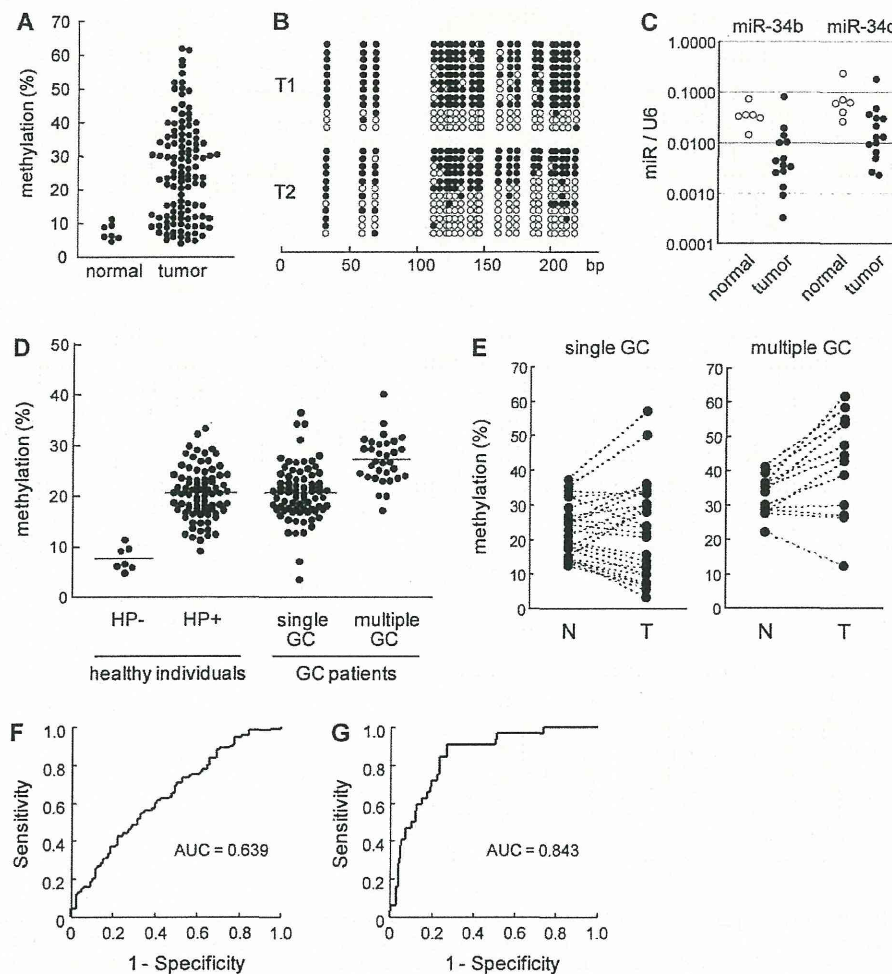

Fig. 4. Analysis of *miR-34b/c* CpG island methylation in samples of primary GC and non-cancerous gastric mucosa. (**A**) Summarized results of bisulfite pyrosequencing in normal stomach tissues from healthy individuals without *Helicobacter pylori* infection (n = 7) and primary GC tumors (n = 118). (**B**) Representative results of bisulfite sequencing in GC tumors (T1 and T2). (**C**) Summary of the results of a TaqMan RT–PCR analysis of *miR-34b* and *miR-34c* in normal stomach from healthy individuals (*n* = 7; white circles) and primary GC tissues (*n* = 14; black circles). (**D**) Summarized results of bisulfite pyrosequencing in non-cancerous gastric mucosae from patients with multiple GC (n = 32) or single GC (n = 77) and normal gastric mucosae from healthy individuals with (n = 78) or without *H. pylori* (HP) infection (n = 7). (**E**) Results of bisulfite pyrosequencing in paired non-cancerous gastric mucosa (N) and GC tumors (T) from patients with a single GC (*n* = 26) or multiple GCs (*n* = 12). (**F**) Receiver operator characteristic curve analysis of *miR-34b/c* methylation. The area under the receiver operator characteristic curve for each site conveys its utility for distinguishing non-cancerous gastric mucosae from GC patients from normal stomach from healthy individuals in terms of its sensitivity and specificity. (**G**) Receiver operator characteristic curve analysis distinguishing non-cancerous gastric mucosae from patients with multiple GC from mucosae from patients with single GC or from healthy individuals.

