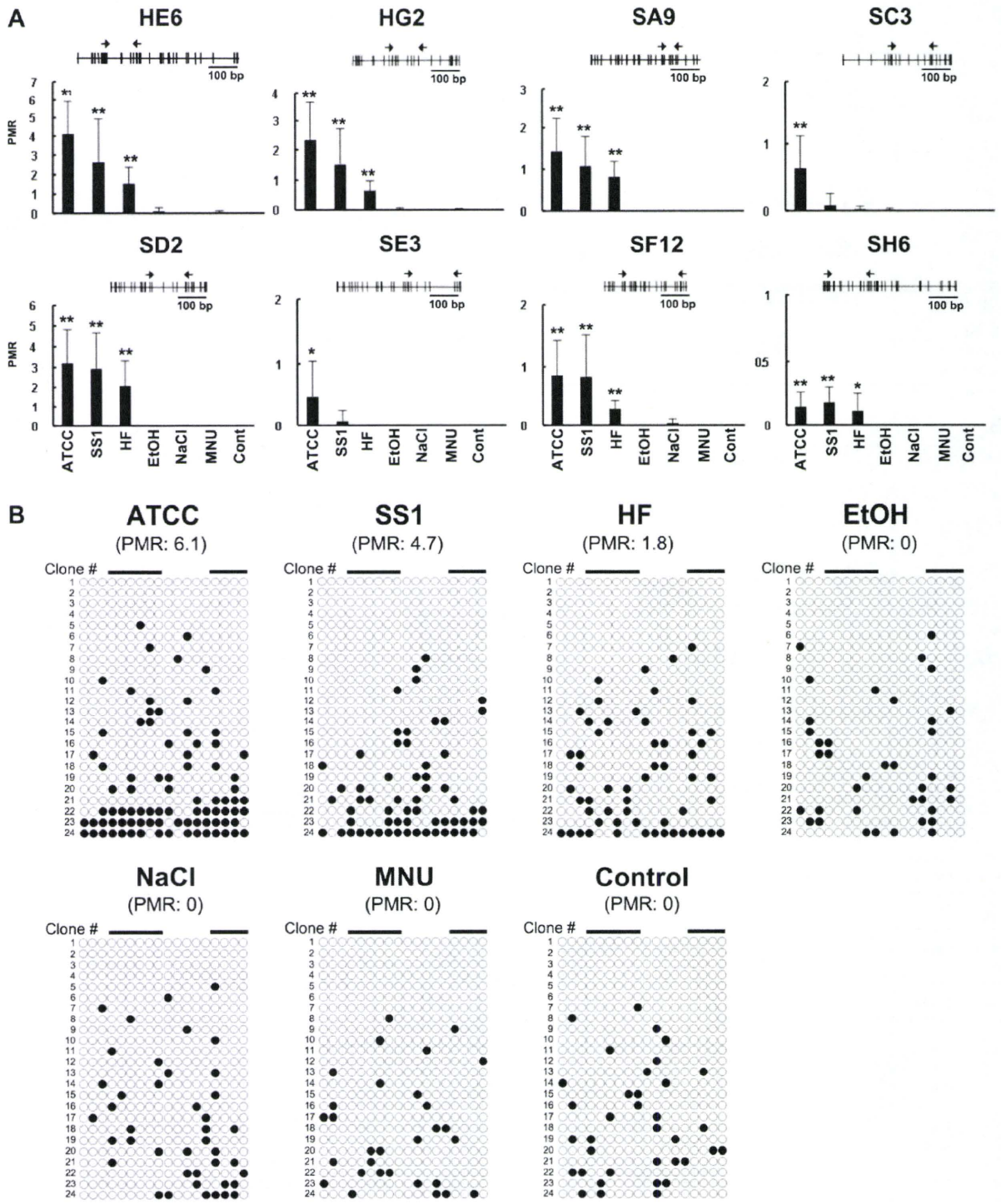

Fig. 2. Methylation induction in GECs by the three *Helicobacter*-induced inflammation but not by EtOH- or NaCl-induced inflammation. (**A**) Methylation levels of eight CGIs assessed by quantitative methylation-specific PCR. Upper panels show CpG maps, and lower panels show methylation levels in percentage of methylated reference. In the upper panel, vertical lines and arrows show individual CpG sites and positions of methylation-specific PCR primers, respectively. Values are shown as mean + SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group. (**B**) Bisulfite sequencing of HE6 in GECs. Numbers in parentheses indicate percentage of methylated reference of the sample assessed by quantitative methylation-specific PCR. Bars, CpG sites on quantitative methylation-specific PCR primers.

