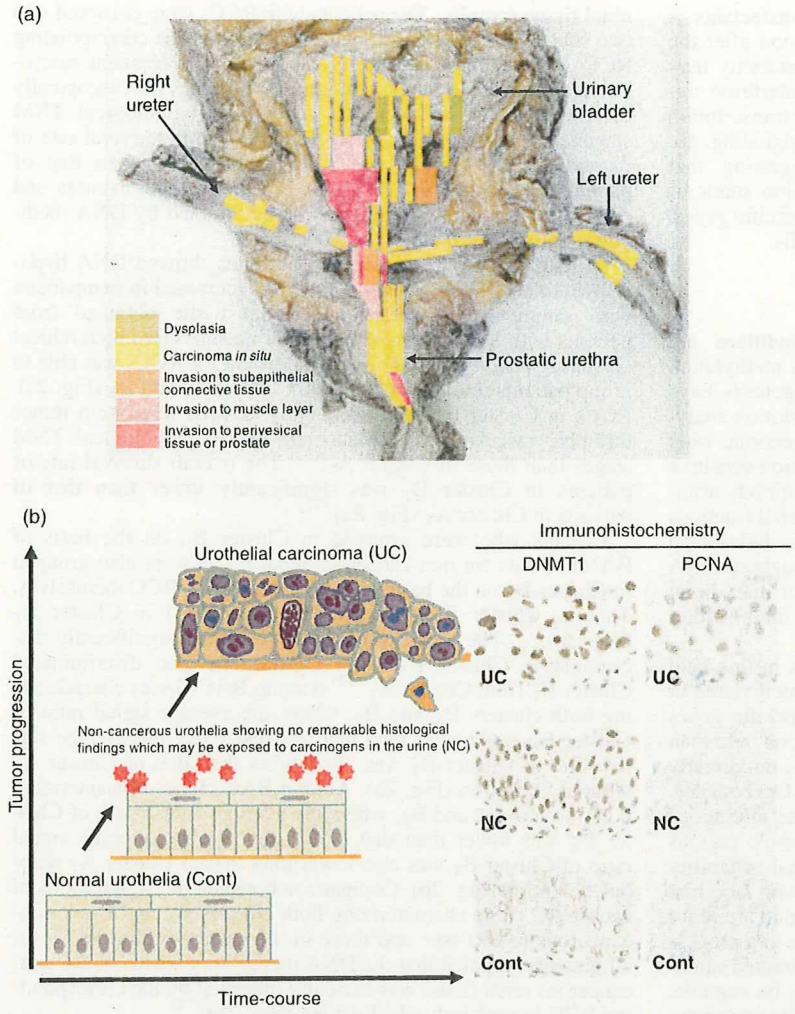

Fig. 1. Overexpression of DNA methyltransferase (DNMT) 1 protein during multistage urothelial carcinogenesis. (a) Specimen obtained by radical cystectomy for multiple urothelial carcinomas (UCs) of the urinary bladder, bilateral ureters, and prostatic urethra. UCs are clinically remarkable because of their multicentricity and tendency to recur: synchronously or metachronously multifocal UCs often develop in individual patients.[38] A possible mechanism for such multiplicity is the "field effect." Even non-cancerous urothelia showing no remarkable histological changes obtained from patients with UCs can be considered precancerous, because they may be exposed to carcinogens in the urine. (b) Immunohistochemical examination for DNMT1 and proliferating cell nuclear antigen (PCNA) in tissue specimens. The incidence of nuclear DNMT1 immunoreactivity had already increased in non-cancerous urothelia showing no remarkable histological changes obtained from patients with UCs (NC), where the PCNA labeling index had not yet increased, compared to that in normal urothelia obtained from patients without UCs (Cont), indicating that DNMT1 overexpression preceded any increase of cell proliferative activity.[56] The intensity of nuclear DNMT1 immunoreactivity was further increased in UCs.[56]

DNMT1 overexpression preceded increased cell proliferative activity (Fig. 1b).[56] The incidence of nuclear DNMT1 immunoreactivity showed a further and progressive increase in dysplastic urothelia, and during transition to UCs (Fig. 1b).[56] Among all examined microdissected specimens of non-cancerous urothelia showing no remarkable histological changes from patients with UCs, or dysplastic urothelia and UCs, accumulation of DNA methylation of C-type CpG islands was significantly correlated with the level of DNMT1 protein expression.[39]

Thus DNMT1 overexpression participates not only in the precancerous stage but also in the malignant progression of various cancers, and has a prognostic impact on patients. DNMT1 overexpression is frequently associated with CIMP of cancers. Although the maintenance activities of DNMT1 are related to its *in vitro* preference for hemimethylated substrates, excessive amounts of DNMT1 in comparison to PCNA may participate in *de novo* methylation of CpG islands. The molecular mechanisms that target DNMT1 to unmethylated substrates in cancers need to be clarified.

Splicing alteration of DNMT3b and DNA hypomethylation in pericentromeric satellite regions. DNA hypomethylation in pericentromeric satellite regions is known to result in centromeric decondensation and enhanced chromosome recombination. In HCCs[57] and UCs,[58] DNA hypomethylation of these regions is correlated with copy number alterations on chromosomes 1 and 9, respectively, where satellite regions are rich. DNMT3b is required for DNA methylation of pericentromeric satellite regions in early mouse embryos, and 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.[59] The major splice variant of DNMT3b in normal liver tissue samples is DNMT3b3, which possesses the conserved catalytic domains.[60] DNMT activity of human DNMT3b3 has been confirmed *in vitro*.[61] In contrast, DNMT3b4 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.[60] 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, are significantly correlated with the degree of DNA hypomethylation in pericentromeric satellite regions.[60] DNA demethylation on satellite 2 has been observed in DNMT3b4-transfected human epithelial 293 cells.[60] As 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.

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Furthermore, the growth rate of DNMT3b4 transfectants is approximately double that of mock-transfectants soon after the introduction of DNMT3b4, when chromosomal instability may not yet have accumulated.[62] Genes implicated in interferon signaling including signal transducer and activator of transcription (STAT) 1, which acts as an effector of interferon signaling, are upregulated in DNMT3b4 transfectants,[62] suggesting that DNMT3b may act to maintain the DNA methylation status of not only pericentromeric satellite regions but also specific genes, probably in cooperation with DNMT1, in cancer cells.

Genome-wide DNA methylation profiling

DNA methylation profiles in precancerous conditions are inherited by cancers. The above findings that DNA methylation alterations are associated with multistage carcinogenesis have prompted us to carry out genome-wide DNA methylation analysis of tissue specimens. Recently, analysis on a genomic-wide scale has become possible using DNA methylation-sensitive restriction enzyme-based or anti-methyl-cytosine antibody affinity techniques that enrich methylated and unmethylated fractions of genomic DNA.[63,64] These fractions can then be hybridized to DNA microarrays or sequenced. Ultra-high-throughput DNA sequencing technologies are being introduced for the direct sequencing of enriched, methylated fragments or for bisulfite-converted genomic sequencing.[65]

We have used BAMCA.[66–69] Many researchers in this field use the 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. DNA methylation status in genomic regions not directly participating in gene silencing, such as the edges of CpG islands, may be altered at the precancerous stage before the alterations of the promoter regions themselves occur.[70] 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.[71] Therefore, we used a BAC array that may be suitable, not for focusing on specific promoter regions, but for overviewing the DNA methylation status of individual large regions among all chromosomes.

When BAMCA methods were applied to samples of non-cancerous renal tissue obtained from patients with clear cell RCCs, many BAC clones showed DNA hypo- or hypermethylation in comparison to normal renal tissue samples from patients without any primary renal tumors.[72] RCCs are usually well demarcated and covered by a fibrous capsule, and hardly ever contain fibrous stroma between cancer cells (Fig. 2a). We were therefore able to obtain cancer cells of high purity from surgical specimens, avoiding contamination with either non-cancerous epithelial cells or stromal cells (Fig. 2a). Therefore, the DNA methylation alterations observed in samples of non-cancerous renal tissue from patients with RCCs cannot be attributable to contamination during sampling. Moreover, DNA methylation alterations in non-cancerous renal tissue did not depend on the distance from the RCC itself to the site from which the non-cancerous renal tissue samples were taken. Because of the lack of any remarkable histological changes or any association with chronic inflammation and persistent infection with viruses or other pathogenic microorganisms, precancerous conditions in the kidney have rarely been described. However, from the viewpoint of DNA methylation, we can consider that non-cancerous renal tissue from patients with RCCs is already at the precancerous stage, showing genome-wide DNA methylation alterations.

We then carried out two-dimensional unsupervised hierarchical clustering analysis based on BAMCA data for non-cancerous renal tissue samples. The patients with RCCs were clustered into two subclasses, clusters A_N and B_N (Fig. 2a). The corresponding RCCs of patients in Cluster B_N showed more frequent macroscopically evident renal vein tumor thrombi, microscopically evident vascular involvement, and higher pathological TNM stages than those in Cluster A_N.[72] The overall survival rate of patients in Cluster B_N was significantly lower than that of patients in Cluster A_N (Fig. 2a).[72] Tumor aggressiveness and even patient outcome might thus be determined by DNA methylation profiles at the precancerous stage.

In RCCs themselves, more BAC clones showed DNA hypo- or hypermethylation, and its degree was increased in comparison with samples of non-cancerous renal tissue obtained from patients with RCCs. Two-dimensional unsupervised hierarchical clustering analysis based on BAMCA data for RCCs was able to group patients into two subclasses, Clusters A_T and B_T (Fig. 2a). RCCs in Cluster B_T more frequently showed renal vein tumor thrombi, vascular involvement, and higher pathological TNM stages than those in Cluster A_T.[72] The overall survival rate of patients in Cluster B_T was significantly lower than that of patients in Cluster A_T (Fig. 2a).[72]

Patients who were grouped in Cluster B_N on the basis of BAMCA data for non-cancerous renal tissue were also grouped in Cluster B_T on the basis of BAMCA data for RCC themselves. That is, Cluster B_N was completely included in Cluster B_T (Fig. 2b).[72] The majority of the BAC clones significantly discriminating Cluster B_N from Cluster A_N also discriminated Cluster B_T from Cluster A_T.[72] Among BAC clones characterizing both clusters B_N and B_T, where the average signal ratio of Cluster B_N was higher than that of Cluster A_N, the average signal ratio of Cluster B_T was also higher than that of Cluster A_T without exception (Fig. 2b). Among BAC clones characterizing both clusters B_N and B_T, where the average signal ratio of Cluster B_N was lower than that of Cluster A_N, the average signal ratio of Cluster B_T was also lower than that of Cluster A_T without exception (Fig. 2b). Comparison between the signal ratios of each BAC clone characterizing both clusters B_N and B_T in non-cancerous renal tissue and those in the corresponding RCCs for all patients revealed that the DNA methylation status of the non-cancerous renal tissue was basically inherited by the corresponding RCC in each individual patient (Fig. 2b).[72]

In non-cancerous renal tissue showing no remarkable histological changes and consisting mainly of renal tubules with specialized functions, no progenitor cell is able to gain a growth advantage, and clonal expansion is unable to occur. Therefore, the distinct DNA methylation profile of Cluster B_N, which is clinicopathologically valid, cannot be established through the selection of one of a number of random DNA methylation profiles in non-cancerous renal tissue in patients with clear cell RCCs, and instead may be established through distinct target mechanisms. As the DNA methylation profiles in Cluster B_T are shared by phenotypically similar patients, who all suffer from clinicopathologically aggressive tumors and show a poor outcome, DNA methylation alterations in at least a proportion of the BAC regions characterizing Cluster B_T cannot be passenger changes. It is clear that cancer itself can induce alterations in DNA methylation. However, DNA methylation alterations of BAC regions characterizing Cluster B_T may significantly participate in carcinogenesis, as the DNA methylation profile in Cluster B_N was established at a very early and precancerous stage of carcinogenesis and inherited during progression of the cancers themselves as Cluster B_T. At least a proportion of DNA methylation alterations at the precancerous stage may be "epigenetic gatekeepers"[21] and which allow time for further epigenetic and genetic alterations including genetic gatekeeper mutations (Fig. 3).