Table I. Mean levels of *miR34b/c* methylation in non-cancerous gastric mucosae and clinicopathological features of healthy individuals and GC patients

	N	Methylation (%)		Age		Grade of gastritis[a]			
		Mean	SD	Mean	SD	Inflammation	Activity	Atrophy	Metaplasia
Healthy, HP−	7	7.8	2.4	60.4	17.3	1.0	0.0	0.0	0.0
Healthy, HP+	78	20.6	5.1	57.7	12.1	1.5	1.3	1.4	0.6
Single GC	77	20.8	5.4	67.4	9.4	1.4	1.2	1.7	0.8
Multiple GC	32	27.3	4.6	72.6	8.7	1.1	0.8	1.9	1.4

HP, *Helicobacter pylori*; SD, standard deviation.
[a]Scored using the Updated Sydney System.

with single GC (27.3 versus 20.8%; $P < 0.001$) (Figure 4D, Table I). Because there were age differences between these two groups, we also calculated the age-adjusted levels of *miR-34b/c* methylation and compared them between these groups using analysis of covariance with *post hoc* multiple comparisons. We found the same tendency in both the crude and the age-adjusted models, indicating that age-related differences in methylation did not account for the results (supplementary Table 9 is available at *Carcinogenesis* Online).

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Paired non-cancerous gastric mucosae and GC tissues were available from several patients (26 with a single GC and 12 with multiple GCs), which enabled us to compare the methylation levels between the two tissues. Interestingly, although the methylation levels of non-cancerous and cancerous tissues did not significantly differ in patients with a single GC (22.1 versus 22.8%; $P = 0.710$), cancer tissues showed significantly higher levels of methylation than their non-cancerous counterparts in patients with multiple GCs (32.3 versus 41.4%; $P = 0.011$) (Figure 4E).

To assess the association between *miR-34b/c* methylation and GC, we categorized the gastric mucosa specimens into four quartiles of methylation (Table II). As compared with individuals who had the least *miR-34b/c* methylation (≤17.5%), having the highest methylation (≥25.4%) was not significantly associated with GC (age-adjusted OR 2.1; $P = 0.125$; 95% confidential interval 0.8–5.4), though it was strongly associated with multiple GC (age-adjusted OR 27.7; 95% confidence interval 3.3–228.9) (Table II). Moreover, when we calculated the ORs for multiple GC adjusted for age, gender, *H. pylori* status and gastritis grade, we found them to be even more significant (Table II), which suggests hypermethylation of *miR-34b/c* in non-cancerous gastric mucosae is an independent additive risk for multiple GC.

We also generated a receiver operator characteristic curve to assess the clinical utility of DNA methylation for the prediction of GC (Figure 4F and G). Although *miR-34b/c* methylation failed to distinguish between non-cancerous gastric mucosa from GC patients and normal gastric mucosa from healthy individuals (area under the curve = 0.639) (Figure 4F), it was highly discriminative between gastric mucosa from patients with multiple GC and mucosa from patients with single GC or from healthy individuals (area under the curve = 0.843) (Figure 4G). The most discriminating cutoff of *miR-34b/c* methylation for multiple GC was 23.1% (sensitivity 90.6% and specificity 72.8%). This suggests that methylation of *miR-34b/c* may be a useful marker with which to screen individuals at a high risk of GC.

Discussion

Dysregulation of miRNA expression is commonly observed in wide variety of cancers, and epigenetic mechanisms have been shown to be key mediators underlying the downregulation of miRNA expression. To screen for epigenetically silenced miRNA genes in GC, we first performed a microarray analysis to identify miRNAs upregulated by demethylation and histone deacetylase inhibition. Consistent with recent reports, these treatments significantly upregulated expression of the C19MC in GC cell lines. The C19MC is composed of 46 miRNA genes, forming a cluster spanning ~100 kb on chr19q13.41. These miRNA genes are interspersed among Alu repeats and, with the exception of the placenta, are silenced in human tissues (23).