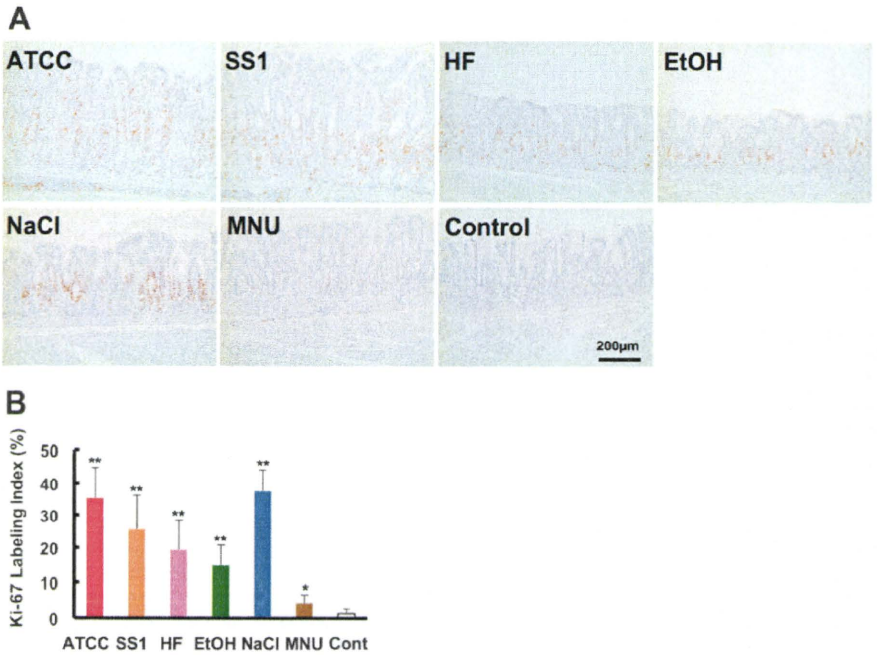

Fig. 3. Cell proliferation of gerbil GECs after the treatment. (**A**) Representative microscopic appearance of Ki-67 immunohistochemistry. (**B**) Ki-67 labeling index. Values are shown as mean + SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group. The NaCl group showed a marked increase of cell proliferation.

