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 *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

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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 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

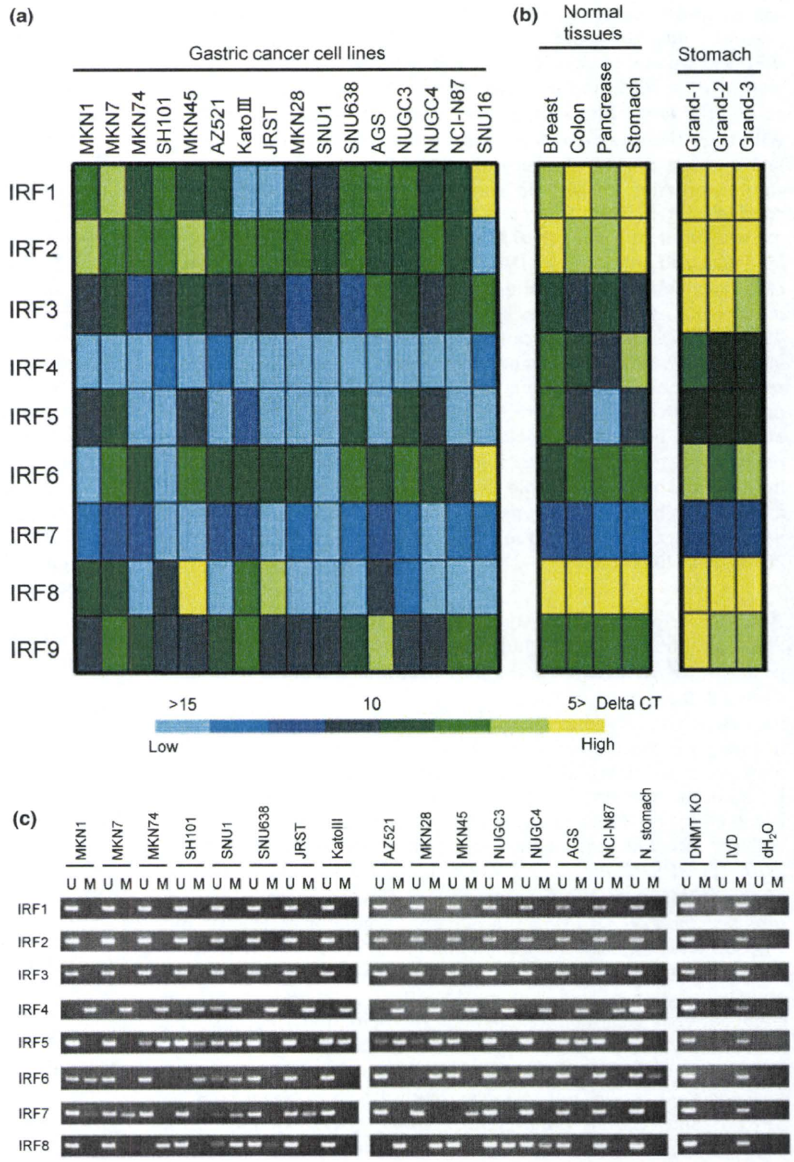

Fig. 1. Down-regulation of interferon regulatory factors (*IRFs*) in gastric cancer cell lines. The heat map shows the expression profiles in 16 gastric cancer cell lines (a) and four normal tissue specimens (b). Levels of expression are normalized to *GAPDH*, and delta-CT values are shown. (c) Analysis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

(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*

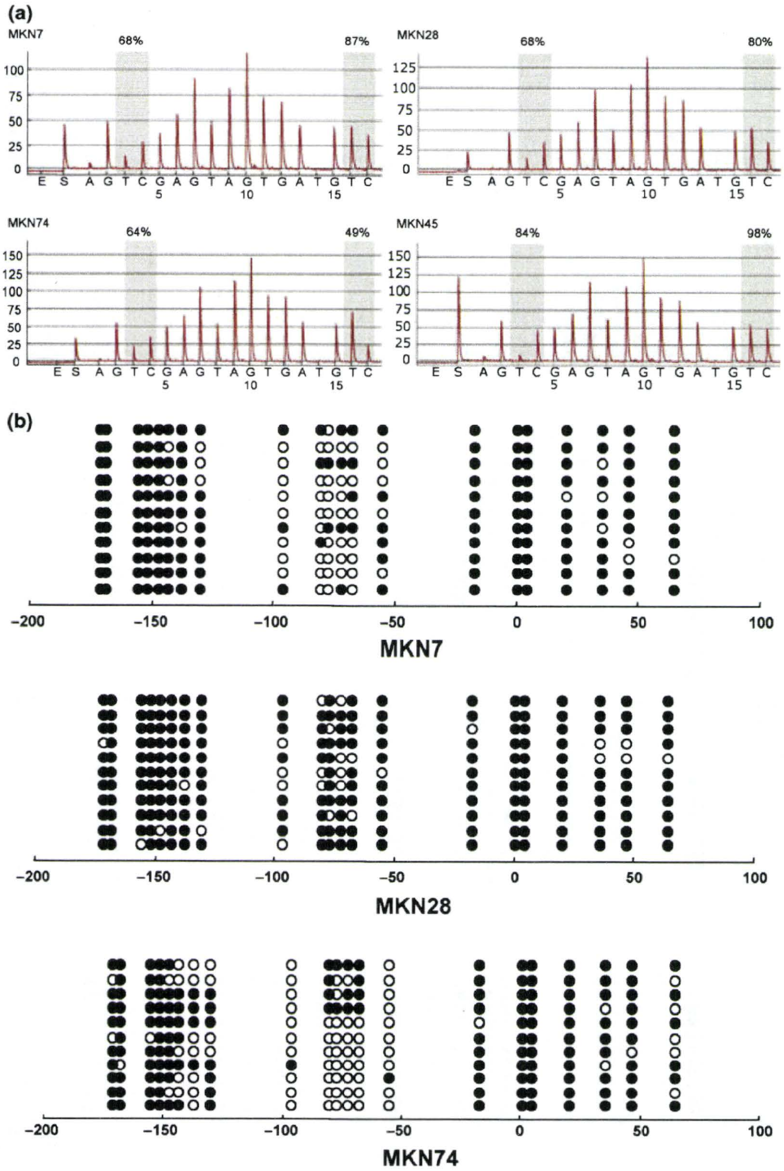

Fig. 2. Methylation analysis of interferon regulatory factor (*IRF*)*-4* in gastric cancer cell lines. (a) Representative pyrosequencing results. Gray columns depict regions of CpG sites, and the percentage methylation at each CpG site is shown on the top. (b) Representative bisulfite-sequencing results. 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