Our microarray analysis also identified a number of miRNAs whose silence is reportedly associated with DNA methylation in cancer. For example, *miR-127* is the first miRNA gene known to be activated by epigenetic drug treatment in cancer cells (6). In addition, methylation of *miR-9* and *miR-148a* has been observed in human metastatic cancer cell lines (24), and *miR-203* is epigenetically silenced in hematopoietic malignancies, which leads to enhanced expression of *ABL1* and *BCR-ABL1* (25). We found that these miRNA genes are also hypermethylated in cultured and primary GCs (data not shown), though further study is needed to clarify their role in gastric carcinogenesis.

MiR-34s have been strongly implicated in cancer. For example, *miR-34a* reportedly acts as a tumor repressor in colon cancer and neuroblastoma (22,26). All three *miR-34* family members are directly regulated by p53, and ectopic expression of *miR-34s* induces cell cycle arrest and/or apoptosis in human cancer cells (20,21,27–30). Conversely, expression of *miR-34s* is frequently downregulated in human malignancies, including lung, colon and ovarian cancer (8,20,22,31). Genes-encoding *miR-34a* and *miR-34b/c* are located in 1p36.23 and 11q23.1, respectively, and both are targets of epigenetic silencing in cancer (8,32). In particular, we and others recently showed that silencing of *miR-34b/c* is associated with CpG island hypermethylation in colon and oral cancers (8,33). In addition, Lujambio *et al.* (24) identified *miR-34b/c* methylation by screening cell lines derived from metastatic colon cancer, melanoma and head and neck cancer and Corney *et al.* (31) recently reported downregulation of *miR-34b/c* in metastatic ovarian cancer, which suggests inactivation of *miR-34b/c* may be associated with cancer metastasis.

In the present study, we found that *miR-34b* and *miR-34c* are significantly upregulated by epigenetic drug treatment in GC cells, whereas *miR-34a* is abundantly expressed without treatment, which was consistent with our earlier observation in colon cancer cells (8). Apparently, the gene-encoding *miR-34b/c* is epigenetically silenced in a majority of GC cell lines, and silencing is associated with hypermethylation of the neighboring CpG island. In addition, our functional study suggests that *miR-34b/c* may be a useful therapeutic target in GC as their ectopic expression significantly downregulated their target genes (e.g. *CDK4* and *MET*) and suppressed GC cell proliferation. Microarray analysis revealed that *miR-34b/c* induces dramatic changes in the gene expression profiles in GC cells and that cell cycle-related genes are the most significantly affected, which is consistent with earlier observations in colon and lung cancer cells (8,20,21). Methylation of *miR-34b/c* was observed in 70% of primary GC specimens, though no correlation between *miR-34b/c* methylation and p53 mutation was found. The high rate of *miR-34b/c* methylation in GC and our functional analysis suggest that they act as tumor suppressors in response to gastric tumorigenesis. Thus, reactivation of *miR-34b/c* could be an effective therapeutic strategy for the treatment of GC.

Helicobacter pylori is a major carcinogenic factor in the stomach, and a number of studies have shown that it induces aberrant DNA methylation in gastric epithelial cells (34–37). Recently, Ando *et al.* (13) reported that *miR-124a* family genes (*miR-124a-1*, *-2* and *-3*) are frequently methylated in primary GC and in normal gastric mucosa from healthy individuals with *H. pylori* infections. Among *H. pylori*-negative individuals, methylation levels are significantly higher in non-cancerous gastric mucosae from GC patients than gastric mucosae from healthy individuals, which suggest methylation of miRNA genes contributes to a field defect contributing to the pathogenesis of GC (13). We also observed that *miR-34b/c* methylation is significantly associated with *H. pylori* infection among healthy

Table II. Methylation of *miR-34b/c* in non-cancerous gastric mucosa and its association with multiple GC