In fact, when the DNA methylation status of C-type CpG islands was examined,[73] the average number of methylated

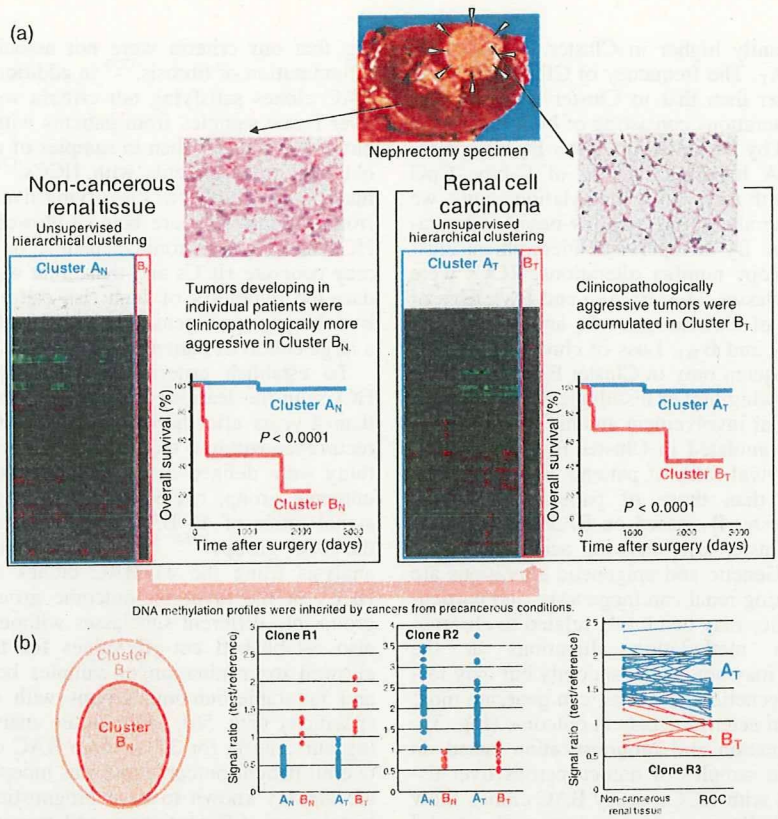

Fig. 2. DNA methylation profiles in precancerous conditions and renal cell carcinomas (RCCs). (a) Bacterial artificial chromosome array-based methylated CpG island amplification (BAMCA) data for tissue samples obtained from patients with RCCs (arrowheads). Using unsupervised hierarchical clustering analysis based on BAMCA data for samples of their non-cancerous renal tissue, patients with RCCs were clustered into two subclasses, Clusters A_N and B_N.[72] Clinicopathologically aggressive RCCs were accumulated in Cluster B_N, and the overall survival rate of patients in Cluster B_N was significantly lower than that of patients in Cluster A_N.[72] Using unsupervised hierarchical clustering analysis based on BAMCA data for their RCCs, patients were clustered into two subclasses, Clusters A_T and B_T.[72] Clinicopathologically aggressive clear cell RCCs were accumulated in Cluster B_T, and the overall survival rate of patients in Cluster B_T was significantly lower than that of patients in Cluster A_T.[72] (b) Correlation between DNA methylation profiles of precancerous conditions and those of RCCs. Cluster B_N was completely included in Cluster B_T (left panel). The majority of the bacterial artificial chromosome (BAC) clones, 724 in all, significantly discriminating Cluster B_N from Cluster A_N, also discriminated Cluster B_T from Cluster A_T.[72] In 311 of the 724 BAC clones, where the average signal ratio of Cluster B_N was higher than that of Cluster A_N, such as Clone R1 in the middle panel, the average signal ratio of Cluster B_T was also higher than that of Cluster A_T without exception.[72] In 413 of the 724 BAC clones, where the average signal ratio of Cluster B_N was lower than that of Cluster A_N, such as Clone R2 in the middle panel, the average signal ratio of Cluster B_T was also lower than that of Cluster A_T without exception.[72] As shown in the scattergram of the signal ratios in non-cancerous renal tissue samples and RCCs for all examined patients for a representative BAC clone, Clone R3, the DNA methylation status of the non-cancerous renal tissue was basically inherited by the corresponding RCC in individual patients (right panel).[72]

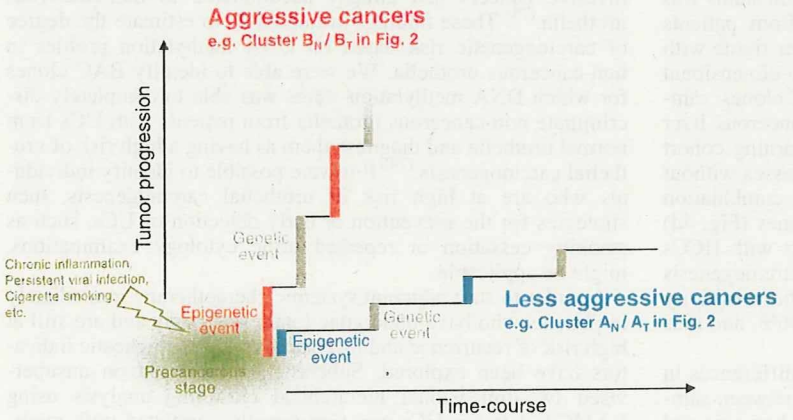

Fig. 3. Significance of DNA methylation alterations at the precancerous stage. Chronic inflammation, persistent infection with viruses or other pathogenic microorganisms, cigarette smoking, exposure to chemical carcinogens, and other unknown factors may participate in the establishment of particular DNA methylation profiles, such as Cluster B_N in Fig. 2. Such DNA methylation alterations in precancerous conditions may not occur randomly, but may be prone to further accumulation of epigenetic and genetic alterations (regional DNA hypermethylation of C-type CpG islands and copy number alterations were accumulated in Cluster B_T in Fig. 2),[72] thus generating more malignant cancers, such as the renal cell carcinomas in patients belonging to Cluster B_T.

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CpG islands was significantly higher in Cluster B_T based on BAMCA than in Cluster A_T. The frequency of CIMP in Cluster B_T was significantly higher than that in Cluster A_T. Genome-wide DNA methylation alterations consisting of both hypo- and hypermethylation revealed by BAMCA in Cluster B_T were associated with regional DNA hypermethylation of C-type CpG islands. For comparison with their DNA methylation status, we also examined copy number alterations by array-based comparative genomic hybridization. By unsupervised hierarchical clustering analysis based on copy number alterations, RCCs were clustered into the two subclasses, clusters A_{TG} and B_{TG}. Loss of chromosome 3p and gain of chromosomes 5q and 7 were frequent in both clusters A_{TG} and B_{TG}. Loss of chromosomes 1p, 4, 9, 13q, and 14q was frequent only in Cluster B_{TG}, and not in Cluster A_{TG}.[74] RCCs showing higher histological grades, renal vein tumor thrombi, vascular involvement and higher pathological TNM stages were accumulated in Cluster B_{TG}. The recurrence-free and overall survival rates of patients in Cluster B_{TG} were significantly lower than those of patients in Cluster A_{TG}.[74] A subclass of Cluster B_T based on BAMCA data was completely included in Cluster B_{TG} showing accumulation of copy number alterations. Genetic and epigenetic alterations are not mutually exclusive during renal carcinogenesis, and particular DNA methylation profiles may be closely related to chromosomal instability. DNA methylation alterations at the precancerous stage, which may not occur randomly but may foster further epigenetic and genetic alterations, can generate more malignant cancers and even determine patient outcome (Fig. 3).

Carcinogenetic risk estimation and prognostication based on DNA methylation status. In samples of non-cancerous liver tissue obtained from patients with HCCs, many BAC clones show DNA hypo- or hypermethylation in comparison with normal liver tissue from patients without HCCs (Fig. 4a).[75] 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 is a priority for patients with HBV or HCV infection. To reveal the baseline liver histology, microscopic examination of liver biopsy specimens is carried out in patients with HBV or HCV infection prior to interferon therapy.[76,77] Carcinogenetic risk estimation using such liver biopsy specimens is advantageous for close follow-up of patients who are at high risk of HCC development. To establish an indicator for carcinogenetic risk estimation, we first omitted potentially insignificant BAC clones associated only with inflammation and/or fibrosis and focused on BAC clones for which DNA methylation status was altered at the precancerous stage in comparison to normal liver tissue and was inherited by HCCs themselves from the precancerous stage (Fig. 4b). Among the BAC clones studied, a bioinformatics approach further identified the top 25 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.[75] By two-dimensional hierarchical clustering analysis using these 25 BAC clones, samples of normal liver tissue and samples of non-cancerous liver tissue obtained from patients with HCCs in the learning cohort were successfully subclassified into different subclasses without any error (Fig. 4c). The criteria established using a combination of the DNA methylation status of the 25 BAC clones (Fig. 4d) diagnosed non-cancerous liver tissue from patients with HCCs in the learning cohort as being at high risk of carcinogenesis with a sensitivity and specificity of 100%.[75] The sensitivity and specificity in the validation cohort were both 96%, and thus our criteria were successfully validated.[75]

It was confirmed that there were no significant differences in the number of BAC clones satisfying our criteria between samples of non-cancerous liver tissue showing chronic hepatitis and samples of non-cancerous liver tissue showing cirrhosis, indicating that our criteria were not associated with the degree of inflammation or fibrosis.[75] In addition, the average numbers of BAC clones satisfying our criteria were significantly lower in liver tissue samples from patients with HBV or HCV infection but without HCCs than in samples of non-cancerous liver tissue obtained from patients with HCCs.[75] Therefore, our criteria may be applicable for classifying liver tissue samples obtained from patients who are being followed up because of HBV or HCV infection, chronic hepatitis, or cirrhosis into those that may generate HCCs and those that will not. 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 with HBV or HCV infection.

To establish criteria for prognostication of patients with HCCs, in the learning cohort, patients who had survived more than 4 years after hepatectomy and 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.[75] Two-dimensional hierarchical clustering analysis using the 41 BAC clones successfully subclassified HCCs in the favorable-outcome group and the poor-outcome group into different subclasses without any error (Fig. 5a). We also established cut-off values for the 41 BAC clones that allowed discrimination of samples between the poor-outcome and favorable-outcome groups with sufficient sensitivity and specificity (Fig. 5b). Multivariate analysis revealed that satisfying our criteria for 32 or more BAC clones was a predictor of overall patient outcome and was independent of parameters that are already known to have prognostic significance,[75] such as histological differentiation, and presence of portal vein tumor thrombi, intrahepatic metastasis, and multicentricity.[33] The cancer-free and overall survival rates of patients with HCCs satisfying the criteria for 32 or more BAC clones in the validation cohort were significantly lower than those of patients with HCCs satisfying the criteria for less than 32 BAC clones (Fig. 5c).[75] Such prognostication using liver biopsy specimens obtained before transarterial embolization, transarterial chemoembolization, and radiofrequency ablation may be advantageous even for patients who undergo such therapies.