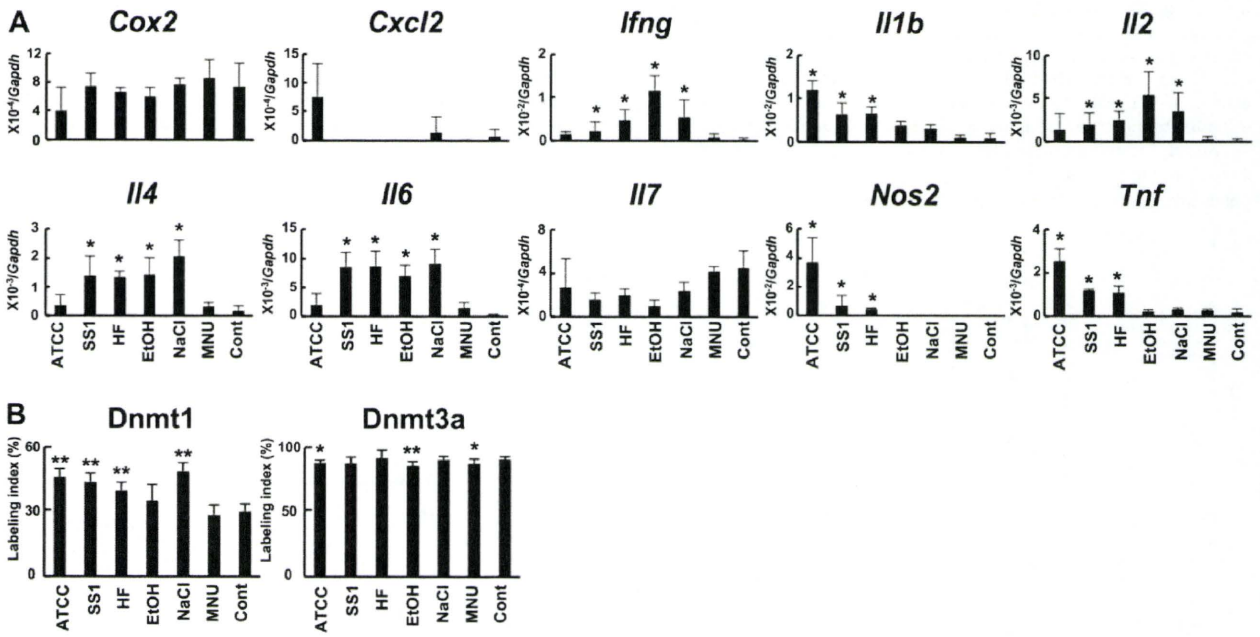

Fig. 4. Expression of inflammation-related genes and Dnmts in the gerbil stomach. (**A**) messenger RNA levels of inflammation-related genes in gerbil gastric tissues containing both mucosal and submucosal layers. Expression levels of *Il1b*, *Nos2* and *Tnf* were elevated only in the three *Helicobacter* groups. (**B**) The fractions of GECs expressing Dnmt proteins in gastric glands by immunohistochemistry. Values are shown as mean + SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control group.

Human relevance of inflammation-related gene expression

To address whether upregulation of specific inflammation-related genes are common in the human stomach, we conducted qRT–PCR of *COX2*, *IFNG*, *IL1B*, *IL6*, *NOS2* and *TNF* using human gastric mucosa samples with and without *H.pylori* infection. Expression levels of *NOS2* and *TNF* were markedly upregulated (27- and 3-fold,

respectively) also in human gastric mucosae (Figure 5). However, *IL1B* expression tended to be lower in gastric mucosae of *H.pylori*-infected individuals.

Discussion

Among the five groups with inflammation, aberrant methylation was induced only in the three *Helicobacter* groups, which showed inflammation with infiltration of mononuclear cells, increased expression of *Il1b*, *Nos2* and *Tnf* and increased cell proliferation. In the EtOH and NaCl groups, these agents were administered repeatedly for 20 weeks, and increased cell proliferation was present at the end of the experiment. The increased proliferation was considered to have persisted for this period because thickening of lamina propria was observed in these two groups. Nevertheless, aberrant methylation was not induced, at least in the CGIs analyzed here. This showed that cell proliferation alone is not sufficient for methylation induction and suggested that both specific types of inflammation and increased cell proliferation are necessary for induction of aberrant methylation.

The inflammation induced in the *Helicobacter* groups was characterized by infiltration of mononuclear cells (lymphocytes and macrophages). In our previous study, suppression of T-cell activation by cyclosporin A remarkably repressed inflammatory response and methylation induction triggered by *H.pylori* infection (14), showing that T-cell activation is involved in methylation induction in this system. However, our recent study in mouse colon demonstrated that aberrant methylation can be induced even in severe combined immunodeficiency mice, which lack functional T and B cells, by dextran sulfate sodium-induced colitis (Katsurano *et al.*, submitted for publication). It is known that, even in severe combined immunodeficiency mice, colitis with macrophage infiltration can be induced (36). If a common mechanism for methylation induction is present in *H.pylori*-infected gastric mucosae and dextran sulfate sodium-treated colonic mucosae, infiltration of macrophages is a candidate for the proximate effector that transmits signal for methylation induction to epithelial cells. It can be considered that, in *H.pylori*-infected gastric mucosae, activation of T cells is required only for the initiation or maintenance of inflammation capable of inducing aberrant DNA methylation.

Among the inflammation-related genes, *Il1b*, *Nos2* and *Tnf* were specifically upregulated in the three *Helicobacter* groups. These three genes are reported to be overexpressed also in human chronic inflammation associated with cancers, such as ulcerative colitis and hepatitis (37–40). *IL1B* promoter polymorphism is associated with risk of human gastric cancers (28) and aberrant methylation of multiple genes in gastric cancers (29). The lack of its upregulation in human gastric mucosae infected with *H.pylori* could be because most of them had superficial gastritis and had already increased *IL1B* expression. *NOS2*, which encodes nitric oxide synthase, was upregulated *in vitro* by administration of IL1B and nitric oxide donors induced methylation of *FMR1* and *HPRT* (41). These suggest that IL1B and NOS2 might be involved in methylation induction. On the other hand, *Ifng*, *Il2*, *Il4* and *Il6* were upregulated mainly in the EtOH and NaCl groups, in which no methylation was induced, and also in the SS1 and HF groups, in which methylation induction levels were lower than in the ATCC group. This suggested a possibility that some (one) of the genes could suppress methylation induction.