Methylation (%)	Total	Non-GC	GC	OR[a]	95% CI	P
≤17.5	49	29	20			
17.6–21.2	48	22	26	1.5	0.6–3.6	0.397
21.3–25.3	49	20	29	1.3	0.5–3.2	0.588
≥25.4	48	14	34	2.1	0.8–5.4	0.125
						P for trend = 0.174
Methylation (%)	**Total**	**Non-MGC**	**MGC**	**OR1[a]**	**95% CI**	***P***
≤17.5	49	48	1			
17.6–21.2	48	46	2	2.0	0.2–24.4	0.571
21.3–25.3	49	40	9	8.5	1.0–72.8	0.052
≥25.4	48	28	20	27.7	3.3–228.9	0.002
						P for trend < 0.001
Methylation (%)	**Total[b]**	**Non-MGC[b]**	**MGC[b]**	**OR2[c]**	**95% CI**	***P***
≤17.5	32	31	1			
17.6–21.2	32	30	2	5.4	0.3–88.8	0.236
21.3–25.3	32	26	6	10.4	0.8–136.5	0.074
≥25.4	31	18	13	44.8	3.4–598.8	0.004
						P for trend < 0.001

CI, confidence interval; MGC, multiple gastric cancer.
[a]Age-adjusted OR.
[b]Samples in which the Updated Sydney System scores are available.
[c]Age, gender, *Helicobacter pylori*, grades of gastric mucosal atrophy and metaplasia-adjusted OR.

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individuals. Levels of *miR-34b/c* methylation in non-cancerous gastric mucosa from patients with single GC were similar to those in gastric mucosa from *H. pylori*-positive healthy individuals. It is noteworthy, however, that non-cancerous gastric mucosa from patients with multiple GC showed even higher methylation levels, suggesting that *miR-34b/c* methylation may be a useful marker predictive of the risk of GC recurrence.

In summary, we have shown that a novel miRNA gene is often epigenetically silenced in GC. Taken together, the high rate of *miR-34b/c* methylation and the results of our functional study suggest that they are novel tumor suppressor genes in GC. In normal stomachs of healthy individuals, moderate levels of *miR-34b/c* methylation are associated with *H. pylori* infection. Moreover, the higher methylation levels seen in non-cancerous gastric mucosae from patients with multiple GC strongly suggest that methylation is involved in an epigenetic field defect contributing to GC development. Our results therefore suggest that methylation of *miR-34b/c* could serve as a useful tumor marker and that restoration of its expression could be an effective anticancer therapy.

Supplementary material

Supplementary Figures 1–5 and Tables 1–9 can be found at http://carcin.oxfordjournals.org/

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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; 101: 1708–1716)

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 *IRF*s (*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

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5-aza-2′-deoxycytidine (DAC) (Sigma, St. Louis, MO, USA) for 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 BioChain (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