As mentioned above, even non-cancerous urothelia showing no remarkable histological changes obtained from patients with UCs may be exposed to carcinogens in urine. In fact, genome-wide DNA methylation profiles of non-cancerous urothelia obtained from patients with nodular invasive UCs showing an aggressive clinical course were inherited by the nodular invasive UCs themselves, suggesting that DNA methylation alterations that were correlated with the development of more malignant invasive cancers had already accumulated in non-cancerous urothelia.[78] These findings prompted us to estimate the degree of carcinogenetic risk based on DNA methylation profiles in non-cancerous urothelia. We were able to identify BAC clones for which DNA methylation status was able to completely discriminate non-cancerous urothelia from patients with UCs from normal urothelia and diagnose them as having a high risk of urothelial carcinogenesis.[78] If it were possible to identify individuals who are at high risk of urothelial carcinogenesis, then strategies for the prevention or early detection of UCs, such as smoking cessation or repeated urine cytology examinations, might be applicable.

In order to start adjuvant systemic chemotherapy immediately in patients who have undergone total cystectomy and are still at high risk of recurrence and metastasis of UCs, prognostic indicators have been explored. Subclassification based on unsupervised two-dimensional hierarchical clustering analysis using BAMCA data for UCs was significantly correlated with recurrence after surgery due to metastasis to pelvic lymph nodes or

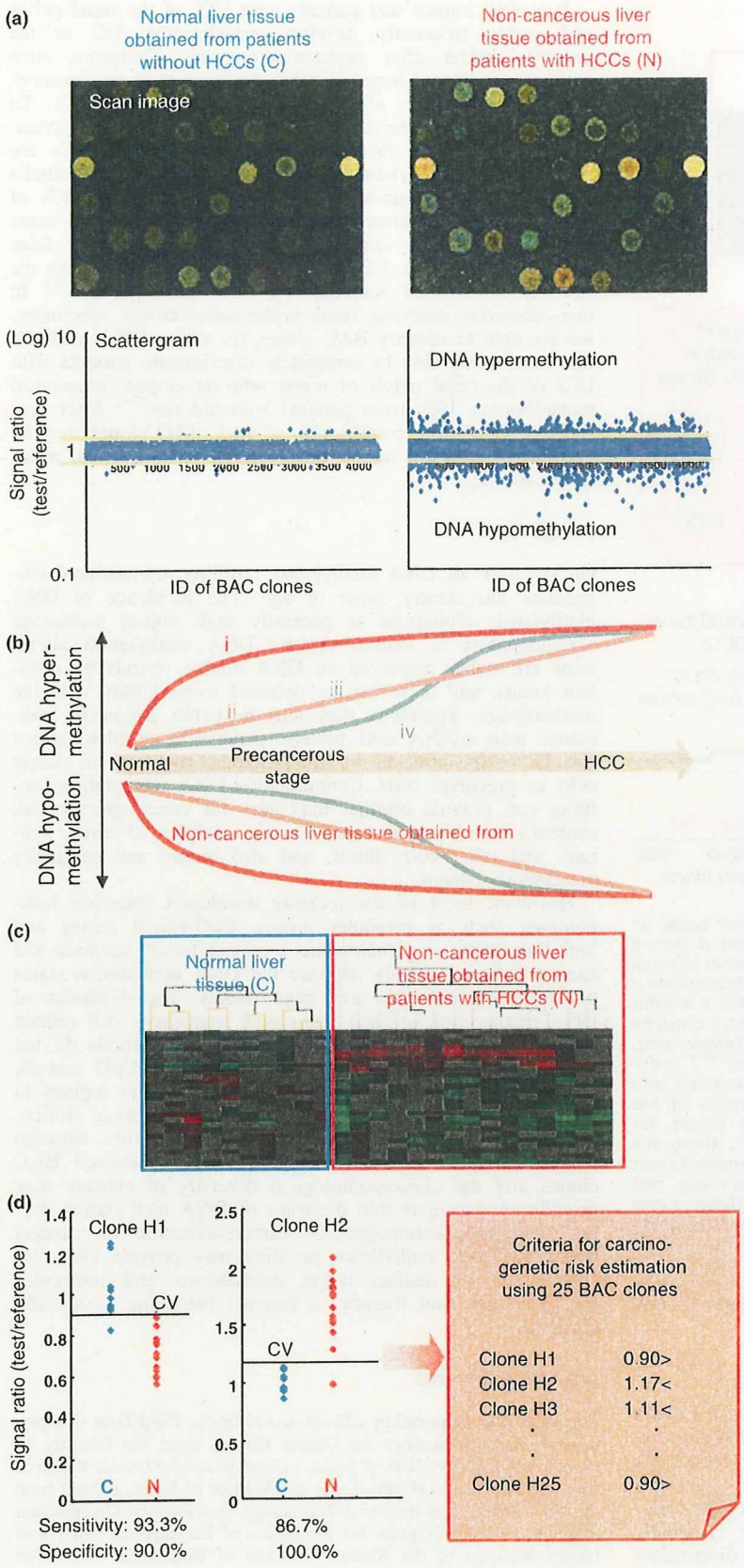

Fig. 4. Risk estimation of hepatocellular carcinoma (HCC) development based on DNA methylation status. (a) Examples of scan images and scattergrams of signal ratios in normal liver tissue obtained from patients without HCCs (C) and non-cancerous liver tissue obtained from patients with HCCs (N). In N samples, many bacterial artificial chromosome (BAC) clones showed DNA hypo- or hypermethylation compared to C samples.[75] (b) Four patterns of DNA methylation alterations seen in BAC clones during multistage hepatocarcinogenesis: (i) DNA methylation alterations occurred at the chronic hepatitis and liver cirrhosis stage, and DNA methylation status did not alter in HCCs from the chronic hepatitis and liver cirrhosis stage; (ii) DNA methylation alterations occurred at the chronic hepatitis and liver cirrhosis stage and further altered in HCCs; (iii) although DNA methylation alterations occurred at the chronic hepatitis and liver cirrhosis stage, the DNA methylation status returned to normal in HCCs; and (iv) DNA methylation alterations occurred only in HCCs. In order to establish criteria for carcinogenetic risk estimation, we focused on BAC clones whose DNA methylation status was inherited by HCCs from the precancerous stage (groups i and ii), whereas group iii may only reflect inflammation and/or fibrosis, and group iv may participate only in the malignant progression stage. (c) Two-dimensional hierarchical clustering analysis using BAC clones that were selected as the top 25 for which DNA methylation status was able to discriminate N from C with sufficient sensitivity and specificity by Wicoxon test and the support vector machine algorithm.[75] C and N samples in the learning cohort were successfully subclassified into different subclasses without any error.[75] (d) Scattergrams of the signal ratios in C and N samples in the learning cohort for representative BAC clones, Clone H1 and Clone H2. Using the cut-off values (CV) in each panel, N samples in the learning cohort were discriminated from C samples with sufficient sensitivity and specificity.[75] Based on a combination of DNA methylation status for the 25 BAC clones, the criteria for carcinogenetic risk estimation were established. Using these criteria, the sensitivity and specificity for diagnosis of N samples in the learning cohort as being at high risk of carcinogenesis were both 100%.[75] The sensitivity and specificity in the validation cohort were both 96%, and thus the criteria were successfully validated.[75]

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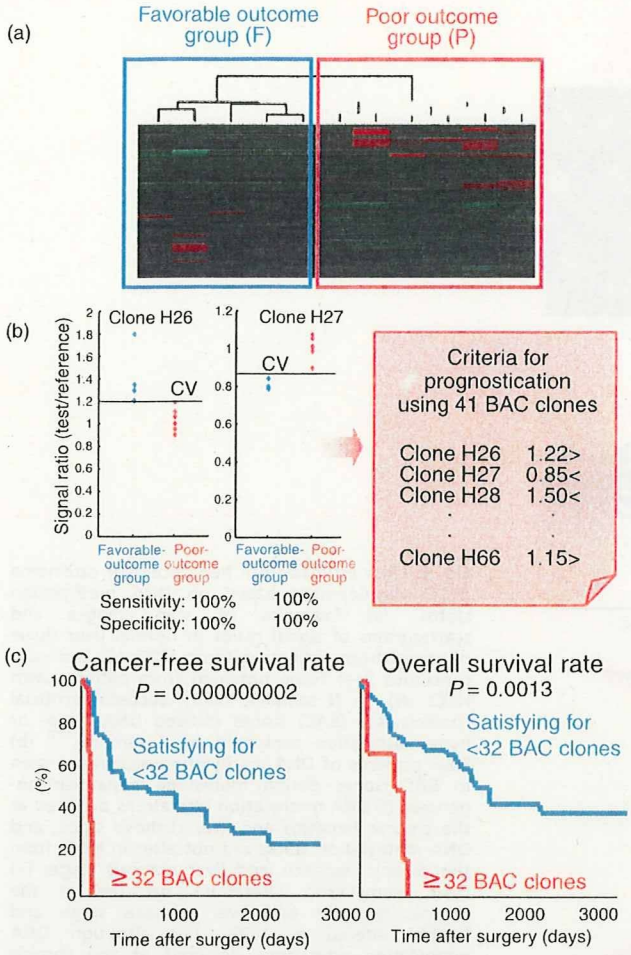

Fig. 5. Prognostication of patients with HCC development based on DNA methylation status. (a) Two-dimensional hierarchical clustering analysis using 41 bacterial artificial chromosome (BAC) clones selected as those for which DNA methylation status was able to discriminate a poor-outcome group (P), who suffered recurrence within 6 months and died within a year after hepatectomy, from a favorable-outcome group (F), who survived for more than 4 years after hepatectomy, with sufficient sensitivity and specificity by Wilcoxon test.[75] F and P patients in the learning cohort were successfully subclassified into different subclasses without any error.[75] (b) Scattergrams of the signal ratios in F and P patients in the learning cohort for representative BAC clones, Clone H26 and Clone H27. Using the cut-off values (CV) in each panel, P patients in the learning cohort were discriminated from F patients with 100% sensitivity and specificity.[75] Based on a combination of the DNA methylation status of the 41 BAC clones, criteria for prognostication were established. (c) The cancer-free and overall survival rates of patients with HCCs in the validation cohort. Patients with HCCs satisfying the criteria for 32 or more BAC clones showed significantly poorer outcome than patients with HCCs satisfying the criteria for less than 32 BAC clones.[75]

distant organs.[78] These data prompted us to establish criteria for predicting recurrence of UCs based on DNA methylation status, and we successfully identified BAC clones for which DNA methylation status completely discriminated patients who suffered recurrence from patients who did not, whereas high histological grade, invasive growth, and vascular or lymphatic involvement were unable to achieve such complete discrimination.[78]

It is well known that patients with UCs of the renal pelvis and ureter frequently develop metachronous UC in the urinary bladder after nephroureterectomy. Therefore, such patients need to undergo repeated urethrocystoscopic examinations for detection of intravesical metachronous UCs. To decrease the need for such invasive urethrocystoscopic examinations, indicators for intravesical metachronous UCs are needed. DNA methylation profiles of non-cancerous urothelia obtained by nephroureterectomy from patients with UCs of the renal pelvis or ureter, which may be exposed to the same carcinogens in the urine as non-cancerous urothelia from which metachronous UCs originate, were correlated with the risk of intravesical metachronous UC development.[78] In non-cancerous urothelia from nephroureterectomy specimens, we are able to identify BAC clones for which DNA methylation status was able to completely discriminate patients with UCs of the renal pelvis or ureter who developed intravesical metachronous UCs from patients who did not.[78] After prospective validation, combination of such BAC clones may be an optimal indicator for the development of intravesical metachronous UC.