SS1 and *H.felis*, which lack CagA, were capable of inducing aberrant methylation although the capacity was weaker than the CagA-positive strain (*H.pylori* ATCC 43504). CagA-positive *H.pylori* strains are known to induce severe gastritis in Mongolian gerbils (16) as confirmed in this study, and this explains their stronger capacity to induce methylation. The three inflammation-related genes associated with methylation induction (*Il1b*, *Nos2* and *Tnf*) had the highest expression in the ATCC group among the three *Helicobacter* groups. CagA-positive *H.pylori* seems to promote methylation induction by maximizing expression of such genes and minimizing expression of genes that suppress methylation induction.

Dnmts are the final effectors to methylate DNA, and their overexpression was observed in various human cancers (35). Immunohistochemical analyses here revealed that Dnmt1 was upregulated in gastric mucosae of gerbils in the three *Helicobacter*-infected groups and the NaCl-treated group. However, the highest expression was observed in the NaCl group, where methylation was not induced. This result indicated that expression of Dnmt1 was not associated with methylation induction but with cell proliferation. Expression of Dnmt3a was significantly but slightly decreased in the ATCC group and this also suggested that the expression itself is not involved in aberrant methylation induction. However, due to the lack of an appropriate antibody, we were not able to exclude the possibility that upregulation of Dnmt3b is involved in methylation induction. Therefore, disturbance in the local balance between Dnmts and factors that protect DNA from aberrant methylation, such as the presence of RNA

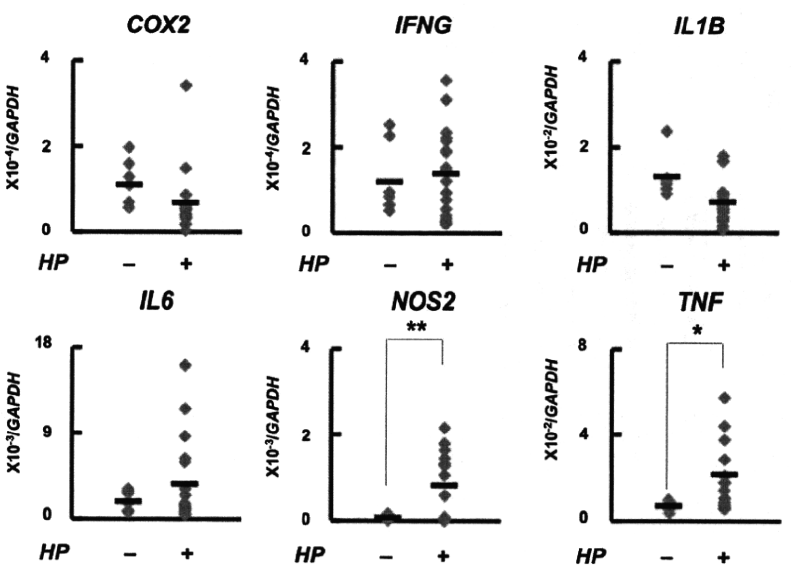

Fig. 5. Human relevance of expression changes in the gerbil stomach. Expression levels of inflammation-related genes were quantified in gastric mucosae of individuals without and with *H.pylori* infection. Bold horizontal bar, the mean expression level; $^{*}P < 0.05$ and $^{**}P < 0.01$.

polymerase II (42) and/or possible overexpression of Dnmt3b might be involved in methylation induction.

In conclusion, inflammation due to infection of *Helicobacter* strains had a high capacity to induce methylation in GECs, regardless of their CagA status. Increased cell proliferation was not sufficient for methylation induction. Therefore, specific types of inflammation, characterized by infiltration of mononuclear cells and expression of specific inflammation-related genes, along with increased cell proliferation were considered to be necessary for methylation induction.

Supplementary material

Supplementary Figures 1–3 and Table 1 can be found at http://carcin.oxfordjournals.org/

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