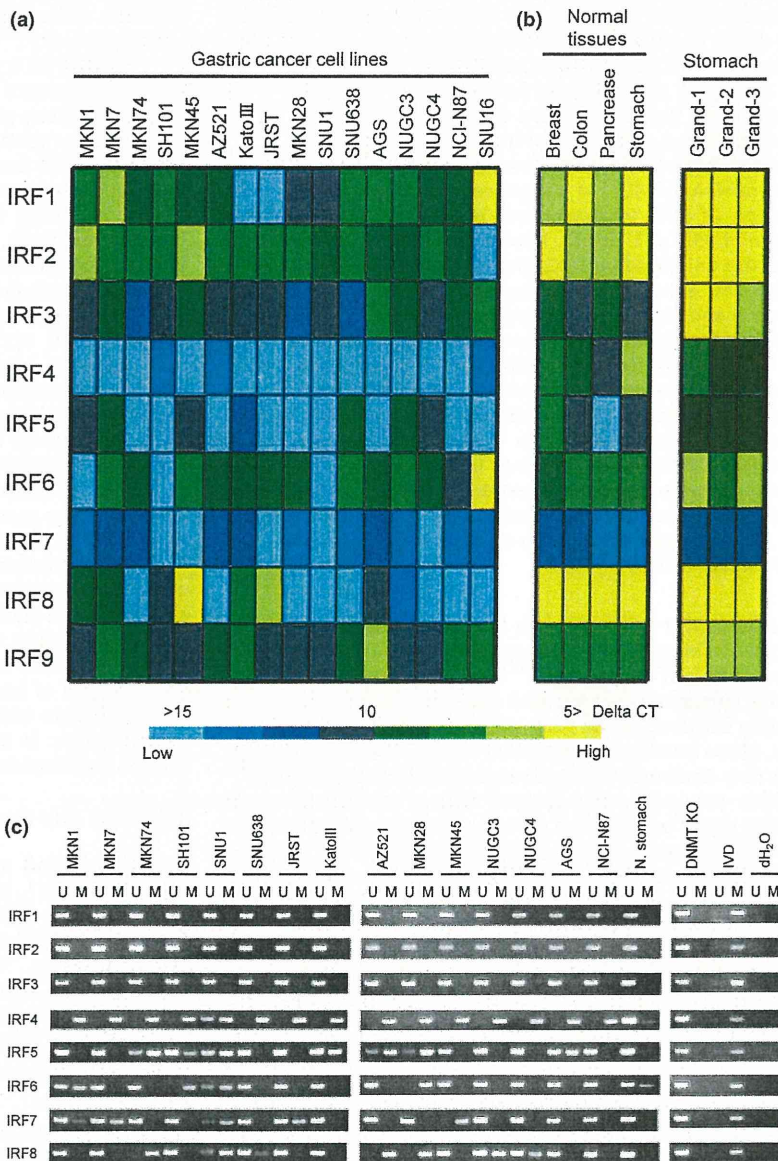

Fig. 1. Down-regulation of interferon regulatory factors (*IRFs*) in gastric cancer cell lines. The heat map shows the expression profiles in 16 gastric cancer cell lines (a) and four normal tissue specimens (b). Levels of expression are normalized to *GAPDH*, and delta-CT values are shown. (c) Analysis 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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(Qiagen, Hilden, Germany). Methylation was determined by 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*