Perspective

On the basis of DNA methylation profiling, translational epigenetics has clearly come of age. The incidence of DNA methylation alterations is generally high during multistage carcinogenesis in various organs. DNA methylation alterations are stably preserved on DNA double strands by covalent bonds, and these can be detected using highly sensitive methodology. Therefore, they may be better diagnostic indicators than mRNA and protein expression profiles, which can be easily affected by the microenvironment of cancer cells or precursor cells. Genome-wide DNA methylation profiling can provide optimal indicators for carcinogenetic risk estimation and prognostication using samples of urine, sputum, and other body fluids, and also biopsy and surgically resected specimens.

However, most of the recently developed detection technologies such as promoter arrays, CpG-island arrays and high-throughput sequencing are sequence-based methods and cannot comprehensively measure the DNA methylation status of repetitive sequences and gene bodies. The dynamics of DNA methylation at such non-unique sequences still remain to be determined.[79] Our BAC array-based methods do not focus only on specific promoter regions and CpG islands, and have successfully identified the chromosomal regions in which coordinated DNA methylation alterations have clinicopathological impact. Evaluation of the correlation between the methylation status of each CpG site in selected BAC clones and the clinicopathological diversity of cancers may provide new insights into the roles of DNA methylation during multistage carcinogenesis. Subclassification of cancers based on DNA methylation profiling may provide clues for clarification of distinct target mechanisms and molecules for prevention and therapy in patients belonging to specific clusters.

Acknowledgments

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Abbreviations

BAC	bacterial artificial chromosome
BAMCA	BAC array-based methylated CpG island amplification
CIMP	CpG island methylator phenotype
DNMT	DNA methyltransferase
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
LOH	loss of heterozygosity
PanIN	pancreatic intraductal neoplasia
PCNA	proliferating cell nuclear antigen
RCC	renal cell carcinoma
UC	urothelial carcinoma

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DNA methylation of interferon regulatory factors in gastric cancer and noncancerous gastric mucosae

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Interferon regulatory factors (IRFs) are transcription factors known to play key roles in innate and adaptive immune responses, cell growth, apoptosis, and development. Their function in tumorigenesis of gastric cancer remains to be determined, however. In the present study, therefore, we examined epigenetic inactivation of *IRF1–9* in a panel of gastric cancer cell lines. We found that expression of *IRF4*, *IRF5*, and *IRF8* was frequently suppressed in gastric cancer cell lines; that methylation of the three genes correlated with their silencing; and that treating the cells with the demethylating agent 5-aza-2′-deoxycytidine (DAC) restored their expression. Expression of *IRF5* in cancer cells was enhanced by the combination of DAC treatment and adenoviral vector-mediated expression of p53, p63, or p73. Interferon-γ-induced expression of *IRF8* was also enhanced by DAC. Moreover, treating gastric cancer cells with DAC enhanced the suppressive effects of interferon-α, interferon-β, and interferon-γ on cell growth. Among a cohort of 455 gastric cancer and noncancerous gastric tissue samples, methylation of *IRF4* was frequently observed in both gastric cancer specimens and noncancerous specimens of gastric mucosa from patients with multiple gastric cancers, which suggests *IRF4* methylation could be a useful molecular marker for diagnosing recurrence of gastric cancers. Our findings indicate that epigenetic *IRF* inactivation plays a key role in tumorigenesis of gastric cancer, and that inhibition of DNA methylation may restore the antitumor activity of interferons through up-regulation of *IRFs*. (*Cancer Sci* 2010)

Gastric cancer arises through the accumulation of multiple genetic changes, including mutation of *adenomatous polyposis coli* (*APC*), *K-ras*, and *p53*.[1] But recent studies have also shown that epigenetic changes such as DNA methylation are also importantly involved in the gene silencing seen in cancer.[2] For instance, genes involved in regulation of the cell cycle and apoptosis are now known to be inactivated by DNA methylation.[3–5] In addition we previously showed that a number of genes involved in signal transduction are epigenetically silenced in cancer. The affected genes include *secreted frizzled-related protein 1* (*SFRP1*), *SFRP2*, *dickkopf 1* (*DKK1*), and *DKK2*, which are negative regulators of WNT signaling,[6,7] *Ras association domain family member 2* (*RASSF2*), a negative regulator of Ras;[8] and *14-3-3σ* and *deafness, autosomal dominant 5* (*DFNA5*), two transcriptional targets of p53.[9,10] Because DNA methylation is an epigenetic change, which does not affect gene sequences, the silenced genes can be reactivated by demethylation, making DNA methylation a useful target of cancer therapy.[11,12]

DNA methylation could also be used as a molecular marker for cancer detection. For instance, methylation of genes such as *SFRP2* and *GATA binding protein-4* (*GATA-4*) has been detected in stool DNA from colorectal cancer patients.[13,14] In gastric cancer, infection by *Helicobacter pylori* (*H. pylori*) induces DNA methylation even in noncancerous tissues.[15] In addition, higher levels of methylation are detected in gastric mucosae from cancer patients than in samples from patients without cancer.[15,16] Thus, DNA methylation in noncancerous tissues could be a potentially useful marker predicting development or recurrence of gastric cancer.

The interferon regulatory factor gene (*IRF*) family encodes a group transcription factors induced by interferon. To date, nine *IRFs* (*IRF1–9*) have been identified (reviewed in ref. 17), and their products have been shown to be involved in variety of processes, including innate and adaptive immune responses, cell growth, apoptosis, and development.[17] Interferon regulatory factor 1 (IRF1) was the first to be identified as a regulatory factor in the interferon system,[18] and several lines of evidence suggest IRF1 acts as a tumor suppressor in human neoplasias. For instance, IRF1 and p53 cooperate via two parallel but independent pathways leading to the induction of cell cycle arrest and p21 gene transcription.[19] In addition, IRF5 is induced by p53 and is involved in growth suppression,[20,21] while both IRF5 and IRF7 are involved in the induction of senescence.[22] And down-regulation of *IRF8* expression contributes to resistance to apoptosis and to the metastatic phenotype in metastatic tumor cells.[23] These findings prompted us to speculate that epigenetic inactivation of *IRF* expression may play a key role in tumorigenesis.

Epigenetic inactivation of *IRF8* has recently been observed in colorectal, nasopharyngeal, esophageal, breast, and cervical cancers,[23,24] and inactivation of *IRF4* was shown to be silenced by DNA methylation in chronic myeloid leukemia.[25] Thus epigenetic inactivation of *IRFs* appears to be centrally involved in the development of human neoplasias. However, there has been no comprehensive analysis of the epigenetic alterations of *IRFs* in gastric cancer. In the present study, therefore, we examined epigenetic inactivation of *IRF1–9* in gastric cancer.

Materials and Methods

Cell lines and specimens. Sixteen gastric cancer cell lines (MKN1, MKN7, MKN28, MKN45, MKN74, KatoIII, AZ521, JRST, SNU1, SNU16, NUGC3, NUGC4, AGS, NCI-N87, SNU16) were obtained from the American Type Culture Collection (Manassas, VA, USA) or the Japanese Collection of Research Bioresources (Tokyo, Japan). In addition, SH101 cells were kindly provided by Dr K. Yanagihara[26] at the National Cancer Center Research Institute and have been described previously. In some cases cancer cell lines were treated with 2 μM 5-aza-2′-deoxycytidine (DAC) (Sigma, St. Louis, MO, USA) for

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72 h, replacing the drug and medium every 24 h. When cells were exposed to DAC and either IFN-α, IFN-β, or IFN-γ, 1000 U/mL IFN-α or IFN-β or 100 U/mL IFN-γ was added to the culture for 48 h following incubation with 0.2 μM DAC. The generation and purification of replication-deficient recombinant adenoviruses encoding p53 (Ad-p53), p63 (Ad-p63), p73 (Ad-p73), or LacZ (Ad-LacZ), as well as the infection procedure, were all described previously.[27,28] At a multiplicity of infection (MOI) of 100, 90–100% of the cells were infected.

Two sets of specimens were used in this study. One set contained a total of 68 primary gastric cancers and 22 corresponding gastric mucosa specimens described previously.[29] The second set contained 35 gastric cancer specimens and 330 noncancerous specimens of gastric mucosa from 165 patients, which were obtained through biopsy during the course of endoscopy. Informed consent was obtained from all patients before collection of the specimens. Genomic DNA was extracted using the standard phenol-chloroform procedure. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and then treated with a DNA-free kit (Ambion, Austin, TX, USA). Total RNA extracted from normal stomach, colon, breast, and pancreas from a healthy individual was purchased from Bio-Chain (Hayward, CA, USA). RNA was also obtained from normal stomach glands using the crypt isolation technique as described previously.[30]

Gene expression analysis. Real-time PCR was carried out using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) and a 7900HT Fast Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. SDS2.2.2 software (Applied Biosystems) was used for comparative delta Ct analysis, and *GAPDH* served as an endogenous control. The primers used in this study are shown in Supplementary Table S1. The TaqMan primers/probes used in this study were: *IRF1*, Hs00971960_m1; *IRF2*, Hs01082884_m1; *IRF3*, Hs00155574_m1; *IRF4*, Hs00180031_m1; *IRF5*, Hs00158114_m1; *IRF6*, Hs00608402_m1; *IRF7*, Hs00242190_g1; *IRF8*, Hs00175238_m1; and *IRF9*, Hs00196051_m1.

Methylation analysis. Samples of genomic DNA (2 μg) were modified with sodium bisulfite using an EpiTect Bisulfite Kit (Qiagen, Hilden, Germany). Methylation was determined by