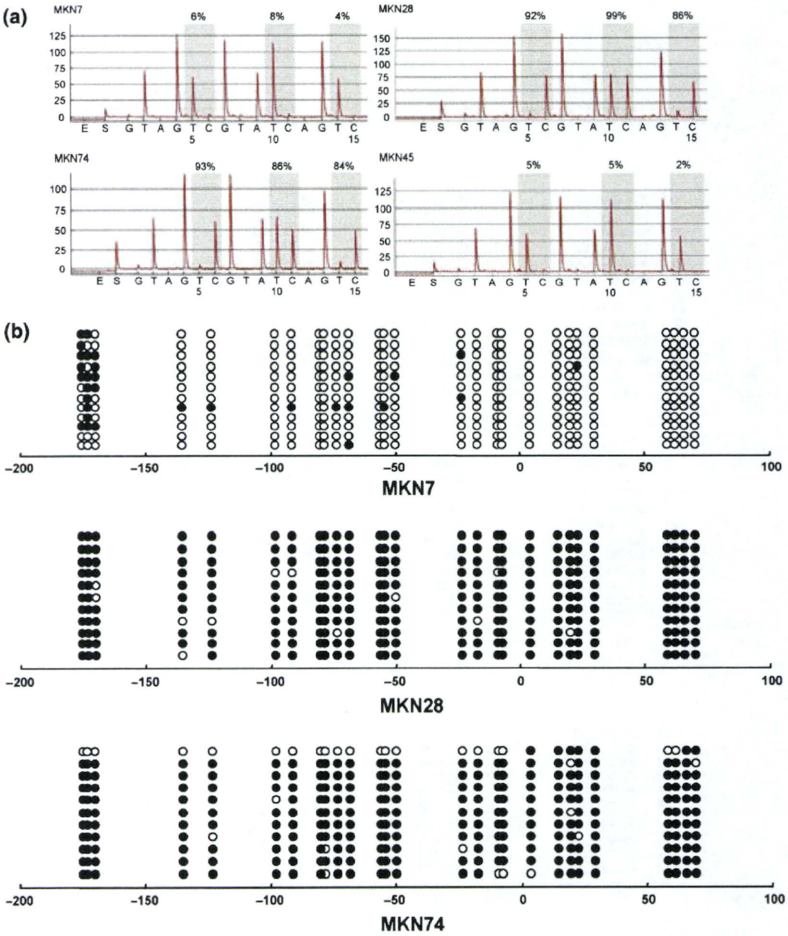

Fig. 3. Methylation analysis of interferon regulatory factor (*IRF*)-*5* in gastric cancer cell lines. (a) Representative pyrosequencing results. (b) Representative bisulfite-sequencing results.

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

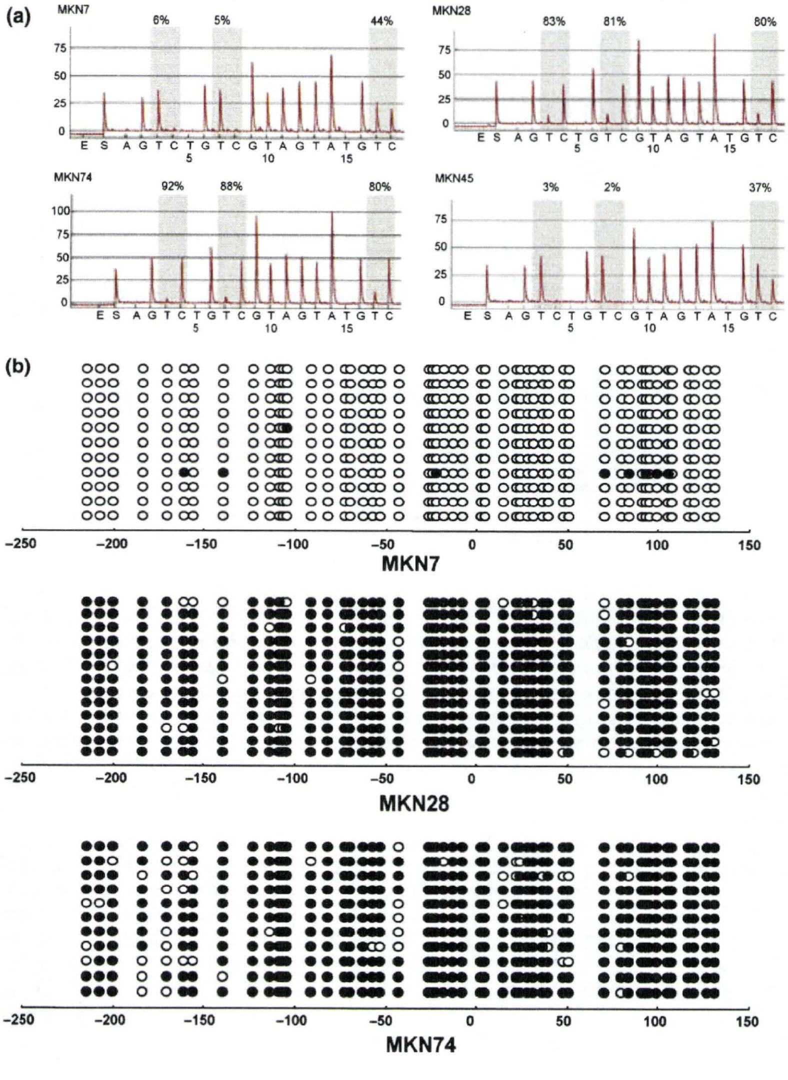

Fig. 4. Methylation analysis of interferon regulatory factor (*IRF*)-*8* in gastric cancer cell lines. (a) Representative pyrosequencing results. (b) Representative bisulfite-sequencing results.