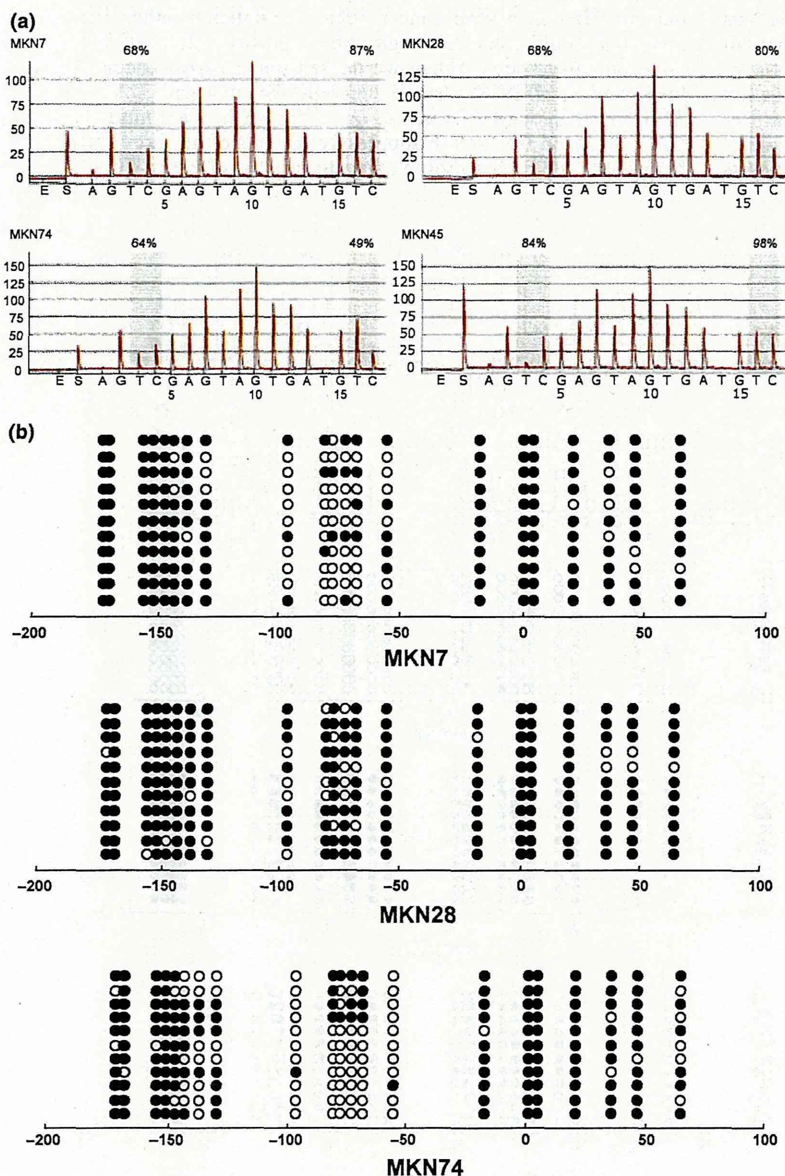

Fig. 2. Methylation analysis of interferon regulatory factor (*IRF*)*-4* in gastric cancer cell lines. (a) Representative pyrosequencing results. Gray columns depict regions of CpG sites, and the percentage methylation at each CpG site is shown on the top. (b) Representative bisulfite-sequencing results.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

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expression were observed (Fig. 1b). To determine whether the 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

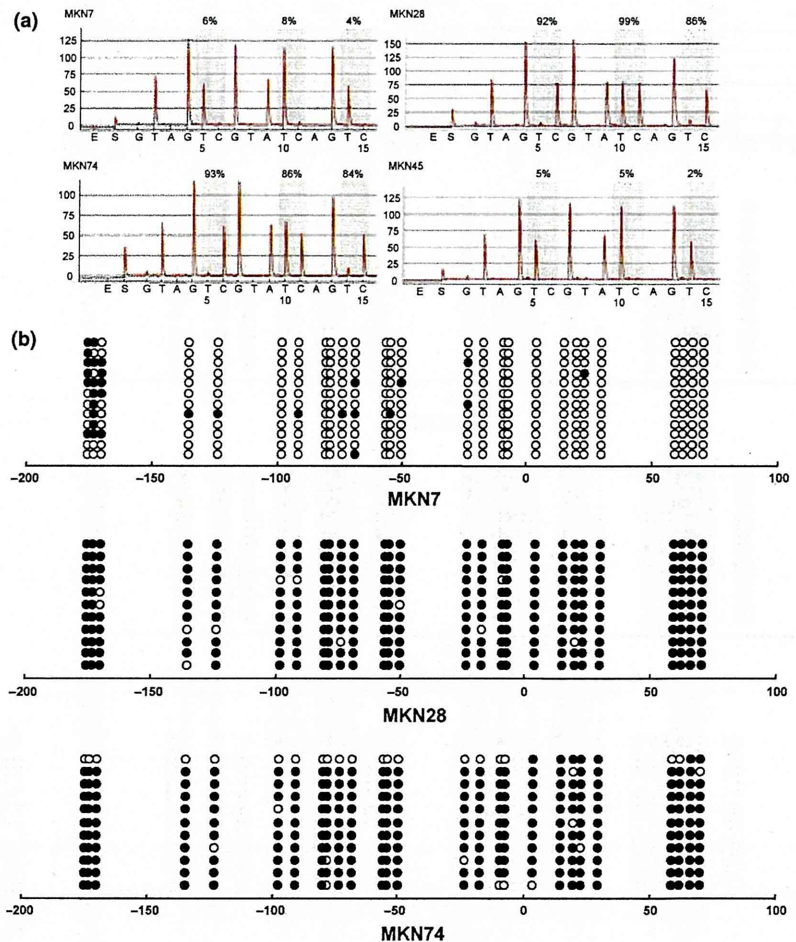

Fig. 3. Methylation analysis of interferon regulatory factor (*IRF*)-*5* in gastric cancer cell lines. (a) Representative pyrosequencing results. (b) Representative bisulfite-sequencing results.

