Table 1
List of primer sequences and PCR conditions for MSP

Genes	Methylation	Forward	Reverse	Annealing temperature (°C)	Length of PCR product (bp)	Number of PCR cycles
GPR150	M	5′-ATT CGT ATA GAT TTA GCG TC-3′	5′-AAT ATT AAA CGC CGA CG-3′	53	151	35
	UM	5′-GATTTAGTGTTGTTTTTTGT-3′	5′-AAAATATTAAACACCAACA-3′	49	144	33
LOC222171	M	5′-TAT AGA AAG CGT TTG TAA CGG C-3′	5′-GAA AAC GAA TCC ACA CCC G-3′	61	117	32
	UM	5′-TTA TAG AAA GTG TTT GTA ATG GT-3′	5′-AAA CAA ATC CAC ACC CA-3′	56	116	33
PRTFDC1	M	5′-GGG TTG TAC GCG ATT ATT C-3′	5′-AAC TAA ACC GCG AAA ACG-3′	58	129	32
	UM	5′-GGG GTT GTA TGT GAT TAT TT-3′	5′-AAC TAA ACC ACA AAA ACA CA-3′	54	130	33
LOC339210	M	5′-GGA GAT TTG CGT CGC GTC-3′	5′-CAA CGT CGT TCT CCT CCT ACG-3′	63	106	32
	UM	5′-TTT TTG GAG ATT TGT GTT GT-3′	5′-AAC ATC ATT CTC CTC CTA CA-3′	56	110	32
ITGA8	M	5′-GCG GGT GGG AGT AGA CGT C-3′	5′-CTA CCC AAA AAC GCG AAC CG-3′	61	138	32
	UM	5′-GGT GGG TGG GAG TAG ATG TT-3′	5′-CTA CCC AAA AAC ACA AAC CA-3′	58	139	33
C9orf64	M	5′-GGA GGT ATC GTC GTT TAT GTC-3′	5′-AAA CGC CTT CGA CAA CG-3′	60	119	32
	UM	5′-GAG GTA TTG TTG TTT ATG TT-3′	5′-AAA ACA CCT TCA ACA ACA-3′	55	119	32
HOXD11	M	5′-ATG CGT TTA GCG GTG ATA GC-3′	5′-AAA CGA CTC CTA ACG CCG-3′	56	143	32
	UM	5′-ATG TGT TTA GTG GTG ATA GT-3′	5′-AAA CAA CTC CTA ACA CCA-3′	55	143	32

aberrant methylation in a minor population of DNA molecules (Laird, 2003; Miyamoto and Ushijima, 2005; Abe et al., 2005). Successful detection of cancer cells has been reported in nipple aspirates, sputum, urine, feces, lymph nodes and other clinical materials. Since one of the causes for the high mortality of ovarian cancers lies in the difficulty in detecting them at early stages (Barnholtz-Sloan et al., 2003), identification of aberrantly methylated genes for marker development is important in ovarian cancers.

In this study, we performed a genome-wide screening for CGIs aberrantly methylated in ovarian cancers, which can lead to identification of novel tumor-suppressor genes and biomarkers. As a procedure for the screening, we adopted methylation-sensitive-representational difference analysis (MS-RDA) (Ushijima et al., 1997; Kaneda et al., 2003; Ushijima, 2005) that has been successfully used to identify aberrantly methylated CGIs in various human cancers (Takai et al., 2001; Kaneda et al., 2002; Hagihara et al., 2004; Miyamoto et al., 2003, 2005; Abe et al., 2005).

Materials and methods

Cell lines, primary tumor samples, and DNA/RNA extraction

OV-90 (serous), TOV-112D (endometrioid), ES-2 (clear cell) and TOV-21G (clear cell) were purchased from the American Type Culture Collection (Manassas, VA). MCAS (mucinous), RMUG-L (mucinous), RMG-I (clear cell), RTSG (poorly differentiated), TYK-nu (undifferentiated), and KURAMOCHI (undifferentiated) were provided by the Japanese Collection of Research Bioresources (Osaka, Japan). Human ovarian surface epithelial (HOSE6-3) cells, established by immortalizing normal human ovarian surface cells with human papilloma virus E6 and E7 (Tsao et al., 1995, 2001), were a

Table 2
List of primer sequences and PCR conditions for RT-PCR

Genes	Forward	Reverse	Annealing temperature (°C)	Length of PCR product (bp)
GPR150	5′-GCTGGCACCTGCAGGTCTA-3′	5′-CGCCACCAGACGGAGAGTA-3′	68	106
LOC222171	5′-GCCTCCGGGTCCTGTTAA-3′	5′-GTCTCCGGCCGTTCACTC-3′	71	107
PRTFDC1	5′-TGTGGTGGGATATGCCTTAGA-3′	5′-TCTGGGACTTTAGTGGTGAGAAT-3′	61	128
LOC339210	5′-GACAGAGAAGCAGGCCAAAC-3′	5′-CAGGTGGTGCATGTATTCCC-3′	72	103
ITGA8	5′-CTGTCAGGCGTTCAACC-3′	5′-CACCAAGACACTCGCTGTG-3′	72	121
C9orf64	5′-TTGGAGCCCTGAAATACTCTGAT-3′	5′-CAAAGCGAGCACCCTCTGAT-3′	67	106
HOXD11	5′-CGCTGTCCCTATACCAAGT-3′	5′-GCATCCGAGAGAGTTGAAGT-3′	69	100
GAPDH	5′-AGGTGAAGGTCGGAGTCA-3′	5′-GGTCATTGATGGCAACAA-3′	68	99

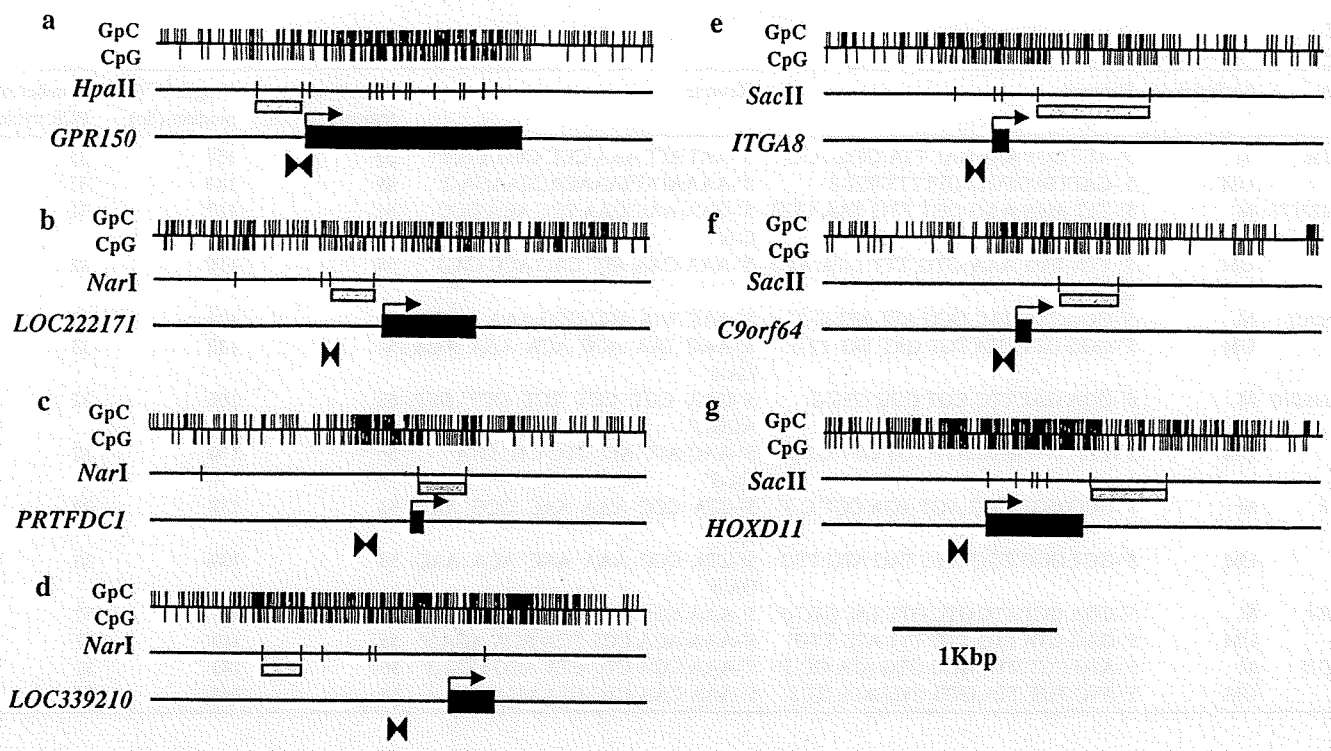


Fig. 1. Genomic structures around the seven CGIs specifically methylated in ovarian cancer cell line(s). Vertical ticks show individual GpC sites (top), CpG sites (middle), and recognition sites (bottom) of restriction enzyme used for MS-RDA (*Hpa*II, *Nar*I or *Sac*II). Gray boxes, DNA fragments isolated by MS-RDA; closed boxes, exons; arrowheads, MSP primers; and arrows, transcription start sites and transcription directions.

kind gift from Dr. Sai-Wah Tsao, University of Hong Kong. Ovarian cancer samples and endometrial cyst samples were obtained from patients who underwent surgery with informed consents. DNA was extracted by a standard phenol/chloroform extraction and ethanol precipitation procedure, and total RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan). Total RNA of the brain and testes was purchased from Ambion (Austin, TX).

MS-RDA and database search

MS-RDA was carried out as in our previous reports (Kaneda et al., 2002, 2003). The genomic origins of the clones obtained by MS-RDA were examined by BLASTN software, and chromosomal positions and relative locations to CGIs and known genes were searched at the GenBank web site (http://www.ncbi.nlm.nih.gov).

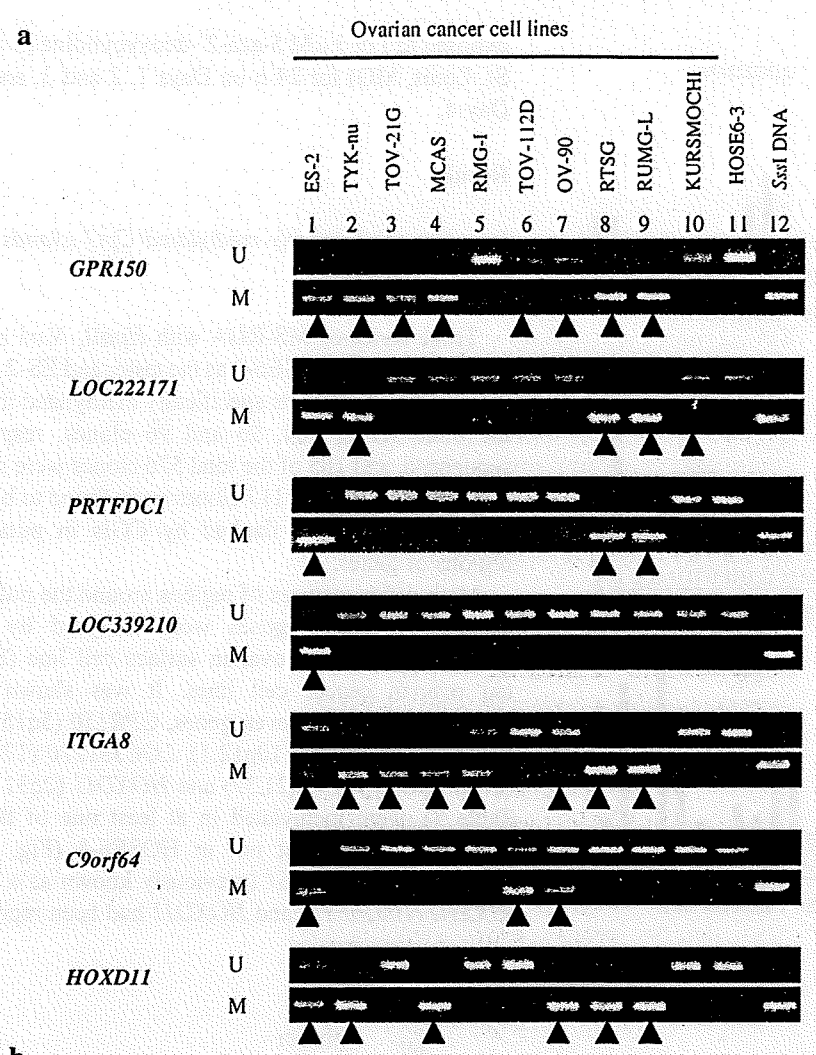
Bisulfite modification and methylation-specific PCR