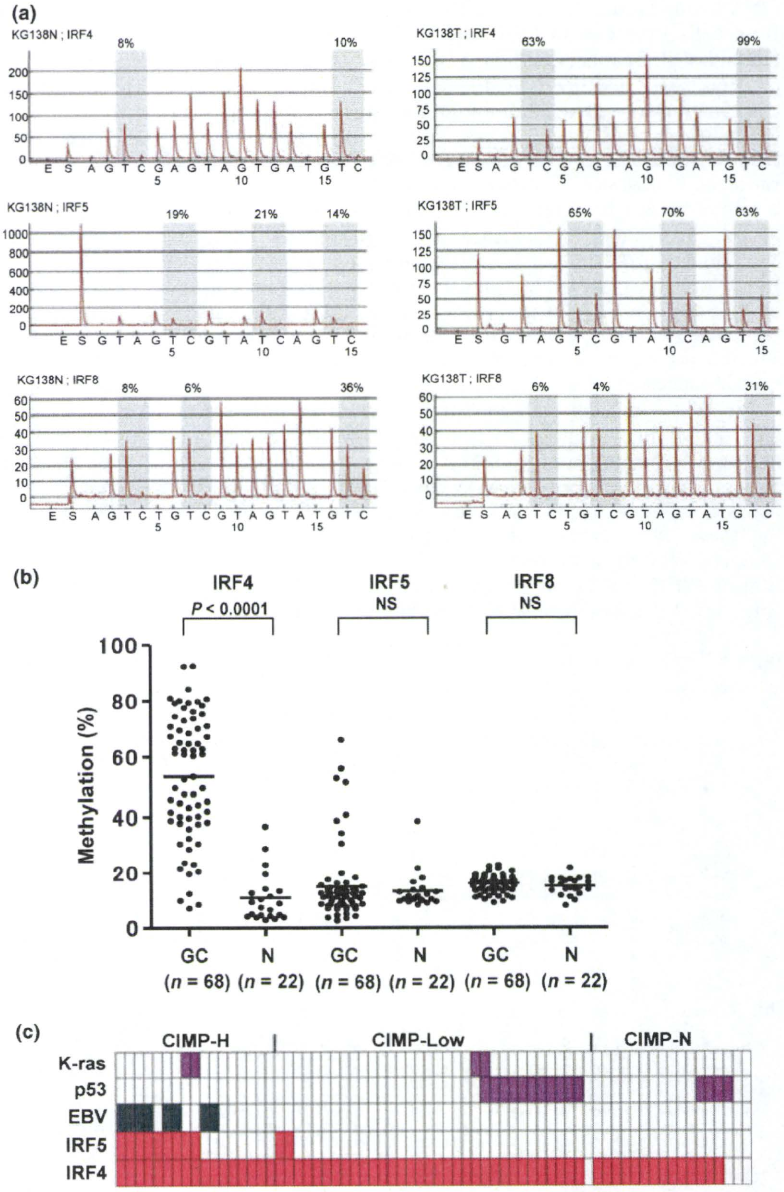

Fig. 5. Methylation of interferon regulatory factor (*IRF*)-*4*, *IRF5*, and *IRF8* in primary gastric cancers. (a) Representative bisulfite-pyrosequencing results. (b) Scatter plot of *IRF* methylation. GC, gastric cancer; horizontal bars, average methylation levels in total cases; N, normal stomach;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

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.

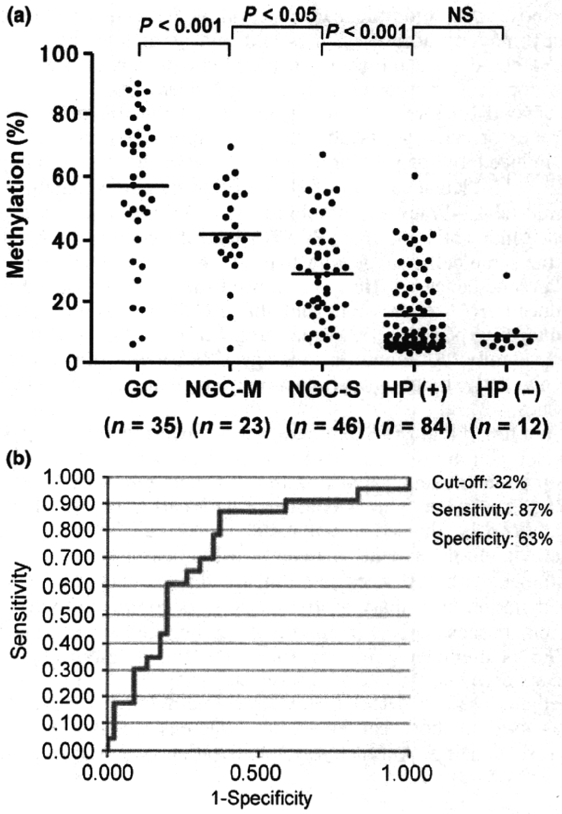

Fig. 6. Methylation levels of interferon regulatory factor (*IRF*)*-4* in gastric cancers and noncancerous gastric mucosae. (a) Distribution of *IRF4* methylation in gastric cancer and noncancerous gastric mucosae. GC, gastric cancer; HP(+), gastric mucosae from *Helicobacter pylori* (*H. pylori*)-positive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

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

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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 RNAeasy (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, apoptosis, and signal transduction were enriched in DAC-treated cells, as compared to mock-treated cells.

Expression analysis of genes identified by microarray

Database analysis revealed that out of 288 genes upregulated by DAC, 155 contain CpG islands in the 5′ end of the gene (Supplementary Table 3). We next selected ten genes known from earlier work to be cancer-related and to have CpG islands in their 5′ ends (Fig. 1). The selected genes were DFNA5, SFRP1, DKK3, PGP9.5, and LOXL4, which were all previously shown to be silenced by DNA methylation in various types of tumors [26–30]; NTN4, which encodes a member of the netrin family involved in the negative regulation of angiogenesis [31]; TRIM50, which encodes an E3 ubiquitin ligase [32]; FKBP6, which encodes an immunophilin family protein [33]; PON1, which encodes an arylesterase and whose polymorphisms are known to be associated with prostate cancer [34]; and OSBPL3, which encodes an oxysterol-binding protein that plays a role in cell adhesion [35]. Real-time PCR analysis revealed that the expression levels of all these genes were low or negligible in MCF7 cells, whereas high levels of expression—i.e., an expression ratio against GAPDH >0.01—were detected for DFNA5, SFRP1, OSBPL3, NTN4, PGP9.5, and LOXL4 in normal breast tissue; cell lines other than MCF7 showed various levels of expression (Supplementary Fig. 2). For DKK3, FKBP6, PON1, and TRIM50, expression was low—i.e., an expression ratio against GAPDH < 0.01—in normal breast tissue, and cell lines showed various levels of expression (Supplementary Fig. 3). Treatment with DAC restored expression of these genes in cell lines in which expression was otherwise low or negligible (Supplementary Fig. 3).

Methylation analysis of ten genes in breast cancer cell lines

To confirm methylation-dependent gene silencing, we next used bisulfite-pyrosequencing to examine the methylation status of the ten genes. This enabled us to quantify the methylation of multiple CpG sites (Fig. 2). The primers and probes were designed to detect methylation in the region around the transcription start sites. Dense methylation of nine genes (SFRP1, DFNA5, DKK3, PGP9.5, OSBPL3, NTN4, TRIM50, FKBP6, and PON1) was detected in MCF7 cells, strongly suggesting that DNA methylation is the cause of gene silencing. Various levels of methylation were detected in four other cell lines and was also associated with gene silencing (Figs. 1, 2; Supplementary Figs. 2, 3). That methylation of LOXL4 was not detected means that LOXL4 is silenced by a mechanism other than DNA methylation.