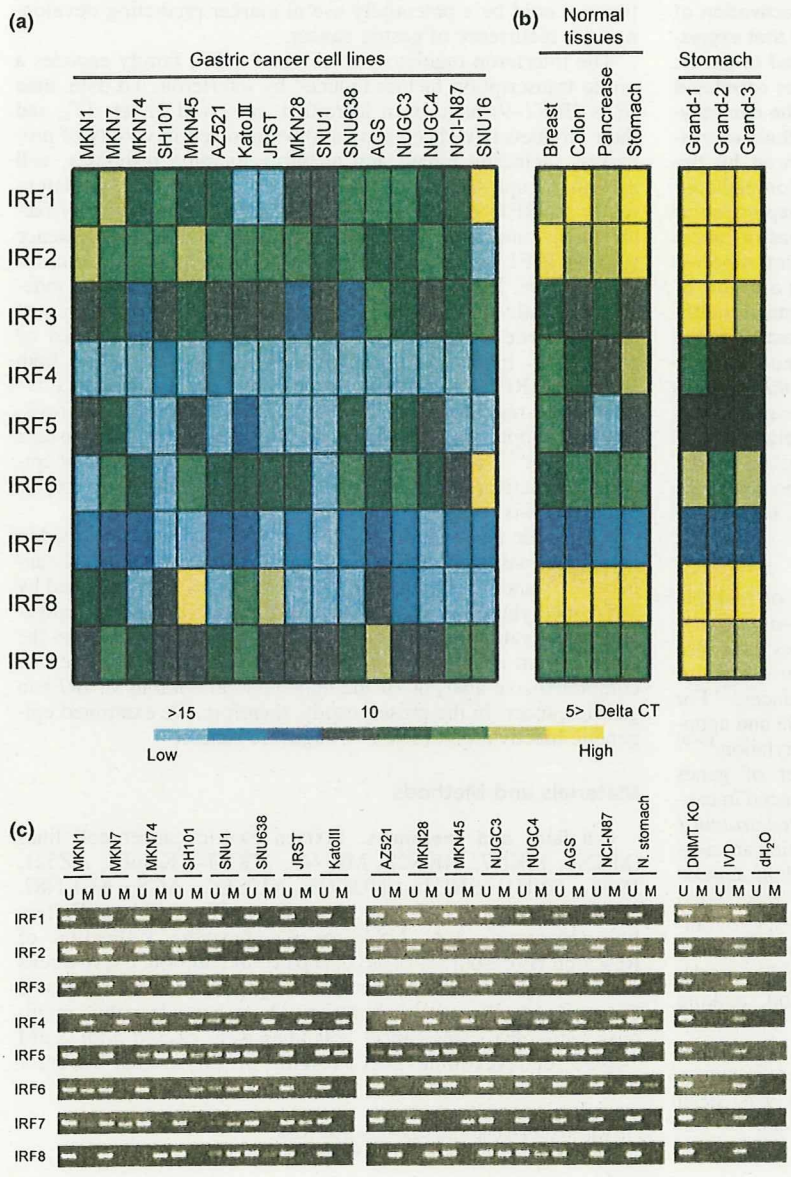

Fig. 1. Down-regulation of interferon regulatory factors (*IRFs*) in gastric cancer cell lines. The heat map shows the expression profiles in 16 gastric cancer cell lines (a) and four normal tissue specimens (b). Levels of expression are normalized to *GAPDH*, and delta-CT values are shown. (c) Analysis of *IRF1–8* methylation in gastric cancer cell lines. Methylation of 5′ CpG islands was examined using methylation-specific PCR. The cell lines examined are shown on the top. DNMT KO: DNMT1$^{-/-}$, DNMT3B$^{-/-}$ HCT116 cell. IVD, *in vitro* methylated DNA; M, methylated; N, stomach: normal stomach; U, unmethylated.

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methylation specific PCR, bisulfite-sequencing, and bisulfite-pyrosequencing, and details of methods are shown in the Supporting Information. The primer sequences are listed in Supplementary Tables S1 and S2.

Statistics. Statistical analyses were carried out using SPSSJ 15.0 (SPSS Japan, Tokyo, Japan). For comparison of methylation levels between cancerous and normal tissues, and for other continuous data, *t*-tests or paired *t*-tests were performed, as appropriate. Fisher's exact test and the Mann–Whitney *U*-test were used to evaluate the association between *IRF* methylation, clinicopathological features, and other genetic and epigenetic alterations. Receiver–operator curves (ROC) were constructed based on *IRF* methylation levels, and *P*-values were calculated by comparing the areas under the curves (AUC) with a reference curve. Values of $P < 0.05$ were considered significant.

Mutation of p53 and KRAS and detection of the presence of CpG island methylator phenotype (CIMP) or Epstein–Barr virus (EBV) were described previously.[31] To determine CIMP status, methylation status of five loci (MINT1, MINT2, MINT12, MINT25, and MINT31) was assessed using combined bisulfite restriction analysis (COBRA). Cases with methylation of four or five loci were defined as CIMP-H. Cases with methylation of one to three loci were defined as CIMP-L. Cases with no methylation were defined as CIMP-N.

Results

Expression of *IRF1–9* in gastric cancer cell lines. To determine whether expression of *IRF1–9* is altered in gastric cancers, we carried out a real-time PCR analysis using a panel of gastric cancer cell lines (Fig. 1a). We found that expression of *IRF4*, *IRF5*, and *IRF8* was frequently down-regulated in these cell lines. Expression of *IRF7* was not detected in normal tissues or in the gastric cancer cell lines, but the remaining *IRFs* were expressed at various levels in normal tissues (Fig. 1a,b). We also examined expression of *IRF1-9* using cDNA prepared using the gastric gland isolation technique, and similar levels of *IRF1–9* expression were observed (Fig. 1b). To determine whether the

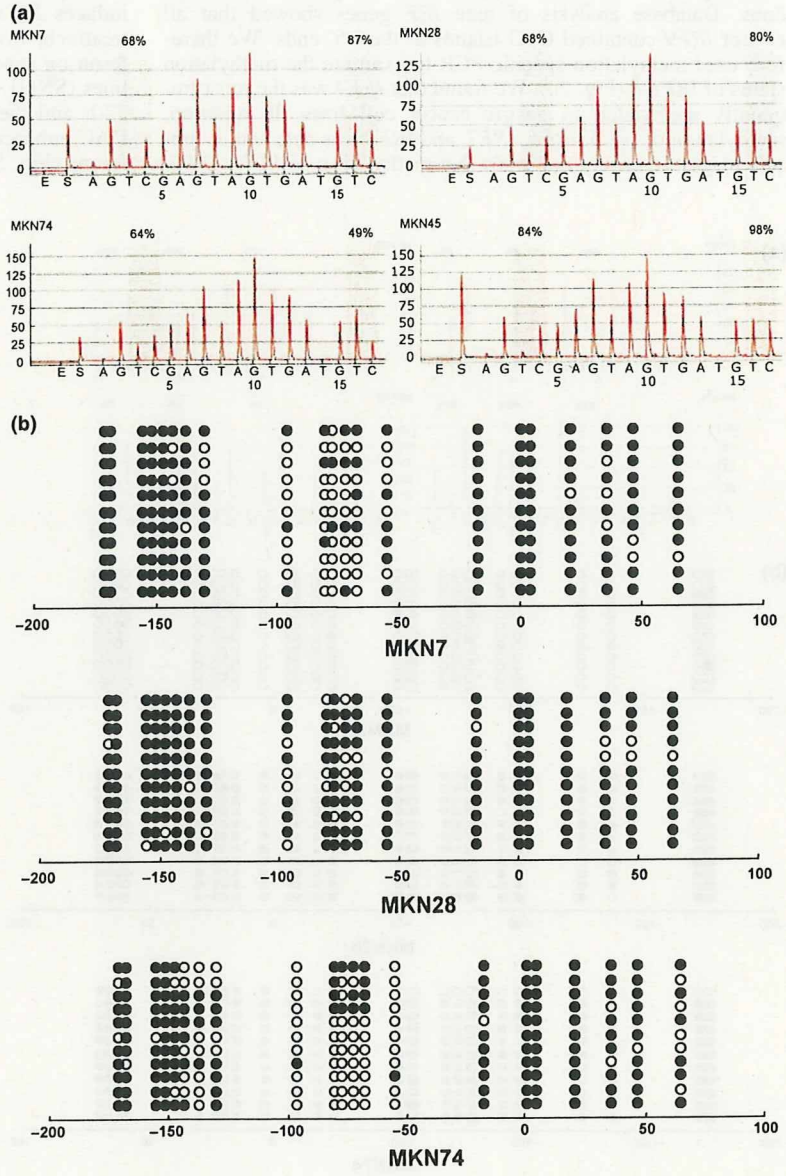

Fig. 2. Methylation analysis of interferon regulatory factor (*IRF*)-*4* in gastric cancer cell lines. (a) Representative pyrosequencing results. Gray columns depict regions of CpG sites, and the percentage methylation at each CpG site is shown on the top. (b) Representative bisulfite-sequencing results. Each circle represents a CpG dinucleotide. Methylation status: open circles, unmethylated; black circles, methylated. The cell lines examined are shown below the columns.

down-regulation of the affected *IRFs* reflected epigenetic modification, we next assessed *IRF* expression following treatment with the demethylating agent DAC. We found that DAC restored *IRF* expression in most gastric cancer cell lines showing *IRF4*, *IRF5*, and/or *IRF8* methylation (Fig. S1). On the other hand, DAC had little effect on several cell lines (i.e. AZ521, AGS, for *IRF5*; NUGC3 for *IRF8*), suggesting other stimuli may be required for full reactivation of *IRFs*.

Treating cancer cells with DAC restored induction of *IRF5* by p53 and of *IRF8* by IFN-γ. Interferon regulatory factor 5 (*IRF5*) and *IRF8* are known to be transcriptional targets of p53[21] and interferon-γ,[32] respectively. We therefore tested whether DAC would enhance the induction of *IRF5* by p53 family members in two gastric cancer cell lines showing *IRF5* methylation. When we infected MKN74 and SNU1 cells with Ad-lacZ, Ad-p53, Ad-p73, or Ad-p63, DAC acted synergistically with the expressed p53 family member to induce *IRF5* expression in the cells (Fig. S2a). In similar fashion, we found that treating MKN28 cells with DAC enhanced the induction of *IRF8* by interferon-γ (Fig. S2b).

Methylation of *IRF4*, *IRF5*, and *IRF8* in gastric cancer cell lines. Database analysis of nine *IRF* genes showed that all except *IRF9* contained CpG islands at their 5′ ends. We therefore used methylation-specific PCR to examine the methylation status of *IRF1-8* (Fig. 1c). We found that *IRF4* was the most frequently methylated in gastric cancer cell lines. In addition, methylation of *IRF5*, *IRF6*, *IRF7*, and *IRF8* was detected in subsets of gastric cancer cell lines. No methylation of *IRF1*, *IRF2*, or *IRF3* was detected in any of the gastric cancer cell lines tested.

We next carried out bisulfite-pyrosequencing to further examine the role of DNA methylation in the down-regulation of *IRF* expression (Figs 2a,3a,4a). Gastric cancer cell lines that exhibited low or negligible *IRF4* expression showed high levels of methylation. Similarly, methylation was well correlated with the down-regulation of *IRF5* and *IRF8* in gastric cancer cell lines.

We then confirmed the methylation status of *IRF4*, *IRF5*, and *IRF8* using bisulfite-sequencing (Figs 2b,3b,4b). High levels of *IRF4* methylation were detected in all of the cancer cell lines tested. In the gastric cancer cell lines, for example, heterogeneous methylation was observed in the region spanning positions −50 to −100 from the transcription start site. High levels of *IRF5* methylation were detected in two (MKN28 and MKN74) of the cancer cell lines showing low or negligible expression, but only sparse methylation was detected in a third (MKN7). MKN7 cells expressed *IRF8* and did not show methylation of that gene. By contrast, MKN28 and MKN74 cells did not express *IRF8* and showed dense methylation of the gene.