Sodium bisulfite modification was performed as reported (Kaneda et al., 2004), using 500 ng genomic DNA restricted with *Bam*HI, and the product was dissolved in 20 μl of TE buffer. Methylation-specific PCR (MSP) was performed on 1 μl of the modified template and primers specific to the methylated or unmethylated sequences. DNA from HOSE6-3 and DNA methylated *in vitro* by *Sss*I-methylase (New England Biolabs) were used as controls for fully unmethylated and

Table 3
Seven CGIs in promoter regions methylated in human ovarian cancers

Genes			Chromosomal location	Accession number	Map start position	CpG island		
Symbol	Description	Accession number				Length (bp)	%GC	Obs/Exp CpG[a]
GPR150	G protein-coupled receptor 150	NM_199243	5q15	AC110002	59000#	2999	58.1	0.8
LOC222171	Hypothetical protein LOC222171	NM_175887	7p15.1	AC007255	101001	3000	56.6	0.74
PRTFDC1	Phosphoribosyl transferase domain containing 1	NM_020200	10p12.1	AL512598	13500#	1000	70.9	0.82
LOC339210	Hypothetical protein LOC339210	XM_378687	17q23.2	AC015912	23000#	3001	62	0.9
ITGA8	Integrin, alpha 8	NM_003638	10p13	AL359645	124000#	2000	59.4	0.68
C9orf64	Chromosome 9 open reading frame 64	NM_032307	9q21.32	AL354733	159000#	1200	58.7	0.78
HOXD11	Homeo box D11	NM_021192	2q31.1	AC009336	90001	2000	66.1	0.83

[a] Obs/Exp CpG: observed CpG/expected CpG ratio; #: Reverse strand.



b

Histologic type	Normal (Tester)	Clear cell (Driver)	Serous	Endo-metrioid	Mucinous		Clear cell		Poorly differ-entiated	Undifferentiated	
Cell / Gene	HOSE	ES-2	OV-90	TOV-112D	MCAS	RMUG-L	TOV-21G	RMG-I	RTSG	TYK-nu	KURAMOCHI
GPR150		■	▒	▒	■	■	■		■	■	
LOC222171		■				■			■	■	▒
PRTFDC1		■				■			■		
LOC339210		■									
ITGA8		▒	▒		■	■	■	▒	■	■	
C9orf64		■	▒	▒							
HOXD11		▒	■		■	■			▒	■	

Fig. 2. Methylation analysis of the seven genes in ovarian cancer cell lines. (a) Results of MSP. Samples 1–10, ovarian cancer cell lines; 11, HOSE6-3 (immortalized normal human ovarian surface epithelial cells); and 12, DNA methylated by *Sss*I methylase (fully methylated DNA). U and M, primer sets specific to unmethylated and methylated DNA molecules, respectively. Arrowheads show bands obtained by the M primer set. (b) Methylation profile of the seven genes in ovarian cancer cell lines. Closed, gray, and open boxes show the presence of only methylated DNA, both methylated and unmethylated DNA, and only unmethylated DNA, respectively.

methylated DNA, respectively. Primer sequences and PCR conditions are summarized in Table 1. To avoid overestimation of the presence of methylated DNA, the numbers of PCR cycles did not exceed 35 cycles. MSP products were separated on 2% agarose gels, and visualized after ethidium bromide staining.

Quantitative real-time PCR

cDNA was synthesized from 2.5 μg of total RNA treated with *DNase*I (Ambion, Austin, TX) using oligo-dT primer (Promega) and SuperscriptII reverse transcriptase (Life Technologies). Quantitative real-time PCR was performed using the

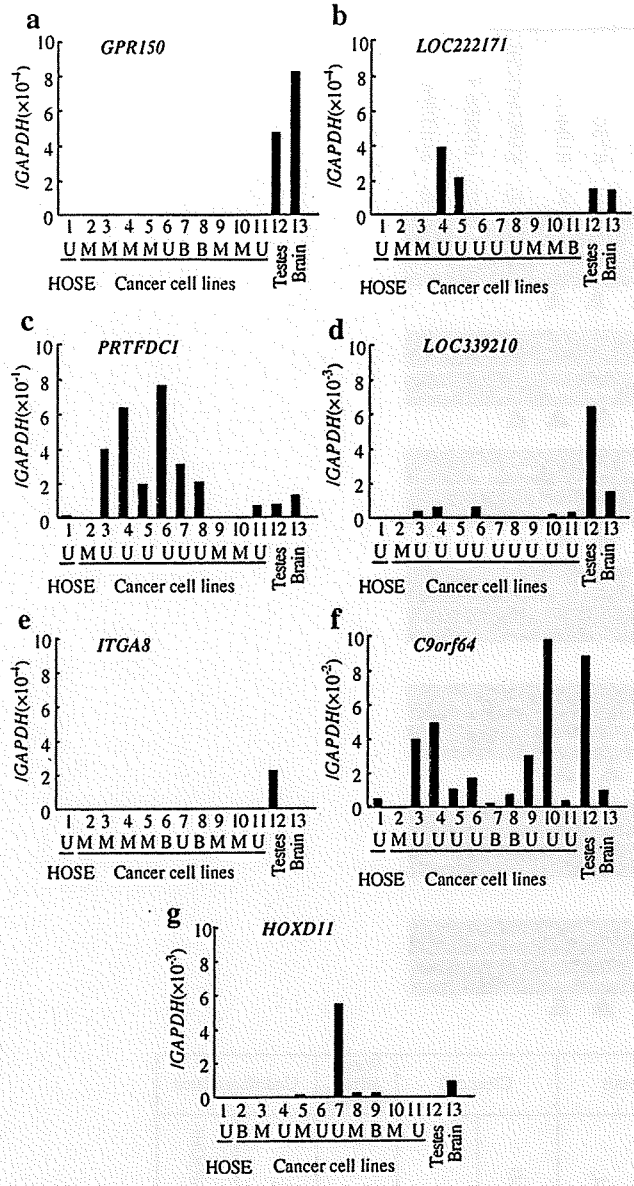


Fig. 3. Expression levels of the seven genes in the normal and ten cancer cell lines. Expression levels were analyzed by quantitative RT-PCR. Sample 1, HOSE6-3; 2–11, 10 ovarian cell lines (ES-2, TYK-nu, TOV-21G, MCAS, RMG-I, TOV-112D, OV-90, RTSG, RUMG-L and KURAMOCHI); 12, the normal testes; and 13, the normal brain. Results of MSP are repeated from Fig. 2, M, B and U representing the presence of only methylated DNA, both methylated and unmethylated DNA, and only methylated DNA, respectively. All the seven genes were not expressed in cell lines without unmethylated DNA molecules.

SYBR Green Real-time PCR Master Mix (Toyobo Co., Ltd., Osaka, Japan) and the 7300 Real Time PCR System (Applied Biosystems, Foster City, CA). The primer sequences and PCR conditions are shown in Table 2. The numbers of target cDNA molecules were normalized to those of *GAPDH* cDNA molecules.

5-Aza-2′-deoxycytidine treatment

Cells were seeded on day 0 at a density of 3×10^5 cells/10 cm dish (RTSG) or 1×10^5 cells/10 cm dish (ES-2). They were exposed to 1 or 3 μM 5-aza-2′-deoxycytidine (5-aza-dC; Sigma, St. Louis, MO) for 24 h on Days 1, 2 and 3, and harvested on Day 4.

Results

Isolation of aberrantly methylated CpG islands by a genome-wide screening

Three series of MS-RDA with *Hpa*II, *Nar*I and *Sac*II were performed using HOSE6-3 as the tester and ES-2 as the driver to isolate DNA fragments specifically methylated in ES-2 cells. In the three series, 384, 96 and 96 clones, respectively, were sequenced, and 185 of the total 576 clones were non-redundant. After BLAST search, 117 clones were found to be derived from CGIs, and 33 were flanked by CGIs in putative promoter regions of genes.

Methylation statuses of regions around the putative promoter regions of the 33 genes were analyzed by MSP in the immortalized human ovarian surface cell line (HOSE6-3) and ten ovarian cancer cell lines. It was shown that putative promoter regions of seven genes, *GPR150* (5q15), *LOC222171* (7p15.1), *PRTFDC1* (10p12.1), *LOC339210* (17q23.2), *ITGA8* (10p13), *C9orf64* (9q21.32) and *HOXD11* (2q31.1) (Fig. 1 and Table 3), were methylated in at least one of the ten ovarian cancer cell lines, but not in HOSE6-3 (Fig. 2a). Aberrant methylation of *C9orf64* (previously known as a homologue of *RIKEN2210016F16*) and *HOXD11* had been reported in gastric

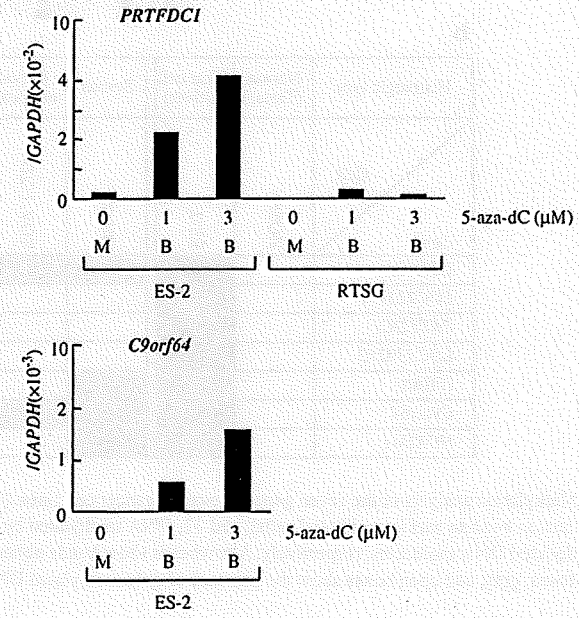
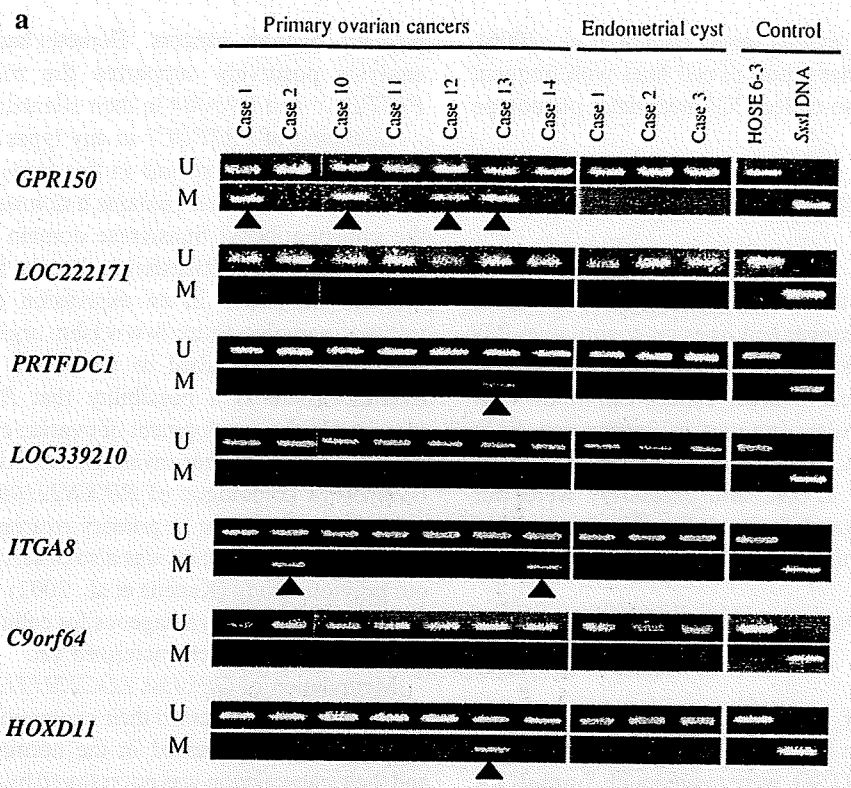