Fig. 1 Real-time PCR analysis of genes upregulated by DAC. The ▶ expression status of DFNA5, SFRP1, DKK3, FKBP6, LOXL4, OSBPL3, NTN4, PGP9.5, PON1, and TRIM50 was confirmed by real-time PCR. The cell lines and tissues examined are shown below the columns. Cell lines were treated for 72 h with either mock (−) or 1.0 μM DAC. The integrity of the cDNA was assessed by comparing the CT values for the genes of interest with that of GAPDH. *Columns* means of three experiments, *bars* SE

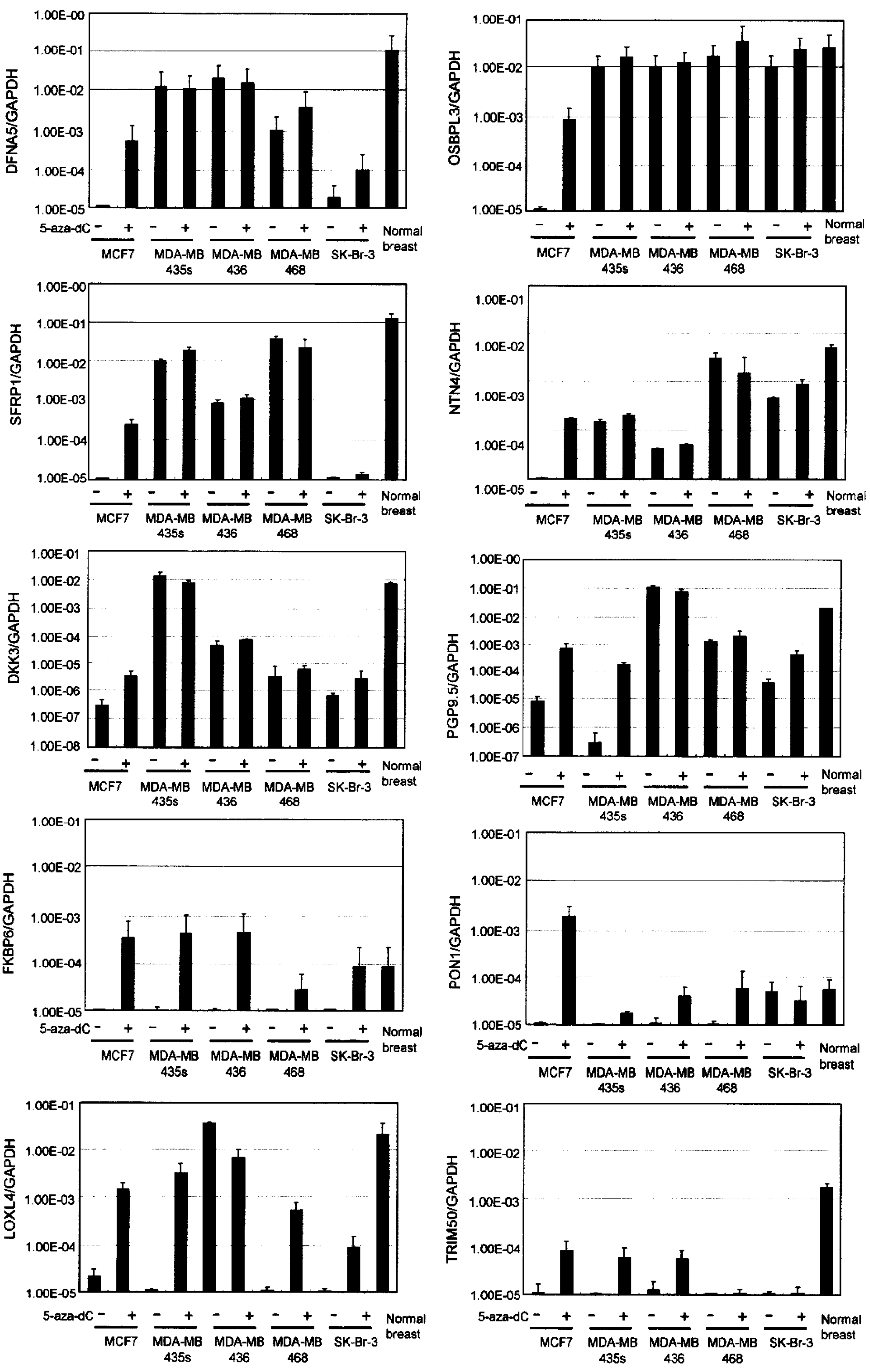
We next performed bisulfite-sequencing analysis to obtain detailed methylation profiles of the CpG sites in the region around the transcription start site of DFNA5 gene. We examined 45 CpG sites and found that DFNA5 was densely methylated in MCF7 cells, which do not express DFNA5. By contrast, little or no methylation was detected in MDA-MB435s, MDA-MB436, MDA-MB-468, and SK-Br-3 cells, which do express DFNA5 (Fig. 3). Thus, the results obtained with bisulfite-sequencing are consistent with both the bisulfite-pyrosequencing data and the DFNA5 expression status.

Restoration of p53-dependent transcription of DFNA5 by demethylation

It was recently reported that DFNA5 is a target gene for p53 [36]. We therefore tested whether demethylation of DFNA5 in MCF7 cells would restore its transcriptional activation by p53 and/or by two other p53 family genes, TAp63γ and TAp73β. When cells were infected with Ad-p53, Ad-p63γ, or Ad-p73β, expression of FLAG-tagged p53 family proteins was detected (Fig. 4a). In addition, p21, a cyclin-dependent inhibitor, was induced by all three vectors (Fig. 4a). We then examined expression of DFNA5 in MCF7 cells with or without treatment with DAC. We found that treating MCF7 cells with DAC restored induction of DFNA5 by p53 family genes, especially by p63, suggesting that DFNA5 is a target of the p53 family, not specifically p53, itself (Fig. 4b). We then performed ChIP assays to determine whether p63γ directly interacts with the p53 response element of DFNA5 (RE-DFNA5) (Fig. 4c). PCR amplification of the ChIP products revealed that one DNA fragment containing RE-DFNA5 was present in the immunoprecipitated complex with p63γ. As a control, we confirmed that p63γ binds to the p53 response element of MDM2 in vivo. These results indicate that DFNA5 can be upregulated by p63γ through direct interaction with RE-DFNA5.