Suppression of cell growth by DAC + IFN. Given that DAC induces *IRFs* in gastric cancer cells, we tested whether DAC treatment would enhance the growth suppressive effect of interferon on cancer cells. When we treated four gastric cancer cell lines (SNU1, MKN28, KatoIII, and MKN74) first with DAC for 72 h and then with IFN-α, -β, or -γ for 48 h, we found that DAC enhanced the growth suppressive effects of all three interferons (Fig. S3). This prompted us to test the effect of IFN on

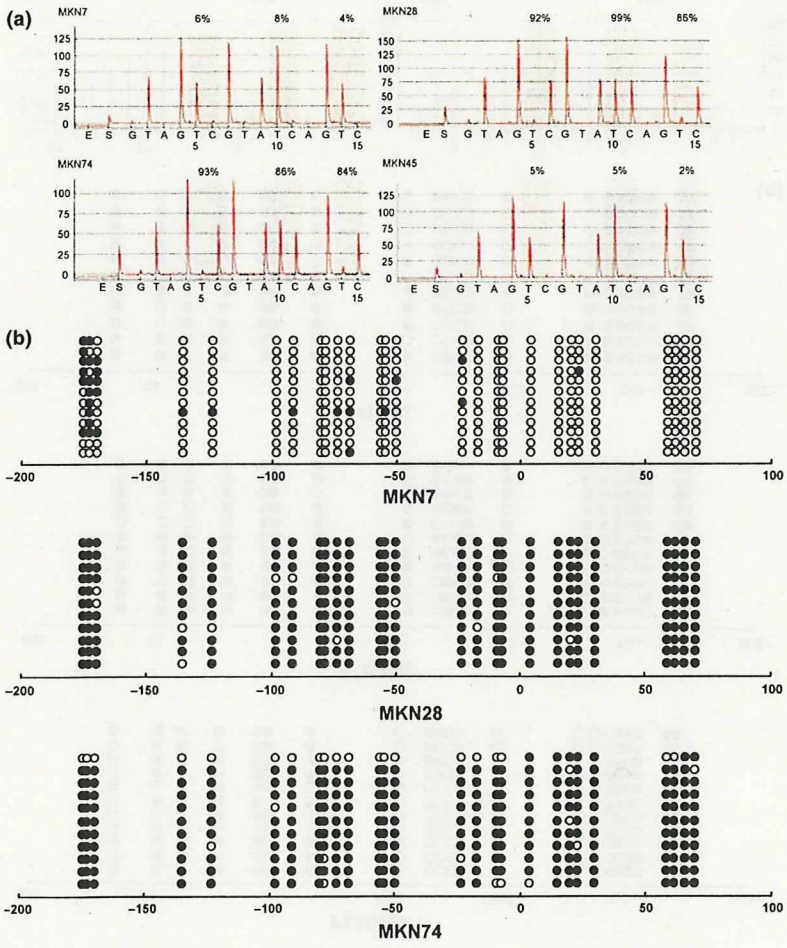

Fig. 3. Methylation analysis of interferon regulatory factor (*IRF*)-*5* in gastric cancer cell lines. (a) Representative pyrosequencing results. (b) Representative bisulfite-sequencing results.

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DNA methylation. Using bisulfite-pyrosequencing with DNA from cells treated with DAC and/or IFN (Fig. S4), we found that although treatment with DAC induced partial demethylation of *IRF4*, *IRF5*, and *IRF8*, treatment with IFN-$\alpha/\beta/\gamma$, alone or in combination with DAC, did not induce further demethylation in MKN74 cells.

Methylation of *IRF4*, *IRF5*, and *IRF8* in primary gastric cancers. To assess *IRF* methylation in primary tumors, we used bisulfite-pyrosequencing to examine primary specimens from 68 gastric cancers and 22 noncancerous gastric tissues (Fig. 5a,b). We found that *IRF4* was frequently methylated in gastric cancer. In addition, we detected high levels of *IRF5* methylation in several gastric cancers, but the average methylation levels did not significantly differ between the cancerous and normal tissues. We did not detect significant methylation of *IRF8* in primary gastric cancers.

We next evaluated the correlation between *IRF* methylation and the clinicopathological features of gastric cancers and between *IRF* methylation and other genetic and epigenetic alterations in gastric cancer. We selected a 13.9% cut-off for *IRF4* and a 16.6% cut-off for *IRF5* methylation based on our finding that these levels represent the 75th percentile among the control samples. With those thresholds, 64 of 68 cases showed methylation of *IRF4*, and 11 of 68 cases showed methylation of *IRF5* (Fig. 5c). Methylation of *IRF4* was detected significantly more frequently in diffuse type and CIMP-H gastric cancers than in CIMP-L or CIMP-N cancers. Methylation of *IRF5* was detected significantly more frequently in gastric cancers positive for EBV and in CIMP-H cancers than in CIMP-L or CIMP-N cancers (Table 1).

Methylation of *IRF4* in noncancerous gastric mucosa is a potential molecular marker for gastric cancer. Several of the cases studied showed high levels of *IRF4* methylation, even in noncancerous gastric mucosa (Fig. 5b). We therefore wondered whether levels of *IRF4* methylation in noncancerous tissues are associated with the presence of gastric cancer. To address that issue, we examined tissue specimens obtained from 165 patients through endoscopic biopsy, including 35 gastric cancer specimens and 330 noncancerous specimens of gastric mucosa (Fig. 6a, Table S3). We found that methylation of *IRF4* in noncancerous gastric tissues was significantly higher in patients with cancer than in those without cancer ($P < 0.001$). In addition, patients with multiple gastric cancers showed significantly higher levels of *IRF4* methylation than patients with a single cancer ($P < 0.05$). Levels of *IRF4* methylation tended to be higher in patients infected with *H. pylori* than in those without *H. pylori*, though the difference was not statistically significant.

The clinical usefulness of DNA methylation for distinguishing cancer patients from noncancer patients was confirmed by ROC analysis. Methylation of *IRF4* gave highly discriminative

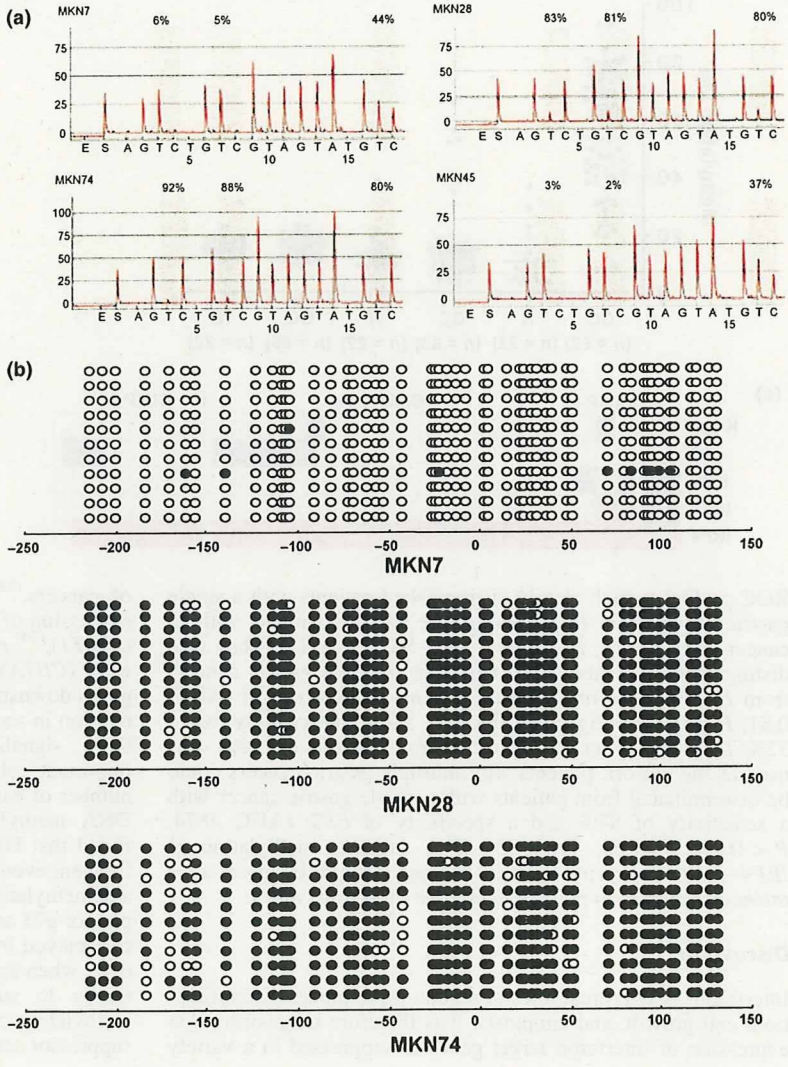

Fig. 4. Methylation analysis of interferon regulatory factor (*IRF*)-*8* in gastric cancer cell lines. (a) Representative pyrosequencing results. (b) Representative bisulfite-sequencing results.

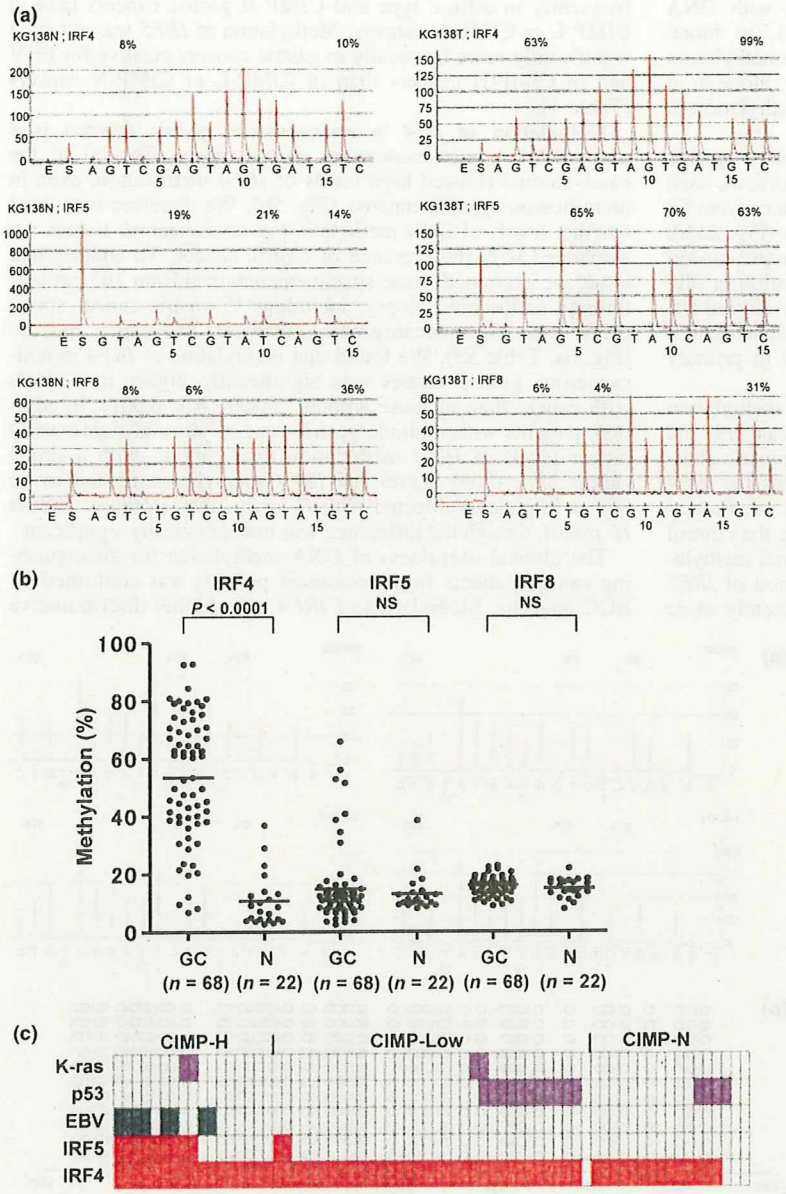

Fig. 5. Methylation of interferon regulatory factor (*IRF*)-*4*, *IRF5*, and *IRF8* in primary gastric cancers. (a) Representative bisulfite-pyrosequencing results. (b) Scatter plot of *IRF* methylation. GC, gastric cancer; horizontal bars, average methylation levels in total cases; N, normal stomach; NS, not significant. (c) Genetic and epigenetic alterations in gastric cancer. Each row represents the separate gene locus shown on the left. Each column is a primary gastric cancer: red rectangles, methylated tumors; purple rectangles, mutated tumors, grey rectangles, Epstein–Barr virus-positive tumors.