Fig. 4. Restoration of the *C9orf64* and *PRTFDC1* transcription by demethylation using 5-aza-dC. The methylation status was analyzed by MSP, and M and B represent the presence of only methylated DNA, and both methylated and unmethylated DNA, respectively. (a) Restoration of *PRTFDC1* in association with demethylation was clear in ES-2, while it was marginal in RTSG. (b) Restoration of *C9orf64* was clear in ES-2 cells, in which *C9orf64* had only methylated DNA molecules.

a



b

Cases					Genes						
No.	Histology	Age	Stage	Lymph node metastasis	*GPR150*	*LOC222171*	*PRTFDC1*	*LOC339210*	*ITGA8*	*C9orf64*	*HOXD11*
1	serous	59	IIIc	N0					■		
2	serous	61	II c	N0							
3	serous	57	IIIc	N1							
4	serous	71	IIIc	NX							
5	serous	53	IIIc	N1							
6	serous	45	IIIc	N1	■						
7	serous	71	IIIc	NX							
8	serous	60	IIIc	N1	■						
9	serous	75	I c	N0	■		■				■
10	serous	50	IV	N1							
11	endometrioid	72	I c	NX							
12	mucinous	33	IIIb	N0							
13	SCC	52	I a	N0					■		
14	clear cell	32	I a	N0							
15	clear cell	55	II c	NX	■						
Frequency					4/15	0/15	1/15	0/15	2/15	0/15	1/15

Fig. 5. Methylation analysis of the seven genes in primary ovarian cancers. (a) MSP results in primary ovarian cancers and endometrial cysts. U and M: primer sets specific to unmethylated (U) and methylated (M) DNA molecules. Arrowheads show bands obtained by the M primer set. (b) Methylation profile of the seven genes in the 15 primary ovarian cancers. Clinicopathological parameters are also included. Gray and open boxes represent the presence and absence, respectively, of methylated DNA molecules. Four genes, *GPR150*, *PRTFDC1*, *ITGA8* and *HOXD11*, were methylated in 1–4 of the 15 primary samples.

cancers and breast cancers, respectively (Kaneda et al., 2002; Miyamoto et al., 2005), and methylation of the other five genes was novel.

When the methylation profiles of the seven genes were analyzed in nine ovarian cancer cell lines, apart from ES-2 (Fig. 2b), *GPR150* and *ITGA8* were most frequently methylated (only methylated DNA molecules in five cell lines, and both methylated and unmethylated DNA molecules in two cell lines), followed by *HOXD11* (four and one cell lines), *LOC222171* (three and one), *PRTFDC1* (two and 0), and *C9orf64* (0 and two). RMUG-L, RTSG, TYK-nu, and MCAS had frequent aberrant methylation (five, five, four and three, respectively), while TOV-21G, TOV-112D, RMG-I, and KURAMOCHI had only one or two genes with aberrant methylation. Those results

were consistent with our previous report (Imura et al., 2006), which showed that the former group of cell lines with frequent methylation of CGIs had a so-called CGI methylator phenotype (CIMP).

Analysis of transcriptional repression of the seven genes identified

Transcription levels of the seven genes were analyzed in HOSE6-3 and ten ovarian cancer cell lines by quantitative RT-PCR (Fig. 3). Two genes, *PRTFDC1* and *C9orf64*, were transcribed in HOSE6-3 and ovarian cancer cell lines with unmethylated DNA molecules, but not in cancer cell lines without unmethylated DNA. In contrast, five genes, *GPR150*, *LOC222171*, *LOC339210*, *ITGA8* and *HOXD11*, were not expressed in HOSE6-3, although they were weakly expressed in ovarian cancer cell lines with unmethylated DNA molecules.

Restoration of the PRTFDC1 and C9orf64 transcription by 5-aza-dC

To examine the role of methylation of the CGIs in the putative promoter regions in the loss of gene transcription, two cancer cell lines, ES-2 and RTSG, were treated with 5-aza-dC, a demethylating agent, and restoration of gene transcription was analyzed (Fig. 4). *PRTFDC1* had only methylated DNA molecules in ES-2 and RTSG, and its transcription was clearly restored with demethylation in ES-2 and marginally restored in RTSG. *C9orf64* had only methylated DNA molecules in ES-2 but only unmethylated DNA molecules in RTSG. Its transcription was restored with demethylation in ES-2, but did not change in RTSG (data not shown). These data supported that methylation of the putative promoter CGIs of *PRTFDC1* and *C9orf64* induced their silencing. In contrast, transcription of the five remaining genes was not restored, while demethylation was detected by MSP.

Methylation of the identified genes in primary ovarian cancers

Methylation of the seven CGIs was analyzed in 15 primary ovarian cancers and seven endometrial cyst samples (Fig. 5a). Four genes, *GPR150*, *PRTFDC1*, *ITGA8* and *HOXD11*, were methylated in four, one, two and one of the 15 samples, respectively, and were not methylated in seven endometrial cyst samples. The other three genes were not methylated at all in primary samples. Among the 15 primary ovarian cancers, six cancers showed aberrant methylation of at least one gene (Fig. 5b).

Discussion

In this study, a genome-wide screening, MS-RDA, was performed to identify CGIs aberrantly methylated in human ovarian cancers, which could lead to identification of novel tumor-suppressor genes and clinical markers. Putative promoter CGIs of seven genes were identified as aberrantly methylated in ovarian cancer cell lines, and four of them, *GPR150*, *ITGA8*, *PRTFDC1*, and *HOXD11*, were aberrantly methylated also in primary ovarian cancers. Demethylation and re-expression analysis positively supported the role of methylation of *PRTFDC1* and *C9orf64* in their silencing.

Silencing of *PRTFDC1* in any types of human cancers was identified for the first time in this study. *PRTFDC1* (10p12.1) encodes a protein that contains a domain of 68% identity with the phosphoribosyl transferase domain of hypoxanthine guanine phosphoribosyltransferase, but the functions of the protein are still unknown. In an expression database dealing with primary samples (http://www.lsbm.org/database/index.html#), *PRTFDC1* is described to be weakly expressed in normal ovaries. There is a possibility that *PRTFDC1* silencing is involved in the development of ovarian cancers, and this should be explored by further investigation. Aberrant methylation of *C9orf64* (a homologue of *RIKEN2210016F16*) was observed only in cell lines, not in primary ovarian cancers, and the cell line-specific methylation was also observed in gastric cancers in our previous study (Kaneda et al., 2002). Although methylation of *C9orf64* can be advantageous for cellular immortalization, its gene function is not characterized yet.

Methylation of *GPR150*, *LOC222171*, *ITGA8*, and *HOXD11* also consistently repressed their expression. However, these four genes were not expressed in the normal cell line, HOSE6-3, and their transcription was not restored by demethylation using 5-aza-dC. This finding suggested that these four genes were physiologically not transcribed in normal ovarian surface epithelium due to mechanisms other than promoter methylation, but that the lack of transcription was one of the promoting factors for their methylation, as observed in our previous studies with pancreatic cancers, breast cancers, and melanomas (Ushijima and Okochi-Takada, 2005; Miyamoto et al., 2005; Hagihara et al., 2004; Furuta et al., 2006). This suggests that these genes could be candidate biomarkers. Although the incidences of methylation of the four genes were not very high for individual genes, a combination of *GPR150* and *ITGA8* detected six of the 15 cancers (40%). Considering that development of tumor markers is important for ovarian cancers, analysis of a larger number of samples seems necessary.

In conclusion, we identified *PRTFDC1* as a gene potentially involved in ovarian cancer development, and aberrant methylation of *GPR150*, *ITGA8*, and *HOXD11* as candidates for biomarker development.

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Marked and independent prognostic significance of the CpG island methylator phenotype in neuroblastomas ☆

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Abstract

The CpG island methylator phenotype (CIMP) was closely associated with poor overall survival (OS) in Japanese neuroblastoma (NBL) cases in our previous study. Here, in German NBL cases, CIMP(+) cases ($n = 95$) showed markedly poorer OS (hazard ratio (HR) = 9.5; $P < 0.0001$) and disease-free survival (DFS) (HR = 5.4; $P < 0.0001$) than CIMP(−) cases ($n = 50$). All the 23 cases with N-*myc* amplification had CIMP. Among the remaining cases without N-*myc* amplification, CIMP(+) cases ($n = 27$) had a poorer OS (HR = 4.5; $P = 0.02$) and DFS (HR = 5.2; $P < 0.0001$) than CIMP(−) cases ($n = 95$). In multivariate analysis, CIMP and N-*myc* amplification had an influence on OS and DFS independent of age and disease stage. CIMP had a stronger influence on DFS than N-*myc* amplification while N-*myc* had a stronger influence on OS.

Keywords: Neuroblastoma; Methylation; CIMP; MS-RDA; N-*myc*

1. Introduction

Neuroblastoma (NBL) is one of the most common pediatric solid tumors, and is characterized by two extreme disease courses, spontaneous regression and life-threatening progression. To implement adequate and necessary therapeutics, NBL cases are stratified into low-, intermediate- and high-risk groups based upon clinical and genetic information, such as disease stage, age at diagnosis, Shimada histology, N-*myc* amplification status, DNA ploidy, and *TrkA* expression level [1–6]. Especially, N-*myc* amplification, present in approximately 20–30% of NBL cases, is a powerful molecular marker for the stratification [1–4]. Nevertheless, more precise risk estimation is necessary for cases currently stratified

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into the intermediate-risk group, and development of a novel prognostic marker is awaited [1,2].

Recently, using a genome-wide screening method for differences in DNA methylation, methylation-sensitive representational difference analysis [7–9], we found that multiple CGIs were methylated in NBL cases with poor prognosis [10]. By analysis of 140 Japanese NBL cases, methylation of the multiple CGIs was shown to be dependent upon each other, and conformed to the concept of the CGI methylator phenotype (CIMP), originally established in colorectal cancers [11]. Cases could be classified as either CIMP(+) or CIMP(−), and a very limited number of cases had an intermediate phenotype. CIMP(+) cases had a markedly poorer overall survival (OS) than CIMP(−) cases with a hazard ratio (HR) of 22.1 [95% confidence interval (95%CI) = 5.3–93.4; $P < 0.0001$]. Its influence was independent of *TrkA* expression status, DNA ploidy, and age at diagnosis. Notably, almost all cases with N-*myc* amplification exhibited CIMP (37 of 38 cases), and, even among the cases without N-*myc* amplification, CIMP(+) cases had a poorer OS than CIMP(−) cases (HR = 12.4; 95%CI = 2.6–58.9; $P = 0.002$). CIMP status was well associated with the methylation level of the *Protocadherin β* (*PCDHB*) gene family, followed by methylation levels of *hepatocyte growth factor-like protein* (*HLP*) gene and *Cytochrome p450 CYP26C1* (*CYP26C1*).