Tumor suppressive activity of NTN4

Netrins and their receptors have been shown to be involved in tumorigenesis [37]. To test whether NTN4 suppresses growth of breast cancer cells, we performed colony formation assays using MCF7 cells, which express negligible levels NTN4. We found that introduction of a plasmid


DFNA5/GAPDH
1.00E-00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
5-aza-dC – + – + – + – + – +
MCF7
MDA-MB 435s
MDA-MB 436
MDA-MB 468
SK-Br-3
Normal breast
OSBPL3/GAPDH
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
SFRP1/GAPDH
1.00E-00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
NTN4/GAPDH
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
DKK3/GAPDH
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
1.00E-06
1.00E-07
1.00E-08
PGP9.5/GAPDH
1.00E-00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
1.00E-06
1.00E-07
FKBP6/GAPDH
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
PON1/GAPDH
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
LOXL4/GAPDH
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
TRIM50/GAPDH
1.00E-01
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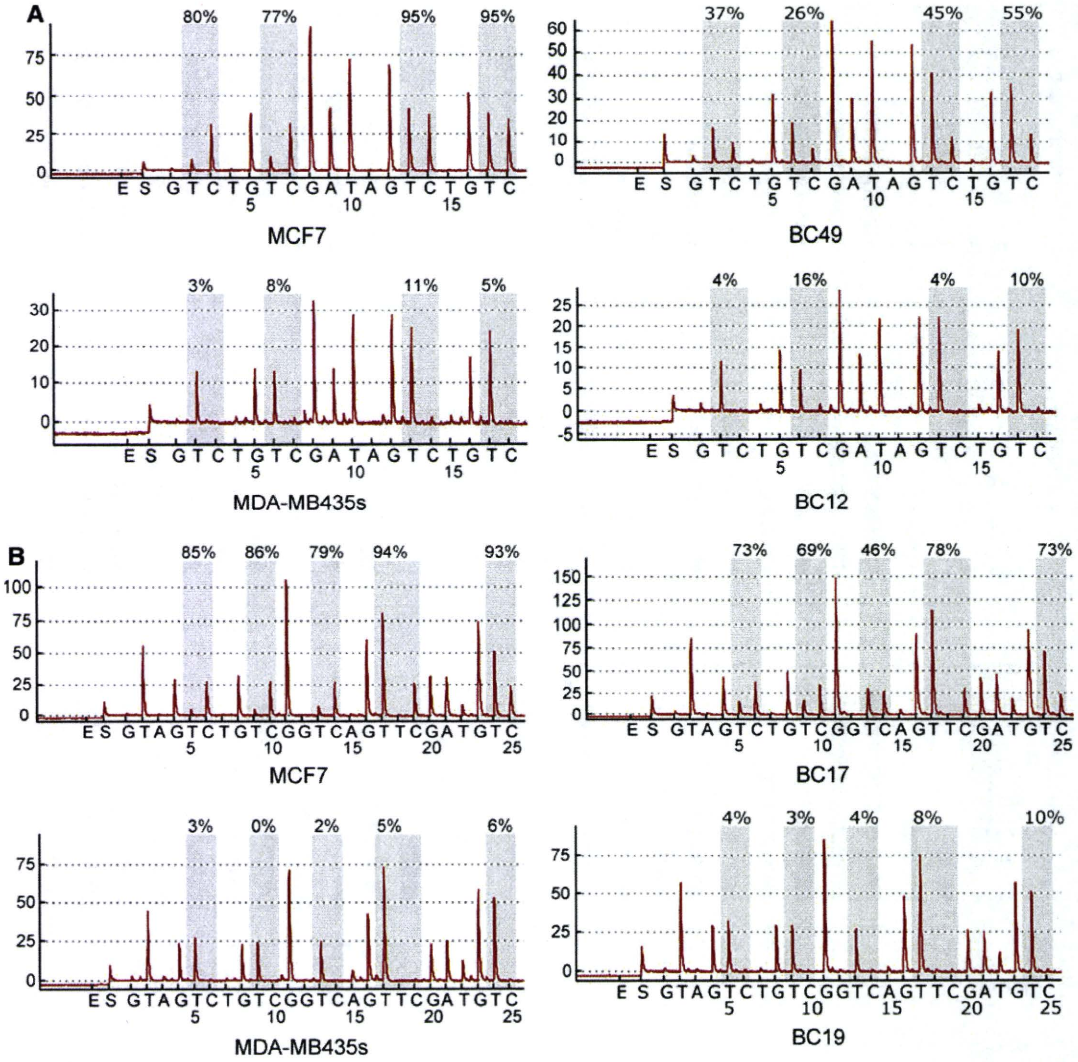


Fig. 2 Representative results of bisulfite-pyrosequencing of DFNA5 (**a**) and SFRP1 (**b**). Bisulfite-pyrosequencing was carried out using DNA from breast cancer cell lines and primary breast cancer specimens. Examined were the regions upstream from the transcription start site (DFNA5: −85 to −97; SFRP1: −65 bp to −44 bp). *Gray columns* depict regions of CpG sites, and the percentage methylation at each CpG site is shown on the *top*. *Y*-axis, signal peaks expressed as a proportion of the number of nucleotides incorporated. *X*-axis, the nucleotides incorporated. Cell lines and specimens are shown below the *columns*

containing NTN4 cDNA significantly suppressed colony growth, suggesting that NTN4 does indeed have tumor suppressive activity (Fig. 5a, b).

Comparison of methylation and clinicopathological features of patients with primary breast cancer

Of the nine aforementioned genes silenced by DNA methylation in primary breast cancer, seven showed significantly higher levels of methylation in cancerous tissues than in normal breast tissues ($P < 0.001$ for NTN4, PGP9.5, DKK3, OSBPL3, SFRP1, DFNA5; $P < 0.01$ for PON1, Fig. 6a; Supplementary Table 7). Methylation was also examined in paired samples of cancerous and adjacent normal breast tissues from 15 patients. Methylation of NTN4 ($P < 0.001$), PGP9.5 ($P < 0.001$), DKK3 ($P = 0.006$), and PON1 ($P = 0.031$) was significantly higher in the tumor tissue than in the adjacent breast tissue (Fig. 6b; Supplementary Table 8). The clinical usefulness of DNA methylation in distinguishing breast cancer from noncancerous tissue was confirmed by analyzing ROC curves (Fig. 6c; Supplementary Table 9). Methylation of NTN4, DKK3, and PGP9.5 showed highly discriminative ROC curve profiles, which clearly distinguished breast cancer from normal breast tissue (NTN4: $p < 0.001$; DKK3: $P < 0.001$; PGP9.5: $P < 0.001$). When we used 16%