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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-α/β/γ, 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

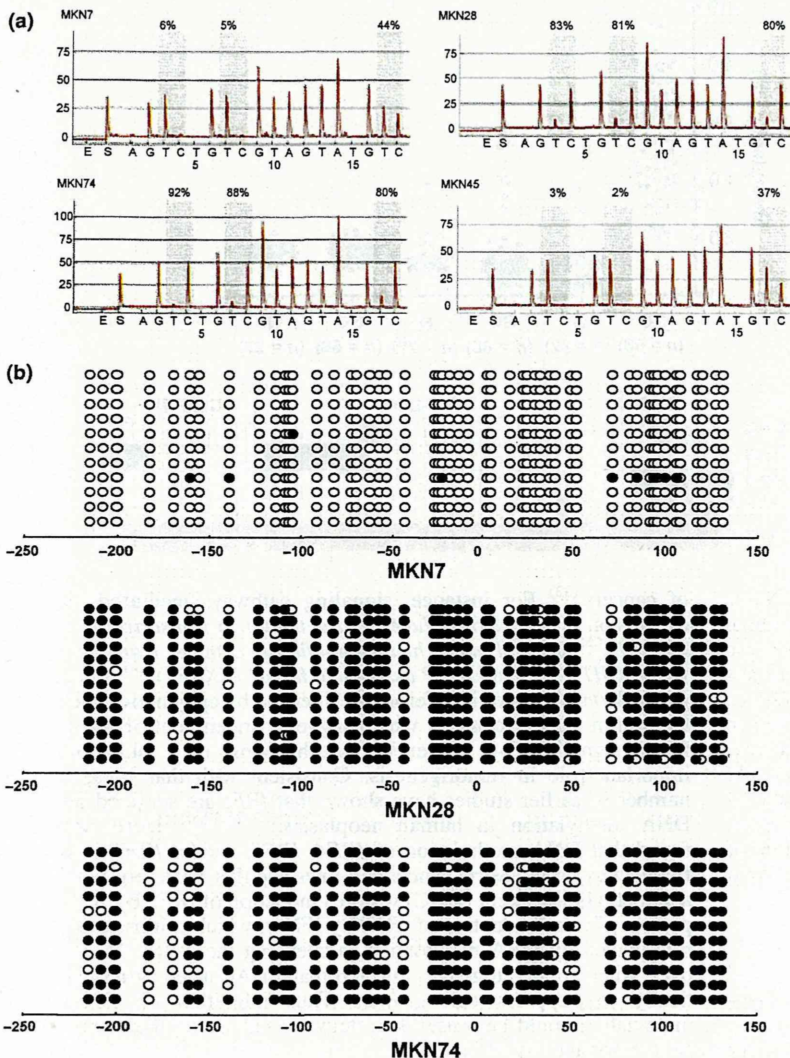

Fig. 4. Methylation analysis of interferon regulatory factor (*IRF*)-*8* in gastric cancer cell lines. (a) Representative pyrosequencing results. (b) Representative bisulfite-sequencing results.