Considering that there could be potential ethnic differences and that genome-wide screenings tend to produce "too good" results [12], here we took advantage of archived materials of German NBL cases. If the strong influence of CIMP on OS is also observed in German cases, we can establish CIMP as a prognostic marker that can be universally used. Also, the German NBL cases have information on disease-free survival (DFS), which was not available for Japanese NBL cases, and the influence of CIMP on DFS can be clarified.

2. Materials and methods

2.1. Tissue samples

A total of 152 cases were collected between 1998 and 2004, and all patients were enrolled in the German NBL Trial. The mean age at initial diagnosis was 1082 days (range 0–9607 days). Thirty-seven, 29, 17, 51 and 17 cases belonged to stages 1, 2, 3, 4, and 4S (International Neuroblastoma Staging System), respectively, although information was not available for one case. The composition of the cohort in terms of stage, N-*myc* status and age at diagnosis was in agreement with the composition of an unselected cohort of 1741 patients diagnosed between 1990 and 2003 in Germany [13]. DNA was extracted by the standard phenol/chloroform procedure, and used for this study under approval of Institutional Review Boards.

2.2. Sodium bisulfite modification and quantitative methylation-specific PCR (MSP)

One microgram of DNA restricted with *Bam*HI underwent sodium bisulfite modification [14], and was suspended in 20 μl of TE buffer. For quantitative MSP, 1 μl of the solution was used for PCR using SYBR Green PCR Core Reagents (PE Biosystems) and an iCycler Thermal Cycler (Bio-Rad Laboratories). PCR was performed separately for methylated (M) DNA molecules and for unmethylated (U) DNA molecules with primers specific to each sequence, and the numbers of M and U molecules in a test sample were determined by comparing their amplification with those of standard samples containing 10–10^6 molecules. Primer sequences and standard DNA were previously described [10]. The "methylation level" was calculated as the fraction of M molecules in the total DNA molecules (# of M molecules + # of U molecules). All the molecular analyses were performed blind to clinical information, and methylation level for a case was obtained as an average of two independent measurements.

2.3. Statistical analysis

Reproducibility of methylation levels between two measurements was assessed using the Pearson correlation coefficient. Survival time was measured from the date of initial diagnosis to the date of death or last contact. Kaplan–Meier analysis and log-rank tests were performed to compare overall survival (OS) and disease-free survival (DFS) between groups. HRs were estimated by the Cox proportional hazards model. These statistical analyses were performed using SPSS, version 13.0 (SPSS Inc., Chicago, IL).

3. Results

3.1. Determination of CIMP statuses in German NBL cases

Methylation levels were measured in 152 German NBLs for three CGI (group)s – (i) the 17 *PCDHB* family genes, (ii) *HLP*, and (iii) *CYP26C1*. They were highly reproducible with a correlation coefficient $\geqslant 0.99$, and the average levels were used hereafter. The methylation level of the *PCDHB* gene family showed a clear bimodal distribution (Fig. 1A). To avoid artificial bias, CIMP statuses were diagnosed before having access to clinical information of the cases. First, since cut-off values between 40% and 60% gave high HRs in our previous

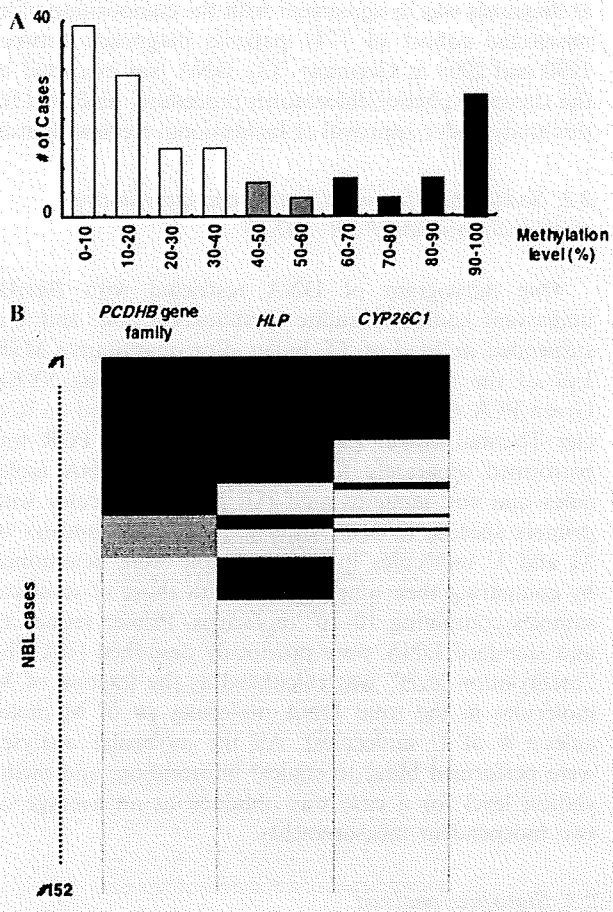

Fig. 1. Bimodal distribution of methylation levels of the *PCDHB* gene family, and diagnosis of CIMP status. (A) Histogram of number of cases according to *PCDHB* methylation levels. The methylation level of the *PCDHB* gene family was measured exactly as in our previous study [10], and its bimodal distribution in German NBLs was confirmed. (B) Methylation statuses of the three CGIs (groups) among the 152 NBLs. Cut-off values for the *PCDHB* gene family, *HLP* and *CYP26C1* were set based on the previous study, which were 40–60%, 10%, and 70%, respectively. Closed and open boxes show high and low methylation levels, and methylation levels of the *PCDHB* gene family between 40% and 60% are shown by grey boxes. Methylation levels of these three CGIs were closely associated with each other.

study [10], cases with methylation levels lower than 40% and higher than 60% were diagnosed as CIMP(−) ($n = 95$) and CIMP(+) ($n = 45$), respectively. Only 12 cases had methylation levels between 40% and 60%.

Then, for these 12 cases, methylation levels of *HLP* and *CYP26C1*, whose predictive powers followed that of the *PCDHB* gene family in our previous study [10], were taken into account. Five of the 12 cases had high levels of methylation of *HLP* and/or *CYP26C1*, and were considered to have CIMP, and seven other cases were left as unknown for CIMP status (Fig. 1B). Cut-off values for *HLP* and *CYP26C1* were set at the same levels as in our previous study, which were 10% and 70%, respectively. As a result, 50, 95, and 7 cases of the 152 cases were diagnosed as CIMP(+), CIMP(−), and unknown, respectively. Methylation statuses of the three CGI (groups) showed close correlation with methylation statuses of the other CGIs.

3.2. Univariate analysis with OS and DFS

In univariate analysis, the 50 CIMP(+) cases exhibited markedly and significantly poorer OS (HR = 9.5; 95%CI = 3.2–28.1; $P < 0.0001$) and DFS (HR = 5.4; 95%CI = 2.9–10.3; $P < 0.0001$) than the 95 CIMP(−) cases. Cases with N-*myc* amplification ($n = 23$) also exhibited markedly and significantly poorer OS (HR = 11.8; 95%CI = 4.9–28.7; $P < 0.0001$) and DFS (HR = 3.1; 95%CI = 1.6–6.0; $P = 0.0007$) than 122 cases without N-*myc* amplification. All of the 23 German cases with N-*myc* amplification had CIMP, as observed in a Japanese population.

Therefore, the German NBL cases were classified into three groups: (a) CIMP(−) cases ($n = 95$), all of which were without N-*myc* amplification, (b) CIMP(+) cases without N-*myc* amplification ($n = 27$), and (c) CIMP(+) cases with N-*myc* amplification ($n = 23$). As for OS (Fig. 2A), the three groups exhibited a step-wise increase of risk, showing the influence of N-*myc* amplification in addition to CIMP. Among the cases without N-*myc* amplification (groups (a) and (b)), CIMP had a significant influence on OS (HR = 4.5; 95%CI = 1.3–16.1; $P = 0.02$). As for DFS (Fig. 2B), CIMP had a significant influence (HR = 5.2; 95%CI = 2.6–10.6; $P < 0.0001$) by comparison of groups (a) and (b). However, additional influence by N-*myc* amplification was unclear by comparison of groups (b) and (c). These suggested that N-*myc* amplification had a strong influence on OS while CIMP had a strong influence on DFS.

3.3. Multivariate analysis

Since CIMP and N-*myc* amplification were dependent upon each other, multivariate analysis was first performed using age at diagnosis, disease stage, and either CIMP or N-*myc* amplification (Table 1A and B). It was confirmed that either CIMP or N-*myc* amplification had a significant influence on OS and DFS independent of age at diagnosis and disease stage.

Then, multivariate analysis was performed using age at diagnosis, disease stage, and both CIMP and N-*myc* amplification to compare the influences of them (Table 1C). As for OS, N-*myc* amplification retained its power while CIMP lost its power. In contrast, as for DFS, CIMP retained its power while N-*myc* amplification lost its power. This result was in accordance with the finding that CIMP had a strong influence on DFS while N-*myc* amplification had a strong influence on OS.

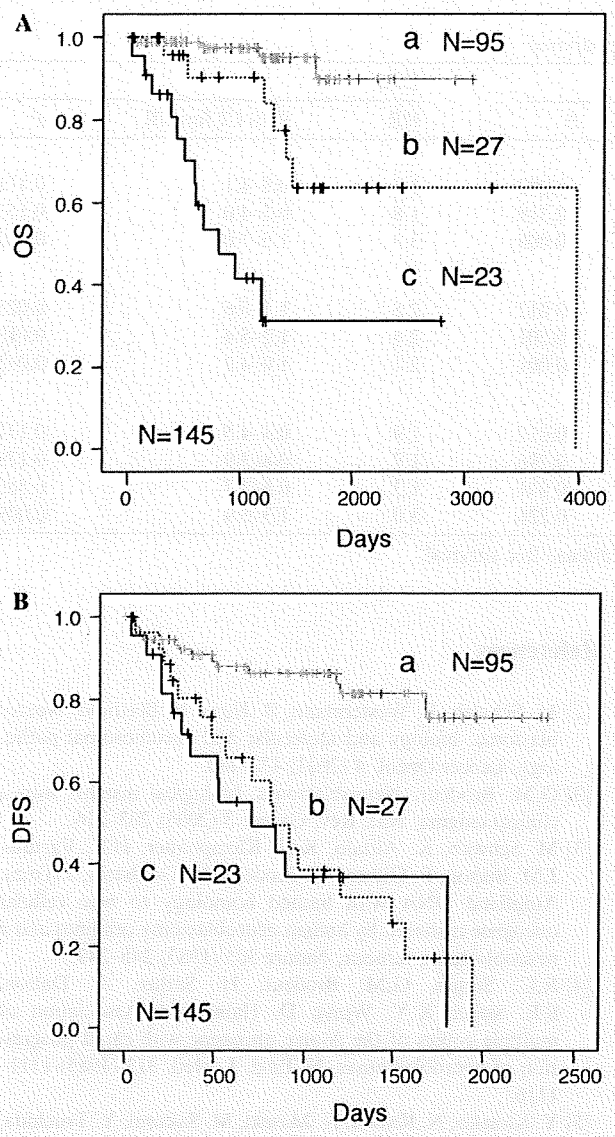

Fig. 2. Kaplan–Meier analysis of (a) CIMP(−) cases without N-*myc* amplification ($n = 95$), (b) CIMP(+) cases without N-*myc* amplification ($n = 27$), and (c) CIMP(+) cases with N-*myc* amplification ($n = 23$). (A) Kaplan–Meier analysis using OS. Using group (a) as a reference, group (b) had a HR of 4.5 (95%CI = 1.3–16.1; and $P = 0.02$), and group (c) had a HR of 21.7 (6.8–69.3; <0.0001). Using group (b) as a reference, group (c) had a HR of 4.8 (1.7–13.6; 0.003). (B) Kaplan–Meier analysis using DFS. Using group (a) as a reference, group (b) had a HR of 5.2 (2.6–10.6; <0.0001), and group (c) had a HR of 5.7 (2.6–12.2; <0.0001). There was no significant difference between groups (b) and (c) ($P = 0.82$).