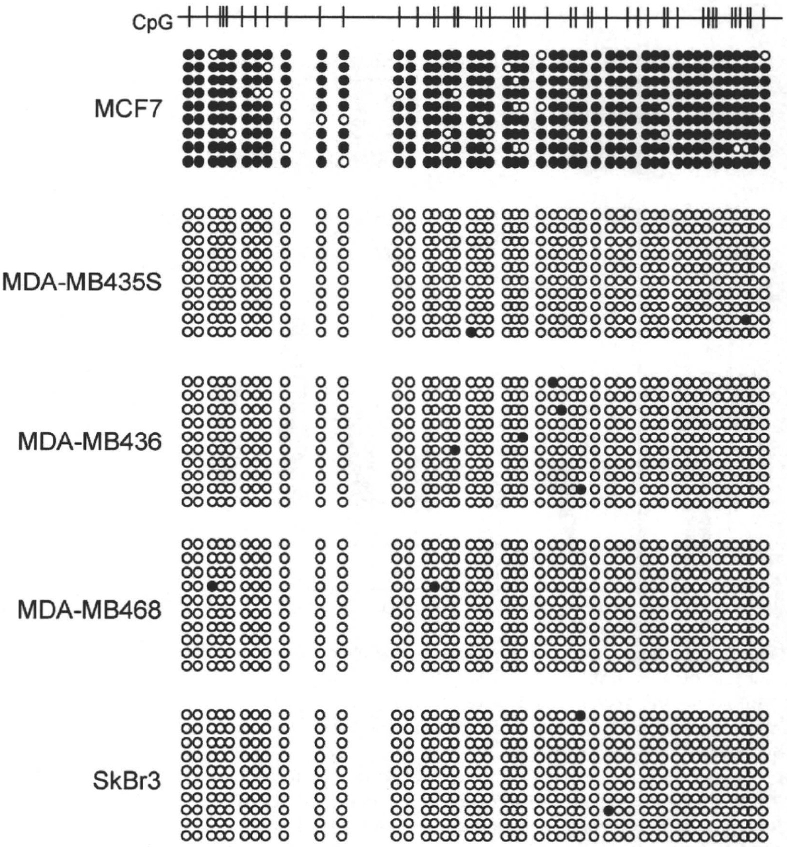


Fig. 3 Bisulfite-sequencing of DFNA5 in breast cancer cell lines. Each *circle* represents a CpG dinucleotide. Methylation status: *open circles* unmethylated, *filled circles* methylated. At least 9 clones were sequenced for each case. The CpG sites in the region spanning −265 to +176 from the transcription start site were analyzed, and are indicated by vertical *bars* (*top*)

methylation as a cut-off value, the sensitivity was 98.6% (95% CI: 92.6–100) and the specificity was 76.5% (95% CI: 50.1–93.2), indicating that methylation of NTN4 could be a useful molecular marker for detection of breast cancer (Supplementary Table 9). We then examined the relation between methylation status and clinicopathological factors (Supplementary Table 10) and found that methylation of FKBP6 is significantly correlated with advanced stages (Fig. 7a; Supplementary Table 11, $P = 0.014$) and tumor size (Fig. 7b, Supplementary Table 12, $P = 0.017$). There was no correlation between methylation and other factors including stages, histological types, number of metastasis positive lymph nodes, vascular invasion.

Discussion

Identification of genes silenced by DNA methylation in breast cancer

In the present study, we performed a microarray analysis to identify genes silenced by DNA methylation in breast cancer. We found that 288 genes were upregulated more than fivefold after treatment with DAC. Among those, SFRP1, DFNA5, and DKK3 are known from earlier studies to be silenced by DNA methylation in breast cancer [23, 24, 38], while PGP9.5 is known to be methylated in a variety of cancer types [39, 40]. Our findings thus confirm that, with our microarray approach, we are able to successfully identify targets of DNA methylation in breast cancer. Gene ontology analysis revealed that genes involved in immune responses, the extracellular region and cytokine activity are significantly upregulated by DAC. Consistent to those findings, Karpf et al. [41] showed that genes regulated via interferon signaling are frequently upregulated by DAC, which suggests that upregulation of genes involved in immune responses, including those involved in antigen presentation, regulation of tumor necrosis factor and/or interferon pathways, may be a general feature of DAC treatment.

Utility of DNA methylation for molecular diagnosis in breast cancer

Although previous studies have identified numerous targets of DNA methylation in breast cancer, the usefulness of the targeted genes for diagnosis remains unclear [13–15]. In fact, those studies confirmed DNA methylation of only a

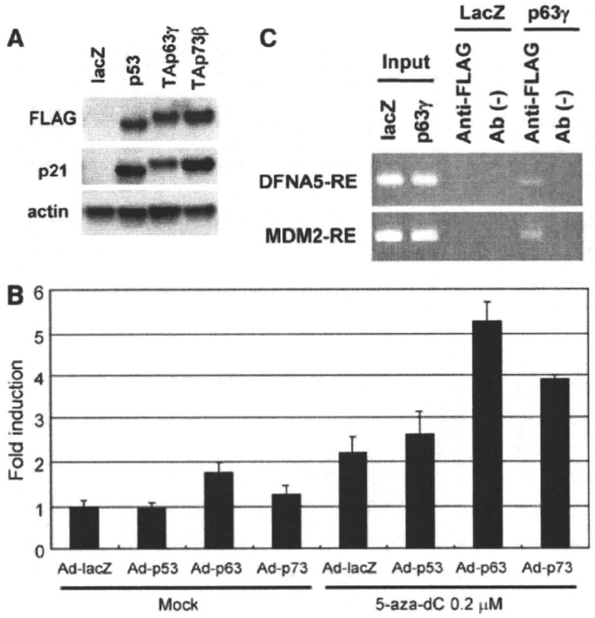


Fig. 4 Induction of DFNA5 by p53 family members. **a** Western blot analysis of p53, TAp63γ, and TAp73β. MCF7 cells were infected with adenoviral vector encoding the protein shown on the *top* and were harvested 24 h after infection. Immunoblot analysis was performed using anti-FLAG antibody. Expression of p21 was examined as a control. **b** ChIP assay. PCR was performed using ChIP products, and one DNA fragment containing the RE-DFNA5 was present in the immunoprecipitated complex with TAp63γ. As a control, fragments MDM2 DNA were amplified. **c** Restoration of p53-dependent DFNA5 expression by DAC. MCF7 cells were treated for 72 h with either mock or 0.2 μM DAC followed by infection with 100 MOI of Ad-lacZ, Ad-p53, Ad-63γ, or Ad-p73β for 24 h. Expression of DFNA5 was examined by real-time PCR. *Columns* mean of three experiments, *bars* SE