ROC profiles, which clearly distinguished patients with a single gastric cancer from *H. pylori*-positive gastritis patients without cancer (AUC: 0.77, $P < 0.001$) (Fig. S5, Table S3). They also distinguished patients with a single or multiple gastric cancers from *H. pylori*-positive gastritis patients without cancer (AUC: 0.81, $P < 0.001$) (Fig. S6, Table S3). More interestingly, when 32% *IRF4* methylation in noncancerous gastric mucosae was used as the cut-off, patients with multiple gastric cancers could be discriminated from patients with a single gastric cancer with a sensitivity of 87% and a specificity of 63% (AUC: 0.74, $P < 0.05$) (Fig. 6b, Table S3). This suggests methylation of *IRF4* in noncancerous gastric mucosae could be used as a molecular marker to predict recurrence of gastric cancer.

Discussion

Interferons play critical roles in regulating immune system function, cell growth, and apoptosis. It is therefore noteworthy that expression of interferon target genes is suppressed in a variety of cancers.[33] For instance, signaling pathways mediated by expression of *signal transducer and activator of transcription 1* (*STAT1*),[34] *class II major histocompatibility complex transactivator* (*CIITA*),[35] and *XIAP associated factor 1* (*XAF1*),[36] three genes downstream of interferon, are silenced by epigenetic inactivation in various cancers, which suggests impairment of interferon signaling by epigenetic mechanisms may play an important role in tumorigenesis. Consistent with that idea, a number of earlier studies have shown that *IRFs* are silenced by DNA methylation in human neoplasias.[23–25,37,38] Here, we found that DNA methylation of *IRF4*, *IRF5*, and/or *IRF8* is a frequent event in gastric cancer cell lines and that treatment with a demethylating agent (DAC) restores induction of *IRF5* by p53, p63, or p73 and induction of *IRF8* by IFN-γ, which confirms the role played by DNA methylation in silencing the genes. Moreover, when applied together, interferon and DAC acted synergistically to suppress cell growth. Thus inhibition of DNA methylation could be a useful strategy for enhancing the tumor suppressor activity of interferon.

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Table 1. Correlation between methylation of IRF4/IRF5 and the clinicopathological features of the patients

Characteristics		IRF4			IRF5		
	Total	*U*	M	*P*-value	*U*	M	*P*-value
n	68	4	64		57	11	
Age							
Mean	64.2	66.3	64.0	0.727	64.5	62.7	0.668
SD	12.1	6.7	12.4		11.7	14.9	
Sex							
Male	45	4	41	0.292	19	4	1.000
Female	23	0	23		38	7	
Location							
Lower	30	2	28	0.929	26	4	0.458
Middle	23	1	22		20	3	
Upper	15	1	14		11	4	
Type							
0	4	0	4	0.605	3	1	0.547
1	5	0	5		4	1	
2	26	3	23		20	6	
3	25	1	24		22	3	
4	8	0	8		8	0	
Histology							
D	38	0	38	0.034	29	9	0.096
I	30	4	26		28	2	
ly							
–	14	0	16	0.566	12	4	0.272
+	44	4	48		45	7	
v							
–	16	0	33	0.115	27	6	0.749
+	52	4	31		30	5	
pT							
pT1	5	0	5	0.225	4	1	0.352
pT2	36	1	35		29	7	
pT3	25	3	22		22	3	
pT4	2	0	2		2	0	
pN							
pN0	18	2	16	0.145	16	2	0.855
pN1	25	2	23		19	6	
pN2	14	0	14		12	2	
pN3	11	0	11		10	1	
pM							
M0	57	4	53	1.000	47	10	0.677
M1	11	0	11		10	1	
Stage (pTNM, 1997, 5th ed)							
1A	3	0	3	0.342	2	1	0.511
1B	12	0	12		11	1	
2	13	3	10		10	3	
3A	12	1	11		9	3	
3B	7	0	7		6	1	
4	21	0	21		19	2	
KRAS							
–	64	4	60	1.000	55	9	0.120
+	4	0	4		2	2	
p53							
–	53	3	50	1.000	42	11	0.105
+	15	1	14		15	0	
EBV							
–	60	4	56	1.000	55	5	<0.001
+	8	0	8		2	6	
CIMP							
H	17	0	14	0.035	8	9	<0.001
L	34	1	33		32	2	
N	17	3	14		17	0	

CIMP, CpG island methylator phenotype; EBV, Epstein–Barr virus; IRF4, interferon regulatory factor 4. ly, lymphatic vessels invasion; pN, pathological node stage; pT, pathological tumor stage; pM, pathological metastasis.

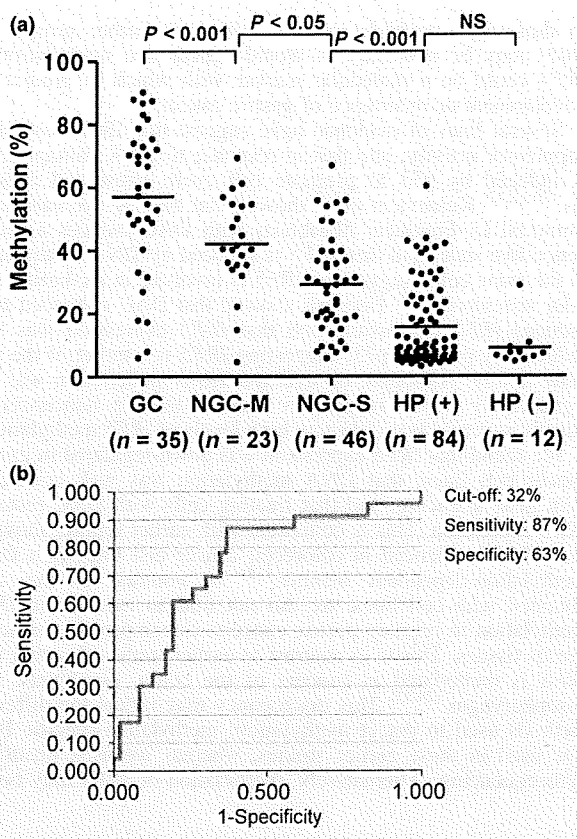

Fig. 6. Methylation levels of interferon regulatory factor (*IRF*)-*4* in gastric cancers and noncancerous gastric mucosae. (a) Distribution of *IRF4* methylation in gastric cancer and noncancerous gastric mucosae. GC, gastric cancer; HP(+), gastric mucosae from *Helicobacter pylori* (*H. pylori*)-positive chronic gastritis patients without cancer; HP(–), gastric mucosae from *H. pylori*-negative chronic gastritis patients without cancer; NGC-M, noncancerous gastric mucosae from multiple gastric cancer patients; NGC-S, noncancerous gastric mucosae from single gastric cancer patients. For noncancerous gastric mucosae, specimens were obtained from the antrum and body, and average methylation levels are shown. Horizontal bars, average methylation levels in total cases. The numbers of cases examined in the study are shown below the column. (b) Receiver–operator curve (ROC) for *IRF4* methylation to discriminate patients with multiple gastric cancers from patients with a single gastric cancer.

It was previously shown that *IRF4* is silenced by DNA methylation in chronic myeloid leukemia.[25] In the present study, we found that *IRF4* is frequently silenced by DNA methylation in both gastric cancers and noncancerous gastric mucosae from cancer patients. Such methylation can be readily detected in serum samples and gastric washing solution,[39,40] and the high frequency of *IRF4* methylation in gastric cancer could be useful for establishing a diagnostic system with DNA methylation as the target. The precise role of *IRF4* methylation in the development and progression of gastric cancer remains unknown. It has been suggested that weakly expressed genes are especially susceptible to methylation changes in cancer.[41] In fact, we found that *IRF4* expression was minimally expressed in gastric epithelium, which consistent with the report that *IRF4* is exclusively expressed in lymphocytic tissues.[17] If that is the case, methylation of *IRF4* may not provide a growth advantage to cells, but may reflect epigenetic defects in the gastric mucosa caused by inflammation. Here we showed that levels of *IRF4* methylation were high in noncancerous gastric mucosae from gastric cancer patients, especially

in those with multiple cancers. Although further prospective study may be necessary, it would appear that methylation of *IRF4* could be a molecular marker with which to predict the development or recurrence of gastric cancer.

Several lines of evidence have suggest that IRF5 has tumor suppressor activity, and that in response to DNA damage *IRF5* is induced by p53 to promote cell cycle arrest and apoptosis.[20,21,42] Kulaeva *et al.*[43] showed that treating spontaneously immortal Li–Fraumeni fibroblasts with DAC induces a senescence-like state, and that *IRF5* is silenced by DNA methylation in the same cells, suggesting IRF5 is involved in mediating cellular senescence.[22] Here we showed that DAC enhanced p53-induced *IRF5* expression, and that *IRF5* expression was also induced by p63 and p73, suggesting *IRF5* is a target of the p53 gene family. Although, on average, *IRF5* methylation was not significantly higher in primary cancers than in noncancerous tissues, several cases did show high levels of *IRF5* methylation.

We found that *IRF8* expression was down-regulated in gastric cancer cell lines; that DNA methylation was well correlated with gene silencing; and that treating cells with DAC restored *IRF8* expression. This is consistent with earlier reports showing that *IRF8* is silenced in colorectal cancer cell lines in a DNA methylation-dependent manner.[23] In contrast to the data obtained with cell lines, we did not find an increase in *IRF8* methylation in primary gastric cancers, as compared to noncancerous tissues. This is in contrast to earlier studies showing that *IRF8* is methylated in cancers of the colon, esophagus, and nasopharyngus.[24,37] This discrepancy may reflect the different methods used to detect methylation: methylation-specific PCR was used in those earlier studies, whereas we used bisulfate-pyrosequencing. Alternatively, methylation of *IRF8* may be an early event in tumorigenesis, which starts in subsets of gastric epithelial cells. Consistent with that idea, Lee *et al.* reported that *IRF8* is methylated only in some esophageal tissues from esophageal cancer patients. Further study will be necessary to clarify the significance of *IRF8* methylation in primary gastric cancers.

In conclusion, we have shown that *IRF4*, *IRF5*, and *IRF8* are epigenetically silenced in gastric cancer cells. Methylation of *IRF5* was associated with CIMP and EBV infection. Moreover, the high degree of *IRF4* methylation in gastric mucosae from cancer patients suggests that DNA methylation of *IRF4* could be a useful molecular marker for gastric cancer diagnosis and risk assessment.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

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

Fig. S1. Real-time PCR analysis of interferon regulatory factor (*IRF*)-*4*, *IRF5*, and *IRF8* expression in gastric cancer cell lines.

Fig. S2. Induction of interferon regulatory factor (*IRF*)-*5* expression by p53 (a) and of *IRF8* expression by interferon (IFN)-γ (b).

Fig. S3. 5-Aza-2′-deoxycytidine (DAC) enhances suppression of cell growth by interferon.

Fig. S4. Methylation analysis of interferon regulatory factor (*IRF*)-*4*, *IRF5*, and *IRF8* after treatment with 5-aza-2′-deoxycytidine (DAC) and/or interferon (IFN).

Fig. S5. Receiver–operator curve (ROC) for interferon regulatory factor (*IRF*)-*4* methylation to discriminate patients with a single gastric cancer from patients with *Helicobacter pylori*-positive chronic gastritis.

Fig. S6. Receiver–operator curve (ROC) curve for interferon regulatory factor (*IRF*)-*4* methylation to discriminate patients with a single or multiple gastric cancers from patients with *Helicobacter pylori*-positive chronic gastritis.