4. Discussion

Methylation levels of the *PCDHB* gene family showed a bimodal distribution in German NBL cases, as in our initial analysis of Japanese NBL cases [10], and the presence of two groups of NBLs from the viewpoint of CIMP was confirmed. The CIMP statuses of individual German NBL cases were determined using criteria established in Japanese NBL cases to avoid falsely "too good" results, which tend to happen in genome-wide analyses [12]. Nevertheless, the strong influence of CIMP on OS in all the NBL cases (HR = 9.5) and also in those without N-*myc* amplification (HR = 4.5) was confirmed. After finishing all the analysis we searched for a *PCDHB* methylation level that would give the highest HR for the 152 German NBL cases, and it was 30% with a HR of 9.8 (95%CI = 2.9–33.0; $P < 0.0001$), followed by 40% with a HR of 9.4 (95%CI = 3.2–27.6; $P < 0.0001$). Based on the precise reproduction of the initial findings in Japanese NBL cases in German NBL cases, CIMP is highly likely to be a novel prognostic marker that can be universally used in cases without N-*myc* amplification. A prospective study is warranted.

A strong influence of CIMP on DFS was revealed for the first time in this study because data on DFS were available only for German NBL cases. In univariate analysis, CIMP had a strong influence on DFS in all the NBL cases (HR = 5.4) and in the cases without N-*myc* amplification (HR = 5.2) (groups (a) and (b) in Fig. 2B). In multivariate analysis involving age at diagnosis, disease stage, and both N-*myc* amplification and CIMP, CIMP retained its power on DFS while N-*myc* amplification retained its power on OS. This suggested that the recurrence of NBL cases was strongly associated with CIMP, but that NBL cases without N-*myc* amplification had higher chances to be induced into the second remission.

The almost complete inclusion of cases with N-*myc* amplification within the CIMP(+) cases in our two independent studies indicates that these two abnormalities are very closely associated with each other. If we assume a single abnormality that underlies a poor prognosis of NBL cases, it is likely that CIMP is caused by it, and some of CIMP(+) NBLs develop N-*myc* amplification. If we assume multiple abnormalities, it is likely that CIMP is consistently associated with the devastating status of NBLs, which can be induced by N-*myc* amplification and other causes. Clarification of what molecular abnormality causes CIMP and how CIMP and N-*myc* amplification are related is important.

The presence of CIMP was considered to lead to a poor prognosis by induction of methylation of promoter CGIs of various tumor-related genes. We

Table 1
Multivariate analysis of prognostic factors for overall and disease-free survival

Variable	OS			DFS		
	HR	95% CI for HR	*P*	HR	95% CI for HR	*P*
(A)						
Age at diagnosis	6.2	0.8–48.7	0.082	1.8	0.8–4.1	0.171
Disease stage	1.8	0.6–5.8	0.319	1.8	0.8–4.0	0.152
CIMP	4.9	1.5–15.8	0.008	3.3	1.5–7.0	0.002
(B)						
Age at diagnosis	13.6	1.8–104.3	0.012	2.5	1.1–5.6	0.025
Disease stage	1.5	0.5–5.0	0.501	2.6	1.2–5.4	0.013
N-*myc* amplification	11.5	3.9–33.8	<0.001	2.1	1.0–4.2	0.043
(C)						
Age at diagnosis	12.1	1.6–94.4	0.017	1.9	0.8–4.5	0.137
Disease stage	1.2	0.3–4.1	0.796	1.7	0.8–3.9	0.179
N-*myc* amplification	8.0	2.5–25.8	<0.001	1.3	0.6–2.7	0.563
CIMP	2.3	0.6–8.9	0.226	3.0	1.3–6.9	0.009

HR, hazard ratio; CI, confidence interval; OS, overall survival; DFS, disease-free survival.

previously observed association between CIMP and promoter methylation of tumor-suppressor *RASSF1A* and *BLU* genes [10]. It is reported that an anti-apoptotic gene, *TMS1*, a homeobox gene, *HOXA9*, a cell cycle gene, *CCND2*, and candidate tumor-suppressor genes, *EMP3* and *NR1I2*, are more frequently methylated in NBL cases with a poor prognosis [15–17]. However, the risk given by methylation of one of these individual genes is much smaller than that given by CIMP. This is in accordance with our hypothesis that CIMP leads to consistent methylation of marker CGIs, such as exonic CGIs of the *PCDHB* gene family, and occasional methylation of promoter CGIs of tumor-related genes. Silencing of an individual gene accounts for a poor prognosis of only a fraction of NBL cases with CIMP. It is known that exonic CGIs are more susceptible to methylation than promoter CGIs [9], and it is expected that they are more useful as a prognostic marker.

In summary, the faithful reproduction in German NBL cases of the highly significant findings obtained in Japanese cases demonstrated that CIMP is a strong and universal prognostic marker for NBL cases, especially for those without N-*myc* amplification. The close association between CIMP and DFS was revealed for the first time in this study.

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Alterations of DNA methylation associated with abnormalities of DNA methyltransferases in human cancers during transition from a precancerous to a malignant state

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Alterations of DNA methylation are one of the most consistent epigenetic changes in human cancers. Human cancers generally show global DNA hypomethylation accompanied by region-specific hypermethylation. Alterations of DNA methylation may result in chromosomal instability as a result of changes in chromatin structure. DNA hypermethylation of CpG islands silences various tumor-related genes. Alterations of DNA methylation are frequently observed in cancers associated with chronic inflammation and/or persistent infection with viruses or other pathogenic microorganisms, such as hepatitis B or C viruses, Epstein–Barr virus, human papillomavirus and *Helicobacter pylori*, or with cigarette smoking. Accumulating evidence suggests that alterations of DNA methylation are involved even in the early and precancerous stages. On the other hand, in patients with cancers, aberrant DNA methylation is significantly associated with poorer tumor differentiation, tumor aggressiveness and poor prognosis. Precancerous conditions showing alterations of DNA methylation may progress rapidly and generate more malignant cancers. DNA methyltransferase (DNMT) 1 over-expression is not a secondary result of increased cell proliferative activity but is significantly correlated with the CpG island methylator phenotype, which is defined as frequent DNA hypermethylation of C-type CpG islands that are usually methylated in a cancer-specific (not age-dependent) manner. Splicing alteration of DNMT3b may result in chromosomal instability through DNA hypomethylation of pericentromeric satellite regions. Alteration of DNA methylation may become an indicator for carcinogenetic risk estimation and early diagnosis of cancers and a biological predictor of poor prognosis in patients with cancers. Correction of DNA methylation status may offer a new strategy for prevention and therapy of cancers.

Introduction

In the earlier days of cancer research, stepwise and orderly progression of genetic alterations causing activation of oncogenes and inactivation of tumor suppressor genes was considered to be the molecular framework responsible for multistage carcinogenesis in humans. However, genetic events alone may not explain the entire process of carcinogenesis: only a few genetic alterations are known to be responsible, especially in the earlier, precancerous stages. Moreover, microscopic observation of cancers frequently reveals histological heterogeneity (e.g. well, moderately or poorly differentiated carcinoma components are simultaneously observed even in tissue sections from any single patient), reflecting complexity of the biological characteristics of tumors. In addition to genetic events, epigenetic events such as alterations of DNA methylation, which can be reversible and underlie the histological heterogeneity of cancers, are another leading player in multistage carcinogenesis.

DNA methylation, a covalent chemical modification resulting in addition of a methyl (CH3) group at the carbon 5 position of the cytosine ring in CpG dinucleotides, plays important roles in chromatin structure modulation, transcriptional regulation and genomic stability, and is essential for the development of mammals (1). The C-terminal catalytic domain of DNA methyltransferases (DNMTs), the major and best known of which is DNMT1, transfers methyl groups from *S*-adenosylmethionine to cytosines (2). DNMT1's preference for hemimethylated over unmethylated substrates *in vitro* and its targeting of replication foci are believed to allow copying of the methylation pattern of the parental strand to the newly synthesized daughter DNA strand (3). Thus, DNMT1 has been recognized as the 'maintenance' DNMT. DNMT1 can interact with the DNMT1-associated protein 1, histone deacetylase 1 and 2 and Rb and can repress gene transcription (4–6). Since DNMT1−/− embryonic stem cells are able to methylate viral DNA *de novo* (7), independently encoded DNMTs have been sought. Among the subsequently identified DNMTs 2 (8), 3a and 3b (9), DNMT activity of DNMT2 has never been demonstrated (10), whereas DNMT3a and DNMT3b do show *de novo* DNA methylation activity *in vitro* (11).

In comparison with normal cells, human cancer cells show a drastic change in DNA methylation status, generally exhibiting global DNA hypomethylation as well as accompanying region-specific hypermethylation (12,13). As 5-methylcytosine is deaminated to thymine, DNA hypermethylation facilitates gene mutation in human cancers. DNA methylation normally promotes a highly condensed chromatin structure through recruitment of DNA-organizing proteins, and DNA hypomethylation in cancer cells causes chromatin decondensation and chromosomal rearrangements that may result in chromosomal instability. Moreover, DNA hypermethylation of CpG islands near gene-regulatory regions silences specific genes, in cooperation with histone modification, including tumor suppressor genes (14).

Association of DNA methylation alterations with both the precancerous stage and malignant progression

In general, candidate tumor suppressor genes, which are located in the commonly deleted chromosomal regions revealed by genomic structural analysis in human cancers, are frequently silenced by alternative two-hit mechanisms consisting of loss of heterozygosity (LOH) and DNA hypermethylation, rather than gene mutation. Thus, DNA hypermethylation of some chromosomal loci is frequently associated with LOH at the same chromosomal loci in human cancers. LOH on chromosome 16 has been frequently detected by classical restriction fragment length polymorphism analysis using Southern blotting in hepatocellular carcinomas (HCCs) which are poorly differentiated, large in size and associated with metastasis (15). Therefore, LOH on chromosome 16 seems to be a late event during multistage hepatocarcinogenesis. At the time of these discoveries, only a few molecular events in the earlier stage of hepatocarcinogenesis were known. Therefore, we first examined DNA methylation status on chromosome 16 in surgically resected tissue specimens.