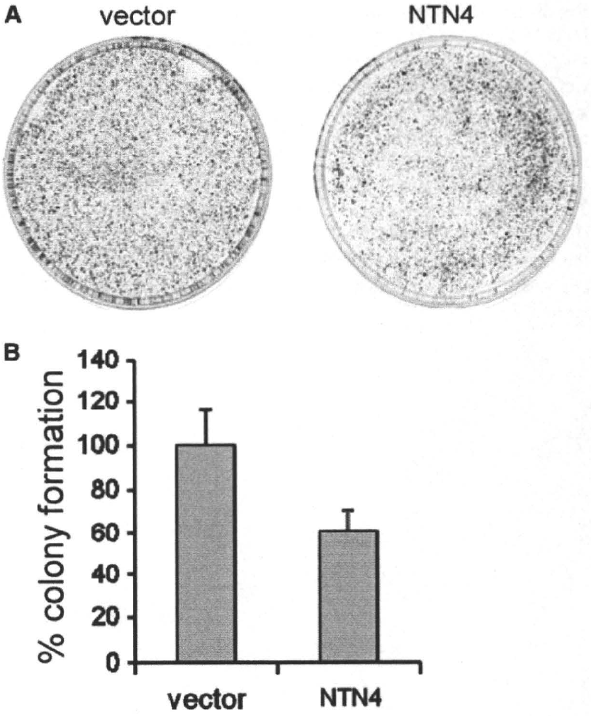
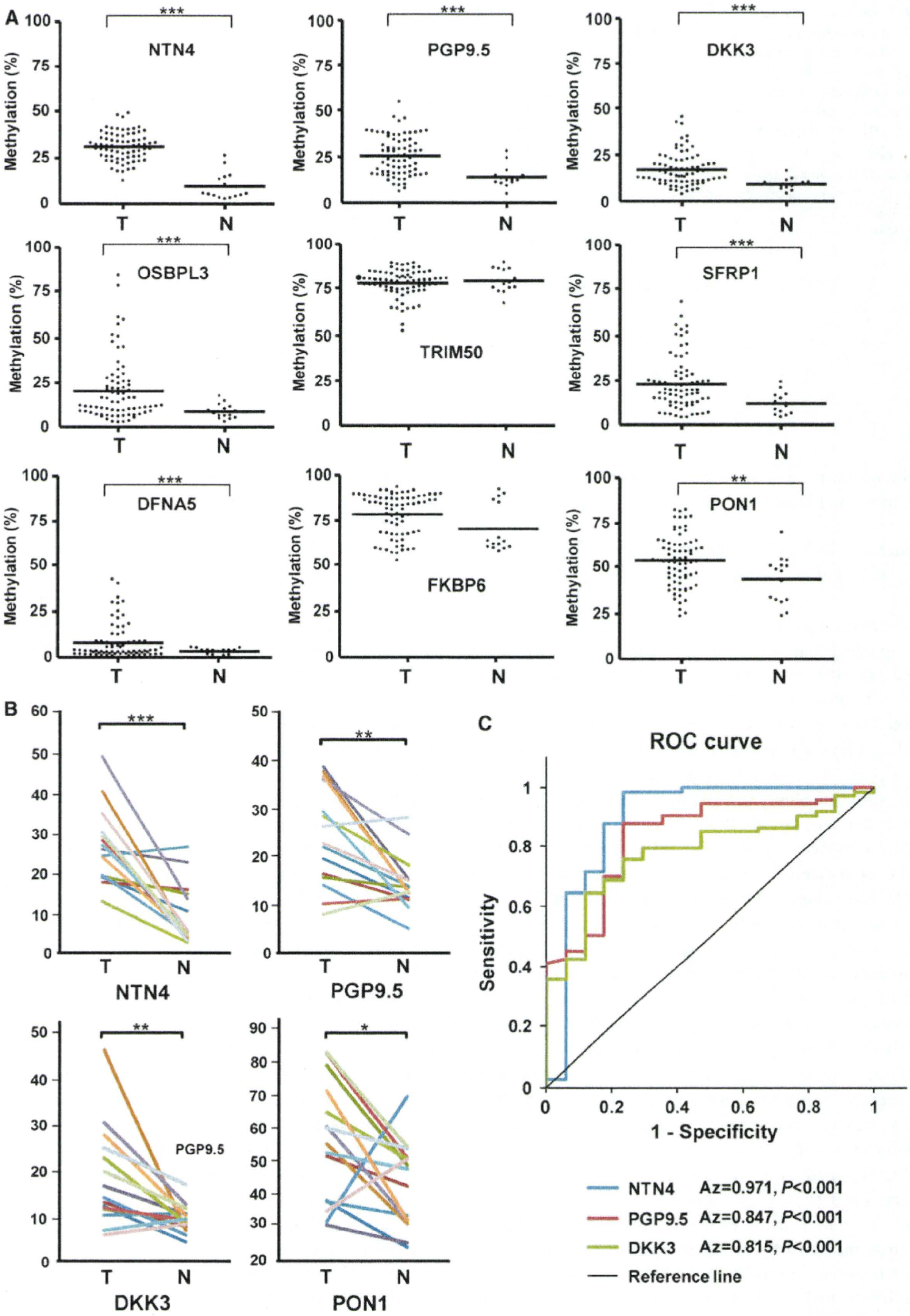


Fig. 5 NTN4 suppresses breast cancer cell growth. **a** Colony formation assay. MCF7 cells were transfected with NTN4 or control vector and plated. After 2 weeks, the cells were fixed with methanol and stained with Giemsa. **b** Relative colony formation efficiencies of MCF7 cells transfected with NTN4 or control plasmid (vector). *Columns* mean of three experiments, *bars* SE

limited number of samples [13, 15], or the methylation analysis was not quantitative [14]. Our findings suggest DNA methylation can be used as a biomarker to detect breast cancer. The cancer can be detected using DNA from biopsy specimens, serum or breast fluid—i.e., any tissue in which genes specifically methylated at a high frequency in cancer can be identified. In the present study, bisulfite-pyrosequencing, a semi-quantitative methylation analysis, revealed that methylation of NTN4, PGP9.5, and DKK3 occurs in a cancer-specific manner. Previous studies have shown that PGP9.5 is silenced by DNA methylation in a variety of tumors [27, 39, 40], and cancer-specific methylation of PGP9.5 has been observed in both head/neck and hepatocellular cancers [39]. On the other hand, normal tissues in the prostate, esophagus, and stomach also show PGP9.5 methylation [39], so that whether or not methylation is cancer-specific is dependent on the cancer and tissue type. Recently, Veeck et al. [24] used methylation-specific PCR to assess the methylation of DKK3 and found that the gene is methylated in 61% of breast cancers. In the present study, we similarly observed that methylation of DKK3 is significantly higher in breast cancers (17.2%) than in normal breast tissues (17.2 vs. 9.8%, $P < 0.001$). Moreover, we showed for the first time that NTN4 is silenced by DNA methylation in cancer. When we used 16% methylation as a cut-off value, the sensitivity was 98.6% (95% CI: 92.6–100) and specificity was 76.5% (95% CI: 50.1–93.2), indicating that methylation of NTN4 could be a good molecular marker for detection of breast cancer.