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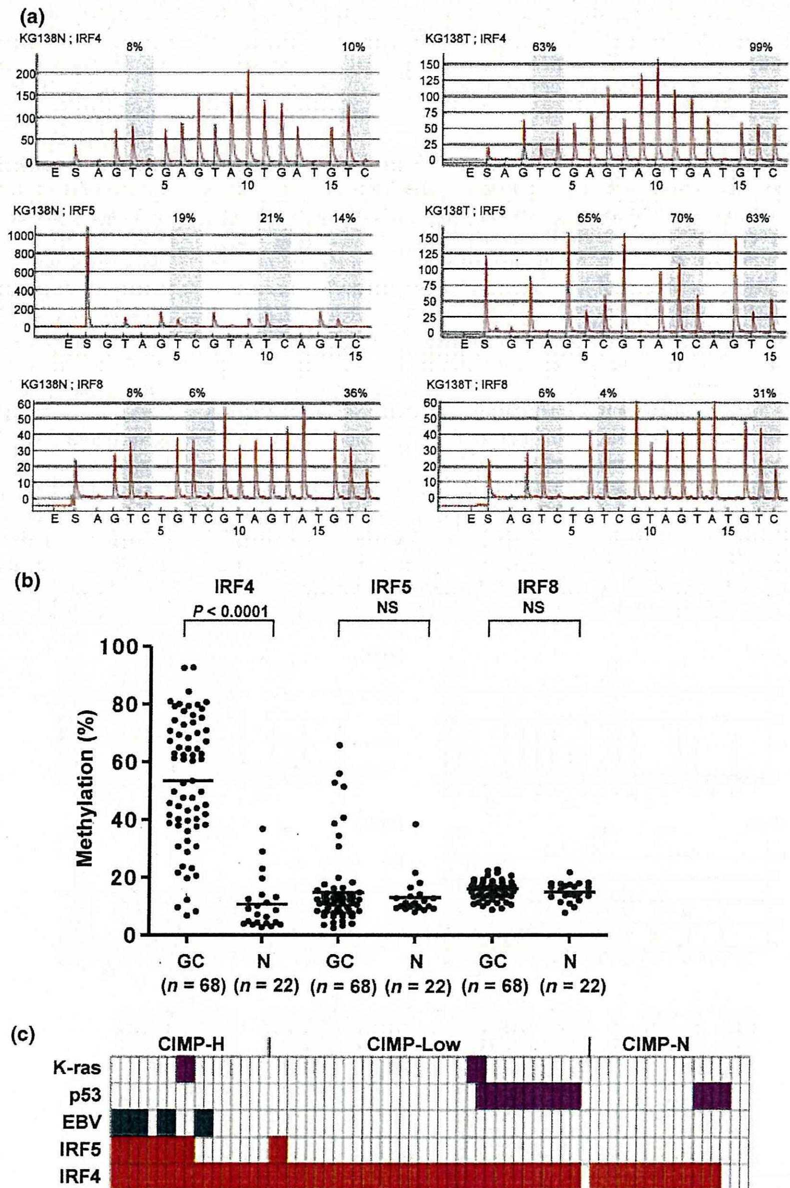

Fig. 5. Methylation of interferon regulatory factor (*IRF*)-*4*, *IRF5*, and *IRF8* in primary gastric cancers. (a) Representative bisulfite-pyrosequencing results. (b) Scatter plot of *IRF* methylation. GC, gastric cancer; horizontal bars, average methylation levels in total cases; N, normal stomach; 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.

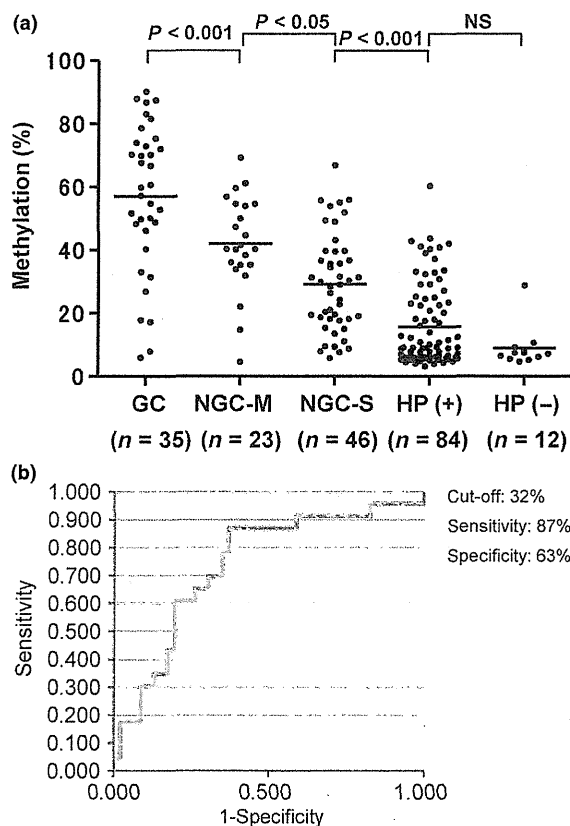

Fig. 6. Methylation levels of interferon regulatory factor (*IRF*)-*4* in gastric cancers and noncancerous gastric mucosae. (a) Distribution of *IRF4* methylation in gastric cancer and noncancerous gastric mucosae. GC, gastric cancer; HP(+), gastric mucosae from *Helicobacter pylori* (*H. pylori*)-positive 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

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