Classical Southern blotting showed that the digestion patterns obtained using HpaII, a DNA methylation-sensitive restriction enzyme, were similar to those obtained using MspI, a DNA methylation non-sensitive restriction enzyme, at the D16S32 (16 pter to p13), TAT (16q22.2) and D16S7 (16q24.3) loci in normal liver tissue obtained from patients with liver metastases from primary colon cancer, indicating that genomic DNA is normally unmethylated in these regions. Surprisingly, DNA hypermethylation at the D16S32, TAT and D16S7 loci, compared with normal liver tissues, was frequently

Abbreviations: CIMP, CpG island methylator phenotype; DNMT, DNA methyltransferase; HCC, hepatocellular carcinoma; LOH, loss of heterozygosity; MBD, methyl-CpG-binding protein; mRNA, messenger RNA; PanIN, pancreatic intra-epithelial neoplasia; PCR, polymerase chain reaction; PCNA, proliferating cell nuclear antigen; RCC, renal cell carcinoma; STAT, signal transducer and activator of transcription; TCC, transitional cell carcinoma.

detected even in non-cancerous liver tissues showing chronic hepatitis or liver cirrhosis, which are widely considered to be precancerous conditions, indicating that alterations of DNA methylation are a very early event during multistage hepatocarcinogenesis (16). This was one of the earliest reports of alterations of DNA methylation in the precancerous stage.

Since the molecular weight of HpaII-digested DNA fragments in HCCs was higher than that in precancerous conditions and the intensity of larger sized bands was increased in HCCs in comparison with precancerous conditions, the numbers of methylated CpG dinucleotides and cells showing DNA hypermethylation may increase progressively as precancerous conditions develop into HCCs. The incidence of DNA hypermethylation at any of the D16S32, TAT or D16S7 loci in progressed HCCs was significantly higher than that in early HCCs and significantly correlated with higher histological grade (16). DNA hypermethylation at any of the D16S32, TAT or D16S7 loci was detected more frequently in HCCs showing associated involvement of the portal vein and intrahepatic metastasis than in HCCs without these features (16). The presence of DNA hypermethylation in both precancerous conditions and progressed HCCs suggests that precancerous conditions with aberrant DNA methylation might generate HCCs rapidly and that the HCCs thus generated might already be at a progressed stage when diagnosed.

Silencing of tumor suppressor genes by DNA hypermethylation

The *E-cadherin* gene is located on 16q22.1 near to the above-mentioned hot spots of both DNA hypermethylation and LOH in HCCs. E-cadherin acts as a Ca^{2+}-dependent cell–cell adhesion molecule in the adherens junctions of epithelial cells (17). Interactions between E-cadherin and cytoskeletal actin proteins through α- and β-catenins confer stability on the adherens junctions. Cell–cell adhesion determines cell polarity and participates in histogenesis. The mutual adhesiveness of cancer cells is significantly weaker than that of normal cells, and this allows cancer cells to disobey the social order, resulting in destruction of histological architecture, which is a morphological hallmark of malignant tumors (18). In signet-ring cell carcinoma of the stomach (19) and lobular carcinoma of the breast (20), in which cancer cells completely lose their mutual adhesiveness even in the *in situ* carcinoma stage, the *E-cadherin* gene is silenced by a two-hit mechanism comprising LOH and gene mutation. A large kindred study of early-onset, diffuse-type stomach cancers in New Zealand revealed a germ line mutation (21), indicating that the *E-cadherin* gene actually satisfies the criteria for a tumor suppressor gene.

On the other hand, suppression of E-cadherin activity is believed to trigger the release of cancer cells from primary cancer nests, resulting in cancer invasion and metastasis. In fact, non-invasive epithelial cells acquired the ability to invade into collagen gels upon addition of antibodies against E-cadherin (22) or plasmids encoding E-cadherin-specific anti-sense RNA (23). Generally, E-cadherin expression is reduced in poorly differentiated cancers that have lost their cell–cell adhesion and show a strong invasive tendency (18). Significant correlations between reduced E-cadherin expression and poor prognosis have been reported in patients with cancers (18). In order to clarify the mechanism responsible for regulation of E-cadherin expression in cancers, we cloned the promoter region of the human *E-cadherin* gene and demonstrated that it showed DNA methylation in human cancer cell lines lacking E-cadherin expression (24). We also observed induction of E-cadherin expression after treatment with the DNMT inhibitor 5-azacytidine in such cell lines (24). Thus, following the *RB* and *VHL* genes, the *E-cadherin* gene became the third example of a tumor suppressor gene that is silenced by DNA hypermethylation.

When assessed by Southern blotting analysis, DNA hypermethylation around the promoter region of the *E-cadherin* gene was detected in 46% of examined non-cancerous liver tissues showing chronic hepatitis or cirrhosis and in 67% of examined HCCs (25). Immunohistochemical examination revealed that hepatocytes in normal liver tissues showed strong E-cadherin immunoreactivity at their cell–cell borders. We found a significant correlation between DNA hypermethylation around the promoter region and reduced E-cadherin expression in primary HCCs (25). This was the first demonstration of a significant correlation between DNA hypermethylation and reduced expression in clinical tissue samples. Heterogeneous E-cadherin expression in non-cancerous liver tissues showing chronic hepatitis or cirrhosis, which is associated with small focal areas of hepatocytes showing only slight E-cadherin immunoreactivity and is not observed in normal liver tissues, might be due, at least partly, to DNA hypermethylation (25). DNA hypermethylation around the promoter region, which increases during progression from precancerous conditions to HCCs, may participate in hepatocarcinogenesis through reduction of E-cadherin expression, resulting in loss of intercellular adhesiveness and destruction of tissue morphology.

DNA hypermethylation of NotI sites at the D17S5 locus has been detected in various human cancers (26). The *hypermethylated-in-cancer-1* gene at this locus (17q13.3) was the first tumor suppressor gene to be identified in commonly methylated chromosomal loci in human cancers (27); human cancer cells transfected with the *hypermethylated-in-cancer-1* gene grew slowly (27) and mice with germ line disruption of one allele of *Hic1* developed different spontaneous malignant tumors (28). DNA methylation at the D17S5 locus was never detected in normal liver tissues but was detected in 44% of examined non-cancerous liver tissues showing chronic hepatitis or cirrhosis and in 90% of examined HCCs (29). In almost all the paired samples showing DNA hypermethylation, the molecular weight of NotI-digested DNA fragments in HCCs was higher than that in precancerous conditions and the intensity of the larger sized bands was higher in HCCs than in precancerous conditions, indicating that the degree of DNA methylation seems to further increase during progression from a precancerous condition to an HCC (29). The level of hypermethylated-in-cancer-1 messenger RNA (mRNA) expression in non-cancerous liver tissues showing chronic hepatitis or cirrhosis was significantly lower than that in normal liver tissues and was further decreased in HCCs (29).

It is now recognized that numerous tumor-related genes, such as *p16, hMLH1, BRCA1, MGMT, GSTP1, TIMP-3 and DAPK-1,* are silenced by regional DNA hypermethylation around their promoter regions in human cancers (14). Several techniques, such as restriction landmark genomic scanning (30), methylation-sensitive representational difference analysis (31) and methylated CpG islands amplification (32), have been developed for cloning genes that are differentially methylated between cancer cells and normal cells (33), and the list of tumor-related genes silenced by DNA hypermethylation is being expanded (14).

Alterations of DNA methylation precede chromosomal instability during multistage carcinogenesis

The hot spot for DNA hypermethylation in HCCs corresponds to a previously reported hot spot of LOH on chromosome 16. It remains to be examined whether alterations of DNA methylation might predispose the locus to allelic loss or whether common or different causes facilitate both alterations of DNA methylation and LOH at certain loci. However, it is at least clear that DNA hypermethylation precedes LOH at the same chromosomal loci during hepatocarcinogenesis: even classical Southern blotting has detected DNA hypermethylation in bulk non-cancerous liver tissues showing chronic hepatitis or cirrhosis, in which LOH has never been detected using the same method (16).

Recently, microdissection techniques and polymerase chain reaction (PCR) using microsatellite markers have been developed for detecting LOH in small numbers of cells from paraffin-embedded tissues. LOH has been reported even in microdissected specimens from non-cancerous lesions, e.g. hyperplastic or dysplastic lesions accompanying non-small cell lung cancers (34) and proliferative lesions adjacent to breast cancers (35). In order to re-examine whether aberrant DNA methylation precedes chromosomal instability during hepatocarcinogenesis, we obtained 308 microdissected specimens and examined LOH and microsatellite instability by PCR using 39 microsatellite markers and DNA methylation status of eight C-type CpG islands that are known to be methylated in a cancer-specific, but not age-dependent, manner, as shown in Table I, by methylation-specific PCR and combined bisulfite restriction enzyme analysis.

Table I. DNA methylation status on C-type CpG islands in tissue samples of various organs

Tissue samples	The incidence of DNA methylation[a] (%)								References
	CpG islands								
	p16	*hMLH1*	*THBS-1*	MINT1	MINT2	MINT12	MINT25	MINT31	
Normal liver tissues	0	0	0	0	0	0	0	0	(36)
Non-cancerous liver tissues obtained from patients with HCCs	15	0	0	10	73	23	0	0	
HCCs	70	0	0	45	85	45	8	65	
Non-cancerous stomach mucosae obtained from patients with stomach cancers	17	14	2	22	1	6	ND	0	(37)
Stomach cancers	22	17	24	37	25	17	ND	10	
Colorectal cancers	23	27	9	23	20	23	20	10	(38)
Normal renal tissues	11	0	0	0	0	11	22	0	(39)
Non-tumorous renal tissues obtained from patients with renal tumors	62	12	29	2	5	10	24	0	
Renal tumors	73	12	43	17	8	17	35	5	
Normal urothelia	0	ND	ND	ND	56	0	25	45	(40)
Urinary bladder cancers	21	ND	ND	ND	76	30	35	79	

MINT, methylated in tumor; ND, not done.
[a]Analyzed by methylation-specific PCR or combined bisulfite restriction enzyme analysis.

In non-cancerous liver tissues showing chronic hepatitis, LOH for at least one marker was found in 20% of informative microdissected specimens, and LOH in at least one microdissected specimen was found in 45% of informative cases (36). In non-cancerous liver tissues showing cirrhosis, LOH for at least one marker was found in 15% of informative microdissected specimens, and LOH in at least one microdissected specimen was found in 40% of informative cases (36). LOH was never detected in normal liver tissues obtained from patients with liver metastases from primary colon cancer and in non-cancerous liver tissue showing no remarkable histological findings from patients with HCCs. Although no degree of DNA methylation of any of the examined CpG islands was ever detected in normal liver tissues obtained from patients with liver metastases from primary colon cancer, DNA hypermethylation was found on at least one CpG island even in 58% of examined microdissected specimens of non-cancerous liver tissue showing no remarkable histological features obtained from patients with HCCs, in which LOH was never detected (36). Thus, aberrant DNA methylation is an earlier event preceding chromosomal instability during hepatocarcinogenesis, even when examined using microdissection techniques (41). The low incidence of microsatellite instability in Japanese patients (42) was compatible with absence of silencing of the *hMLH1* gene by DNA hypermethylation during hepatocarcinogenesis (36).