In contrast to the genes mentioned above, methylation of FKBP6, PON1, and TRIM50 was detected even in normal breast tissues, and increases in promoter methylation reportedly correlate with age in colorectal and prostate tissues [42, 43]. In this regard, methylation of SFRP1, which has been shown to correlate with aging in colon [44], was not high in breast tissue, indicating that age-related methylation is also tissue-specific and that further studies

Fig. 6 Methylation analysis in primary breast cancers. **a** Summary of methylation levels in normal and cancerous breast tissue: *N* normal tissue, *T* cancerous tissue. *** $P < 0.001$, ** $P < 0.01$. **b** Analysis of NTN4, PGP9.5, DKK3, and PON1 methylation in breast cancer and adjacent normal breast tissue. **c** ROC curve analysis of NTN4, PGP9.5, and DKK3 in primary breast cancer. The area under the ROC curve for each site conveys its utility for distinguishing normal breast from breast cancer in terms of its sensitivity and specificity ▶


A
Methylation (%)
100
75
50
25
0
NTN4
PGP9.5
DKK3
OSBPL3
TRIM50
SFRP1
DFNA5
FKBP6
PON1
***
**
T
N
B
NTN4
PGP9.5
DKK3
PON1
*
C
ROC curve
Sensitivity
1 - Specificity
0
0.2
0.4
0.6
0.8
1
NTN4 Az=0.971, P<0.001
PGP9.5 Az=0.847, P<0.001
DKK3 Az=0.815, P<0.001
Reference line

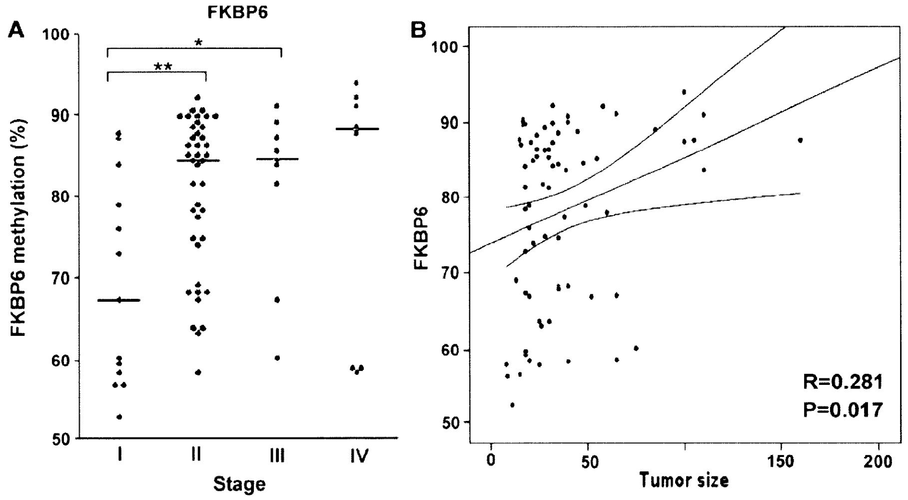


Fig. 7 Correlation between FKBP6 methylation and the clinicopathological features of breast cancer. **a** Scatter plot of FKBP6 methylation in patients with cancers of different stages. ** $P < 0.01$, * $P < 0.05$. **b** Scatter plot in which FKBP6 methylation is plotted against tumor size. *X*-axis: tumor size (mm). *Y*-axis: FKBP6 methylation level (%)

will be necessary to clarify the significance of its methylation in normal breast tissues.

Functional roles of genes silenced by DNA methylation in breast cancer

Our microarray analysis revealed several genes involved in cell signaling pathways to be methylated. SFRP1 and DKK3 are Wnt antagonists frequently silenced by methylation in colorectal and gastric cancers [29, 45, 46], and several lines of evidence suggest that activation of Wnt signaling plays a key role in breast cancer. For example, Bafico et al. [47] found that the unphosphorylated form of β-catenin is frequently present in breast cancer cell lines, and that the Wnt ligands WNT-2, WNT-3, and WNT6 are frequently overexpressed in the same cell lines. Mutation of APC or β-catenin is rare in breast cancer, however, so that the mechanism underlying the activation of Wnt signaling in this disease is not fully understood [48]. In the present study, we found that SFRP1 is inactivated by DNA methylation in MCF7 cells, and that SFRP1 is methylated in 45 of the 75 (60%) primary breast cancers tested. Perhaps inactivation of negative regulators of Wnt is involved in activating Wnt signaling in breast cancer.

DFNA5 was originally identified as a gene involved in nonsyndromic hearing impairment [49]. Since then, DFNA5 has also been identified as a gene downregulated in etoposide-resistant melanoma [50]. Although the role of DFNA5 in mediating the effects of etoposide remains unclear, Lage et al. [51] showed that introduction of the gene into tumor cells increases their susceptibility to apoptosis mediated by activated caspase-3 following etoposide treatment. In addition, DFNA5 was recently found to be inactivated by DNA methylation in gastric cancers, and introduction of DFNA5 into gastric cancer cells suppressed colony formation and induced apoptosis [26].

DFNA5 is reportedly a target gene of p53 [36]. In the present study, we found that DFNA5 is silenced by methylation in MCF7 cells and that treating the cells with DAC restored induction of DFNA5 by p53 family genes, especially p63γ. Thus, DFNA5 does not appear to be targeted selectively by p53, itself, but by p53 family member, p63γ.

Netrins are secreted molecules involved in axon guidance and angiogenesis. Among them, NTN4 is an antiangiogenic factor that acts through its receptor, neogenin [31]. Notably, expression of NTN4 is associated with a good prognosis in breast cancer [52]. In the present study, we showed for the first time that NTN4 is silenced by DNA methylation in breast cancer and that treating breast cancer cells with a demethylating agent (DAC) restores its expression. We also showed that NTN4 has tumor suppressive activity. Identification of NTN4 as a candidate tumor suppressor in breast cancer may be useful for the development of new cancer therapies [53]. The methylation of NTN4 was cancer-specific, suggesting epigenetic changes to the gene could be a useful molecular marker for diagnosis.

FK506-binding proteins (FKBPs) are immunophilins involved in protein folding and cell signaling. Among them, FKBP6 has been identified as a candidate gene underlying Williams syndrome, a developmental disorder caused by haploinsufficiency of genes at 7q11.23 [54], and expression of FKBP6 specifically localizes to meiotic chromosome cores and regions of homologous chromosome synapsis [33]. We found that methylation of FKBP6 was correlated with tumor size and stage. The role of FKBP6 in tumorigenesis remains unknown, but its methylation in normal tissue suggests that methylation of FKBP6 could be an example of so called "passenger