Table S1. Primers used for methylation-specific PCR (MSP) used in this study.

Table S2. Primer sequences used for bisulfite-pyrosequencing and bisulfite-sequencing.

Table S3. High levels of interferon regulatory factor (IRF)-4 methylation are associated with multiple gastric cancers.

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PRECLINICAL STUDY

Genomic screening for genes upregulated by demethylation revealed novel targets of epigenetic silencing in breast cancer

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Abstract Breast cancer arises through the accumulation of multiple genetic alterations and epigenetic changes such as methylation, which silences gene expression in a variety of cancers. In the present study, we applied genomic screening to identify genes upregulated by the demethylating agent 5-aza-2′-deoxycytidine (DAC) in a human breast cancer cell line (MCF7). We identified 288 genes upregulated and 29 genes downregulated more than five-fold after treatment with DAC, and gene ontology analyses revealed the genes to be involved in immune responses, apoptosis, and cell differentiation. In addition, real-time PCR analysis of ten genes silenced in MCF7 cells confirmed that they are upregulated by DAC, while bisulfite-pyrosequencing analysis confirmed that nine of those genes were silenced by methylation. We also found that treating MCF7 cells with DAC restored induction of DFNA5 by p53, as well as by two other p53 family genes, p63γ and p73β. Introduction of NTN4 into MCF7 cells suppressed cell growth, indicating that NTN4 has tumor suppressive activity. In primary breast cancers, we detected cancer-specific methylation of NTN4, PGP9.5, and DKK3, suggesting that methylation of these genes could be useful markers for diagnosis of breast cancer. Thus, DNA methylation appears to be a common event in breast cancer, and the genes silenced by methylation could be useful targets for both diagnosis and therapy.

Keywords DNA methylation · Epigenetics · Gene expression

Tomoko Fujikane, Noriko Nishikawa, Minoru Toyota, Hiromu Suzuki contributed equally to this work.

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Introduction

Epigenetic changes such as DNA methylation and histone modification are now thought to play a significant role in tumorigenesis. Under normal physiological conditions, DNA methylation is involved in such processes as X-chromosome inactivation, genome imprinting, and suppression of repetitive sequences [1], but genome-wide hypomethylation and regional hypermethylation are also common events in tumors [2]. For example, breast cancer, which continues to be one of the most commonly occurring cancers among women, worldwide [3], is known to arise through the accumulation of multiple genetic and epigenetic DNA alterations. Given that more than 1,000 genes are silenced by

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DNA methylation in other types of cancers [4], the targets of epigenetic inactivation in breast cancer have just begun to be identified. To date, analysis of candidate genes for DNA methylation in breast cancer has shown that the targets of epigenetic inactivation include cell cycle regulators such as p16 [5] and 14-3-3 sigma [6], cell adhesion molecules such as E-cadherin [7], cytokines such as HIN-1 [8], genes involved in cell signaling such as RASSF1 [9], proapoptotic genes such as TMS1 [10], genes involved in development such as HOXB13 [11], and transcription factors such as activator protein-2α [12]. Genomic screening approaches using cDNA microarrays, and promoter microarrays identified several novel targets of DNA methylation [13–15]. This makes identification of novel genes epigenetically inactivated in breast cancer an important step toward a better understanding of the pathogenesis of the disease. In the present study, therefore, we applied genomic screening to identify genes silenced by DNA methylation in breast cancer and confirmed the results by quantitative methylation analysis. Our findings suggest that DNA methylation is a common event in breast cancer and that many of the genes silenced by DNA methylation could represent useful targets for both diagnosis and therapy.

Materials and methods

Cell lines and specimens

Five breast cancer cell lines (MCF7, MB435s, MB436, MB468, and SKBR-3) were obtained from the American Type Culture Collection (Manassas, VA) or the Japanese Collection of Research Bioresources (Tokyo, Japan). All cell lines were cultured in appropriate medium supplemented with 10% fetal bovine serum and incubated under a 5% CO_2 atmosphere at 37°C. In addition, 75 breast cancer specimens and 15 breast tissue samples from areas adjacent to tumors were obtaining from Sapporo Medical University Hospital at surgery and stored at −80°C. In accordance with institutional guidelines, all patients gave informed consent prior to collection of the specimens. Genomic DNA was extracted using the phenol/chloroform method. Total RNA was extracted from cell lines using Trizol (Life Technologies, Inc.) according to the manufacturer's instructions.

cDNA microarray analysis

Breast cancer cells (MCF7, MB435s, MB436, MB468, and SKBR-3) were treated with DAC for 72 h, total RNA was extracted and purified using Trizol (Invitrogen) and RNA-easy (Qiagen), after which the RNA samples were quantified using NanoDrop ND-100, the quality was assessed using an Agilent Technologies 2100 Bioanalyzer. The RNA concentration in the samples was >100 ng/μl, and the RNA integrity score was 8–10, with 10 being the highest possible score. Sample amplification and labeling were performed using a Low RNA Input Fluorescent Linear Amplification kit (Agilent Technologies) according to the manufacturer's instructions. Samples (1.65 μg) labeled with Cy3 were hybridized and processed on a 4 × 44 K Whole Human Genome Oligo Microarray. Scanning was performed with an Agilent G2565BA microarray scanner using the settings recommended by Agilent Technologies. After all raw data were normalized, fold-change comparisons and gene set enrichment (BROAD Institute) and gene ontology analyses were performed using GeneSpring GX 10.0. The presence of CpG islands was examined using BLAT (http://genome.brc.mcw.edu/cgi-bin/hgBlat) with previously described criteria [16].

Real-time PCR

Samples (5 μg) of RNA were reverse-transcribed using Superscript III (Invitrogen) to prepare first strand cDNA. For semi-quantitative analysis, real-time PCR was carried out using a 7900 Sequence Detection System (Applied Biosystems). The reaction mixture contained 1× TaqMan Universal PCR Master Mix, primers and probes for each gene and 1 μl of cDNA. GAPDH served as an endogenous control. The Taqman probes used in this study are shown in Supplementary Table 1. Each experiment was done in triplicate.

Methylation analysis

For bisulfite-pyrosequencing, genomic DNA was treated with sodium bisulfite as described previously [17, 18], after which pyrosequencing was performed to assess the methylation status [19]. Bisulfite-PCR primers were designed using PSQ Assay Design software (Biotage, Uppsala, Sweden), and the primers and PCR conditions used were specific for each target gene. After the PCR, the biotinylated strand was captured on streptavidin-coated beads (Amersham Bioscience), and pyrosequencing was performed using PSQ HS Gold SNP reagents and a PSQ HS 96 (Biotage, Uppsala, Sweden). For each gene, the average percentage methylation of the entire CpG island was calculated, and cases in which there was more than 10% methylation were deemed to be positive for methylation. To sequence the bisulfite PCR products, the amplified fragments were cloned into a vector using a TOPO TA cloning kit (Invitrogen), after which a cycle sequencing reaction was carried out using a BigDye terminator kit (Applied Biosystems), and the DNA was sequenced using an ABI 3100 automated sequencer (Applied Biosystems).

Primer sequences used for bisulfite-pyrosequencing and bisulfite-sequencing are shown in Supplementary Table 2.

Infection by adenovirus

The generation, purification, and infection procedures used with replication-deficient recombinant adenovirus containing the p53 (Ad-p53), TAp63γ (Ad-p63γ), TAp73β (Ad-p73β), or the bacterial lacZ gene (Ad-lacZ) were described previously [20]. The relative efficiency of adenoviral infection was determined by X-gal staining of cells infected with the Ad-lacZ (control). At an MOI of 100, 90–100% of the cells were infected (data not shown).

Western blot analysis

Mouse anti-FLAG mAb (M2; Sigma) was used for immunoblotting. Whole cell lysates were prepared by scraping cell monolayers into radioimmunoprecipitation assay buffer without SDS [containing 150 mmol/l NaCl, 50 mmol/l Tris–HCl (pH 7.2), 1% deoxycholic acid, 1% Triton X-100, 0.25 mmol/l EDTA (pH 8.0), protease and phosphatase inhibitors, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 mmol/l phenylmethylsulfonyl fluoride, 5 mmol/l NaF, and 100 μmol/l sodium orthovanadate], and protein concentrations were determined (Lowry reagent, Bio-Rad). Equal amounts of protein were separated by SDS-PAGE and transferred to Immobilon P membranes (Millipore).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed as described previously using a ChIP assay kit (Upstate Biotechnologies). Briefly, 2×10^6 cells were cross-linked using 1% formaldehyde solution for 15 min at 37°C. The cells were then lysed in 200 μl of SDS lysis buffer and sonicated to generate 300- to 800-bp DNA fragments. Following centrifugation, the cleared supernatant was diluted tenfold with ChIP dilution buffer, after which 1/50 of the extract volume was used for PCR amplification as the input control. The remaining extract was incubated with a specific antibody for 16 h at 4°C. Immune complexes were precipitated, washed, and eluted as recommended. DNA–protein cross-links were reversed by heating for 4 h at 65°C, after which the DNA fragments were purified and dissolved in 50 μl of Tris–EDTA. One microliter of each sample was then used as a template for PCR amplification. PCR for histone analysis was carried out as described previously [21] using the primers listed in Supplementary Table 2. PCR amplification of *DFNA5* and *MDM2* containing the putative p53RE was also carried out using primers listed in Supplementary Table 2.

Colony formation assays

Colony formation assays were carried out as described previously [22]. Briefly, MCF7 cells (1×10^5 cells) were transfected with 5 μg of pReceiver-M11-NTN4 (EX-U1401-M11, GeneCopeia) or with empty vector using Lipofectamine 2000 according to manufacturer's instructions. Cells were then plated on 60-mm culture dishes and selected for 14 days in 0.6 mg/ml G418, after which the colonies that formed were stained with Giemsa and counted using National Institutes of Health IMAGE software.

Statistics

To compare methylation levels between tumors and normal tissues, *t* tests were performed for all samples, and paired *t* tests were performed for matched samples from the same patients. Receiver operating characteristic (ROC) curves were constructed based on the levels of NTN4, PGP9.5, and DKK3 methylation, and *P* values were calculated by comparing the ROC curves to a reference curve. ANOVAs with post hoc Games-Howell tests were performed to compare methylation levels at different cancer stages. A scatter plot was constructed by plotting levels of FKBP6 methylation against tumor size, and a Pearson's correlation coefficient was calculated for these values. Values of $P < 0.05$ were considered significant. All statistical calculations were performed using SPSSJ 15.0 (SPSS Japan Inc.).

Results

Identification of genes upregulated by DAC

The global changes in gene expression induced by DAC in MCF7 breast cancer cells were examined using an Agilent Whole Genome microarray that covers 44,000 transcripts of human genes (Supplementary Fig. 1). As compared with mock-treated cells, 288 genes were upregulated and 29 genes were downregulated more than fivefold by DAC in these cancer cells (Supplementary Tables 3, 4). Four genes (SFRP1, DKK3, DFNA5, TAC1) were recently shown to be silenced by DNA methylation in breast cancer [15, 23–25]. To identify biological processes significantly affected by demethylation, we used gene ontology analysis to assess the function of the 288 upregulated genes. Detailed results are shown in Supplementary Table 5. Treating the cells with DAC led to significant upregulation of genes involved in immune responses, the extracellular region, and cytokine activity. We also conducted a gene set enrichment analysis using functional annotation tools (Supplementary Table 6). Among 26 selected gene sets, genes involved in cell differentiation, cell development, defense responses,