Etiologic backgrounds of carcinogenesis and regional DNA hypermethylation

Alterations of DNA methylation are frequently associated with carcinogenesis related to chronic inflammation and/or persistent infection with viruses or other pathogenic microorganisms, such as chronic hepatitis associated with hepatitis B virus or hepatitis C virus infection. As mentioned above, alterations of DNA methylation occur even in non-cancerous liver tissues showing no remarkable histological findings obtained from patients with HCCs, i.e. even before inflammation has become histologically obvious. This phenomenon might be at least partly attributable to hepatitis viral infection. Hepatitis B virus DNA is integrated into the cellular genome, and the integrated viral DNA is known to alter the DNA methylation status in several adjacent cellular genes and DNA segments (43). Epstein–Barr virus infection in stomach cancers is significantly associated with marked accumulation of DNA hypermethylation of C-type CpG islands (37). Induction of latent membrane protein 1 of Epstein–Barr virus has been reported to induce DNMT1 over-expression in cultured cancer cells (44). *Helicobacter pylori* infection, another etiologic factor that is believed to be involved in stomach carcinogenesis, has also been reported to strongly promote regional DNA hypermethylation (45), although the molecular mechanisms by which *H.pylori* infection alters DNA methylation are still unclear and warrant further investigation. Cervical intra-epithelial neoplasia is a precursor lesion for squamous cell carcinoma of the uterine cervix closely associated with human papillomavirus infection. DNMT1 protein expression is increased even in low-grade cervical intra-epithelial neoplasias compared with normal squamous epithelium and further increased in higher-grade cervical intra-epithelial neoplasias and squamous cell carcinomas of the uterine cervix (46). Human papillomavirus-16 E7 protein has been reported to associate directly with DNMT1 and stimulate the methyltransferase activity of DNMT1 *in vitro* (47), and accumulation of DNA hypermethylation on tumor-related genes has also been observed during cervical carcinogenesis (48).

In the same way that HCCs are preceded by chronic hepatitis, ductal carcinomas frequently emerge in pancreases damaged by chronic pancreatitis. Therefore, at least a proportion of peripheral pancreatic duct epithelia with an inflammatory background may be at the precancerous stage. When the DNA methylation status of the *p14, p15, p16, p73, APC, hMLH1, MGMT, BRCA1, GSTP1, TIMP-3, CDH1 and DAPK-1* tumor-related genes was examined, the incidence of DNA hypermethylation of at least one of the genes and the average number of methylated genes were significantly higher in microdissected specimens of peripheral pancreatic duct epithelia with an inflammatory background and in another precancerous lesion, pancreatic intra-epithelial neoplasia (PanIN), compared with that in peripheral pancreatic duct epithelia without an inflammatory background, and was further increased in ductal carcinomas (Figure 1; 50). The *BRCA1, APC, p16 and TIMP-3* genes are frequently methylated in ductal carcinomas of the pancreas (50). With respect to inflammation-related carcinogenesis, cytokine interleukin-6 treatment has been reported to induce DNMT1 over-expression in cultured cells (51), though the significance of cytokine signaling in alterations of DNA methylation has never been confirmed in chronic pancreatitis *in vivo*.

Cigarette smoking is another background factor associated with alterations of DNA methylation during multistage carcinogenesis. DNA hypermethylation at the D17S5 locus was observed in 31% of examined non-cancerous lung tissues, which may contain progenitor cells for cancers, obtained from patients with non-small cell lung cancers and in 33% of corresponding non-small cell lung cancers (52). The incidence of DNA hypermethylation at the D17S5 locus was significantly associated with poorer differentiation of lung adenocarcinomas (52). The incidence of DNA hypermethylation in both non-cancerous lung tissues and non-small cell lung cancers of patients

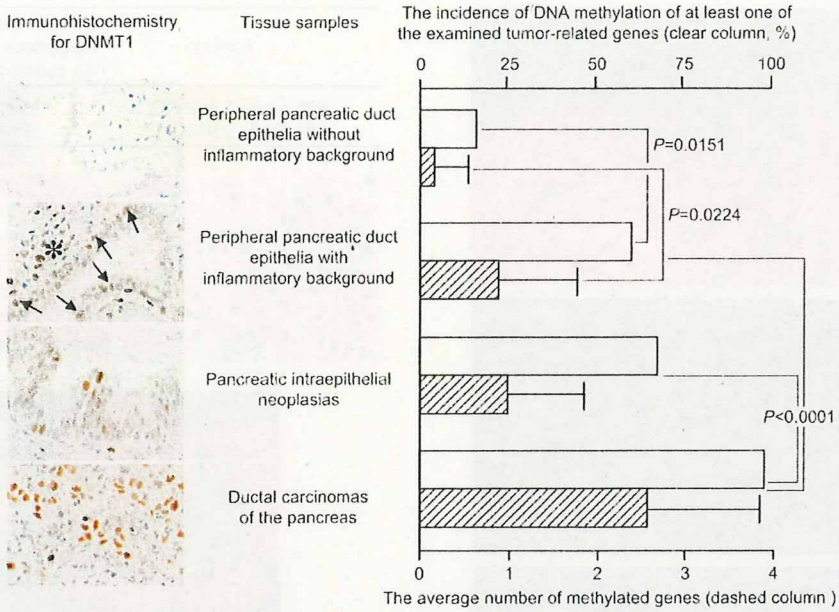

Fig. 1. DNMT1 protein expression and DNA methylation status of CpG islands in tumor-related genes during multistage carcinogenesis of the pancreas. Immunohistochemical examination for DNMT1 was performed in peripheral pancreatic duct epithelia without an inflammatory background, peripheral pancreatic duct epithelia with an inflammatory background (arrows), PanINs and ductal carcinomas (49). Infiltrating lymphocytes (asterisk) were used as an internal positive control for DNMT1 immunoreactivity. Tissue samples were microdissected from surgically resected materials, embedded in agarose beads and subjected to methylation-specific PCR to evaluate the DNA methylation status of the *p14, p15, p16, p73, APC, hMLH1, MGMT, BRCA1, GSTP1, TIMP-3, CDH1 and DAPK-1* genes (50). The incidence of DNMT1 nuclear immunoreactivity, the incidence of DNA methylation of at least one of the 12 genes and the average number of methylated genes increased progressively during multistage carcinogenesis of the pancreas. The average number of methylated genes in ductal carcinomas was significantly correlated with DNMT1 protein expression level ($P = 0.0093$).

who were current smokers was significantly higher than in patients who had never smoked (52). The incidence of DNA hypermethylation in non-cancerous lung tissues obtained from patients with non-small cell lung cancers was significantly correlated with the extent of pulmonary anthracosis, as an index for the cumulative effects of smoking (Figure 2; K. Eguchi, Y. Kanai, K. Kobayashi and S. Hirohashi, unpublished data). Cigarette smoking may participate in alteration of DNA methylation during the development of non-small cell lung cancers. The molecular mechanisms by which carcinogens related to cigarette smoking affect DNA methylation status are still unclear and warrant further investigation.

The incidence of DNA hypermethylation at multiple C-type CpG islands in non-cancerous tissues and cancers from various organs is summarized in Table I. For example, the methylated in tumor-25 clone is methylated in normal renal tissues obtained from patients without renal cancers as frequently as in non-cancerous renal tissues showing no marked histological findings obtained from patients with renal cancers or in renal cancers (39), although it is never methylated in normal liver tissues. DNA methylation profiles of normal tissues tend to be organ specific. Moreover, hot spots of DNA hypermethylation vary among cancers arising in different organs and may reflect the influence of various carcinogenetic factors. The molecular mechanisms responsible for determination of target genes of the CpG island methylator phenotype (CIMP), defined by frequent DNA hypermethylation of C-type CpG islands (53), should be further clarified.

Alterations of DNA methylation are a hallmark of precancerous conditions even in histologically normal tissues

Alterations of DNA methylation are considered to participate in the precancerous stage in various organs, in association with obvious etiological factors, e.g. chronic inflammation, persistent infection with viruses or other pathogenic microorganisms, as mentioned above. Unlike cancers derived from such organs, precancerous conditions in the kidney have been rarely described: pathologists hardly ever observe histological changes in non-cancerous renal tissues obtained from patients with renal cancers. Surprisingly, even in non-cancerous renal tissues showing no marked histological findings obtained from patients with renal cancers, the average number of methylated CpG islands was significantly higher than that in normal renal tissues obtained from patients without renal cancers, regardless of patient age and smoking history (39). The average number of methylated CpG islands was even higher in renal cancers. From the viewpoint of alterations of DNA methylation, the presence of precancerous conditions can be recognized even in the kidney. In other words, regional DNA hypermethylation participates in the early and precancerous stage of multistage renal carcinogenesis. More surprisingly, the average number of methylated CpG islands in non-cancerous renal tissues showing no marked histological change obtained from patients with conventional renal cell carcinomas (RCCs) was significantly correlated with a higher histological grade of corresponding RCCs developing in individual patients (Figure 3; 39), indicating that precancerous conditions showing accumulation of DNA methylation may generate more malignant RCCs.

Regional DNA hypermethylation has a prognostic impact on patients with cancers

Accumulation of DNA methylation at CpG islands in conventional RCCs is significantly correlated with higher histological grade, an infiltrating growth pattern and vascular involvement (39), suggesting that regional DNA hypermethylation is continuously involved in multistage renal carcinogenesis from precancerous conditions to malignant progression. The recurrence-free survival rate of patients with RCCs showing accumulated DNA methylation of CpG islands was significantly lower than that of patients with RCCs not showing this feature (39).

The incidence of increased DNMT1 protein expression in HCCs is significantly correlated with poorer tumor differentiation and portal

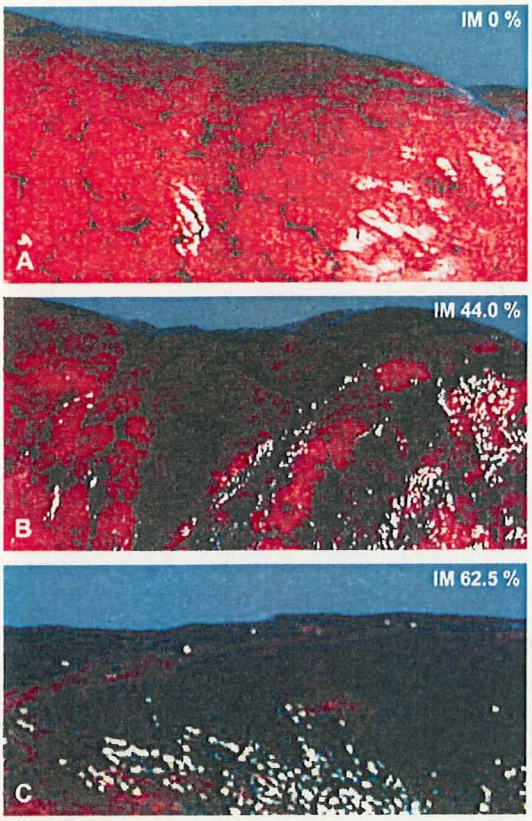

Fig. 2. Correlations between DNA hypermethylation at the D17S5 locus and anthracosis in non-cancerous lung tissues from patients with non-small cell lung cancers. The extent of pulmonary anthracosis in each resected lung was graded macroscopically: grade 1, slight accumulation of charcoal particles in the intra-lobular lymphatics forming a fine reticular pattern scattered in the visceral pleura (**A**); grade 2, the reticular pattern due to charcoal particle accumulation was denser and showed fusion in places (**B**) and grade 3, dense accumulation of charcoal particles was present throughout most of the visceral pleura (**C**). The incidence of DNA hypermethylation at the D17S5 locus (IM) analyzed by Southern blotting using a DNA methylation-sensitive restriction enzyme (NotI) in non-cancerous lung tissues showing grade 3 anthracosis (62.5%) was higher than that in those showing grade 2 (44.0%) or 1 (0%) anthracosis ($P = 0.0011$).

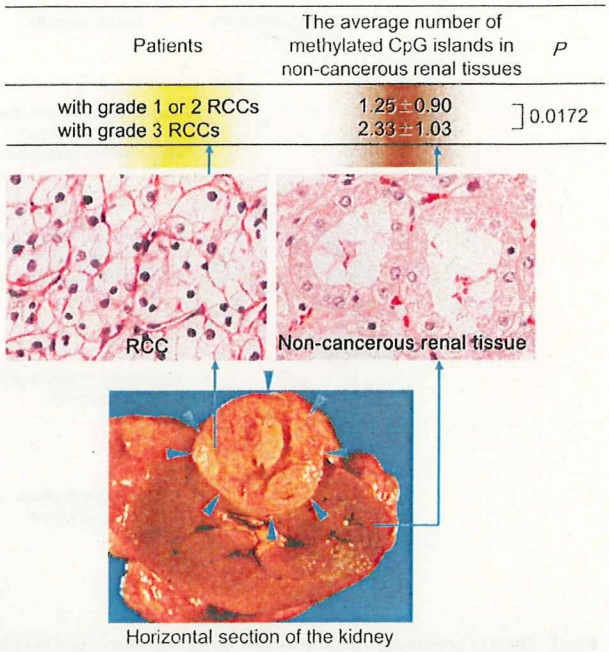

Fig. 3. DNA methylation status in non-cancerous renal tissues obtained from patients with RCC (arrowheads). The average number of methylated CpG islands in non-cancerous renal tissues obtained from patients with histological grade 3 RCCs was significantly higher than that in equivalent tissues obtained from patients with histological grade 1 or 2 RCCs (39): accumulation of DNA methylation of CpG islands in non-cancerous renal tissues was significantly correlated with a higher histological grade of the corresponding RCCs, indicating that precancerous conditions showing regional DNA hypermethylation may generate more malignant RCCs.

vein involvement (54). Moreover, the recurrence-free and overall survival rates of patients with HCCs showing increased DNMT1 protein expression are significantly lower than those of patients with HCCs that do not (54). Increased DNMT1 protein expression in ductal carcinomas of the pancreas is significantly correlated with the extent of cancer invasion to the anterior pancreatic capsule, retroperitoneal tissue and other surrounding organs and with advanced stage (49), suggesting that DNMT1 over-expression is associated with aggressiveness of pancreatic cancers. Moreover, patients with ductal carcinomas of the pancreas showing increased DNMT1 protein expression have a poorer prognosis (49).

Regional DNA hypermethylation and increased DNMT1 protein expression participate not only in the precancerous stage but also in malignant progression, and have a prognostic impact on patients with cancers. Analysis of DNA methylation status at multiple CpG islands and/or immunohistochemical examination for DNMT1 in biopsy specimens obtained for histological diagnostic purposes and/or surgically resected materials may become a useful tool for prognostication in individual clinical cases.

DNMT1 over-expression is not always a secondary result of increased cell proliferative activity but is significantly correlated with regional DNA hypermethylation

We focused on abnormalities of DNMTs underlying alterations of DNA methylation in human cancers. The expression level of DNMT1 mRNA was first examined by quantitative reverse transcription–PCR analysis, and found to be significantly higher even in non-cancerous liver tissues showing chronic hepatitis or cirrhosis than in normal liver tissues and was even higher in HCCs (55,56). With respect to multistage carcinogenesis of the pancreas, the incidence of nuclear DNMT1 immunoreactivity was significantly elevated in peripheral pancreatic ductal epithelia with an inflammatory background and PanINs than in peripheral pancreatic ductal epithelia without an inflammatory background (Figure 1; 49). The incidence of nuclear DNMT1 immunoreactivity was significantly associated with the degree of PanIN dysplasia. The incidence of nuclear DNMT1 immunoreactivity was significantly higher in invasive ductal carcinomas of the pancreas than in PanINs (Figure 1; 49). The average number of methylated tumor-related genes in microdissected specimens of ductal carcinomas of the pancreas was significantly correlated with the expression level of DNMT1 protein examined immunohistochemically in the precisely microdissected areas (50).

The level of DNMT1 mRNA expression was higher in colorectal and stomach cancers than in the corresponding non-cancerous mucosae (38). The average ratio of DNMT1 expression in tumor tissue relative to the corresponding non-cancerous mucosa was significantly higher in CIMP-positive colorectal and stomach cancers than in CIMP-negative colorectal and stomach cancers, but no such association was observed for the expression of DNMT2, DNMT3a or DNMT3b (38). Immunohistochemical examination showed that increased DNMT1 protein expression was significantly associated

with poorer differentiation of stomach cancers, whereas none of the examined non-cancerous stomach epithelia exhibited DNMT1 immunoreactivity (except in the proliferative zones) (37). Significant correlation between DNMT1 over-expression and CIMP in stomach cancers was also confirmed by immunohistochemistry at the protein level (37). The *hMLH1, THBS-1 and E-cadherin* genes may be targets for over-expressed DNMT1 in stomach cancers (37).

Thus, DNMT1 may be responsible for *de novo* methylation of CpG islands during multistage carcinogenesis. The maintenance activities of DNMT1 are related to its preference for hemimethylated substrates *in vitro*. A theoretical explanation for the role of DNMT1 in *de novo* DNA methylation in human cancers with dysfunction of p21WAF1, which competes with DNMT1 for binding with proliferating cell nuclear antigen (PCNA), has been proposed (57). Moreover, it has recently been suggested that DNMT1 is capable of *de novo* DNA-methylating activity *in vivo* as well as having a maintenance function: *de novo* methylation of CpG islands has actually been observed in human fibroblasts over-expressing DNMT1 (58,59). Therefore, it is feasible that, in cancers, DNMT1 over-expression participates in regional DNA hypermethylation.

Transitional cell carcinomas (TCCs) of the urinary bladder are clinically remarkable because of their multicentricity and tendency to recur: synchronously or metachronously multifocal TCCs often develop in individual patients. A possible mechanism for such multiplicity is the 'field effect', whereby carcinogenic agents in the urine cause malignant transformation of multiple urothelial cells. Even non-cancerous urothelia showing no remarkable histological features obtained from patients with urinary bladder cancers can be considered precancerous because they may be exposed to carcinogens in the urine. On the other hand, DNMT1 mRNA is expressed mainly during S phase and because tumor tissues of various organs generally contain a greater proportion of dividing cells than do normal tissues, it has been debatable whether increased DNMT1 expression is due to an increase in the proportion of dividing cells or to an acute increase of DNMT1 expression per individual cancer cell. This uncertainty prompted us to compare DMNT1 immunoreactivity and the PCNA-labeling index during urothelial carcinogenesis. The incidence of nuclear DNMT1 immunoreactivity had already increased independently of cell proliferative activity in non-cancerous urothelia showing no marked histological features obtained from patients with urinary bladder cancers, where the PCNA-labeling index had not yet increased, compared with that in normal urothelia obtained from patients without urinary bladder cancers, indicating that DNMT1 over-expression preceded increased cell proliferative activity during multistage urothelial carcinogenesis (60). A similar discrepancy between DNMT1 immunoreactivity and the PCNA-labeling index was also observed in liver (54), stomach (37) and uterine cervix cancers (46). Excessive amounts of DNMT1 compared with PCNA, which targets DNMT1 to replication foci, may participate in *de novo* methylation of CpG islands. Further examinations are required to clarify whether or not unknown functional protein complexes recruit DNMT1 to specific DNA sequences during carcinogenesis. The incidence of nuclear DNMT1 immunoreactivity was even higher in dysplastic urothelia and TCCs than in non-cancerous urothelia showing no marked histological features obtained from patients with urinary bladder cancers (60). Among all examined microdissected specimens of non-cancerous urothelia showing no marked histological features obtained from patients with urinary bladder cancers, dysplastic urothelia and TCCs, concurrent DNA hypermethylation of three or more examined C-type CpG islands was significantly correlated with increased DNMT1 protein expression (40).

Splicing alteration of DNMT3b may result in chromosomal instability through DNA hypomethylation of pericentromeric satellite regions

Satellite regions are abundant in pericentromeric heterochromatin DNA on chromosomes 1, 9 and 16 and are heavily methylated in normal cells. DNA hypomethylation of these regions is known to result in centromeric decondensation and enhanced chromosome recombination. In 18% of examined non-cancerous liver tissues showing chronic hepatitis or cirrhosis and 67% of examined HCCs, satellites 2 and 3 were hypomethylated (56). Frequent chromosome 1q copy gain with a pericentromeric breakpoint has been reported in HCCs showing DNA hypomethylation of satellite 2 (61). In TCCs of the urinary bladder, the ureter or the renal pelvis, DNA hypomethylation of satellites 2 and 3 was significantly correlated with LOH on chromosome 9 (62). Therefore, DNA hypomethylation of pericentromeric satellite regions may induce chromosomal instability during multistage carcinogenesis.

DNMT3b is specifically required for DNA methylation of pericentromeric satellite regions in embryonic stem cells and early mouse embryos (11). Germ line mutations of the *DNMT3b* gene have been reported in patients with immunodeficiency, centromeric instability and facial anomalies syndrome, a rare recessive autosomal disorder characterized by DNA hypomethylation of pericentromeric satellite regions (63). However, no mutation of any coding exon of the *DNMT3b* gene was detected in examined HCCs (64). When examined by quantitative reverse transcription–PCR analysis using a primer set not discriminating splice variants of DNMT3b, the total level of DNMT3b mRNA was higher in HCCs than in the corresponding non-cancerous liver tissues (64). Thus, it is unlikely that reduced expression of DNMT3b simply causes DNA hypomethylation of these regions during hepatocarcinogenesis.

There are four splice variants in the C-terminal catalytic domain of DNMT3b. DNMT3b3 possesses the N-terminal region and conserved methyltransferase motifs I, IV, VI, IX and X. DNMT activity of human DNMT3b3 has been confirmed *in vitro* (65), and DNMT3b3 is expressed ubiquitously in normal human tissues. Our data obtained by splice variant-specific quantitative reverse transcription–PCR has also indicated that the major variant in normal liver tissues is DNMT3b3 (64). On the other hand, DNMT3b4 probably does not show DNMT activity because it lacks the conserved methyltransferase motifs IX and X, although it retains the N-terminal domain required for targeting to heterochromatin sites through binding to RP58. We have confirmed that normal liver tissues show only a trace level of DNMT3b4 expression (64). The level of DNMT3b4 mRNA in non-cancerous liver tissues obtained from patients with HCCs and in HCCs was significantly correlated with the degree of DNA hypomethylation of pericentromeric satellite regions (64). In addition, the ratio of DNMT3b4 mRNA to DNMT3b3 mRNA in non-cancerous liver tissues obtained from patients with HCCs and in HCCs was also significantly correlated with the degree of DNA hypomethylation of pericentromeric satellite regions (64). DNMT3b4 lacking DNMT activity may compete with the major variant, DNMT3b3, for targeting to pericentromeric satellite regions. This may be the reason why DNMT3b4 over-expression results in DNA hypomethylation of pericentromeric satellite regions in precancerous conditions and HCCs. To confirm this possibility, we introduced DNMT3b4 into human epithelial 293 cells, which express a significant level of DNMT3b3 mRNA and a trace level of endogenous DNMT3b4 mRNA. DNA demethylation on satellite 2 was observed in DNMT3b4 transfectants, depending on the expression level of myc-tagged DNMT3b4 (64). DNMT3b4 over-expression may lead to chromosomal instability through induction of DNA hypomethylation of pericentromeric satellite regions during hepatocarcinogenesis.

The growth rate of DNMT3b4 transfectants was approximately double that of mock transfectants soon after the introduction of DNMT3b4, when chromosomal instability may not yet have accumulated (66). We assumed that this change was caused by altered gene expression. A majority of the genes that were up-regulated in DNMT3b4 transfectants but not in mock transfectants were implicated in interferon signaling (66). Although genes that encoded interferons themselves were not up-regulated, *signal transducer and activator of transcription (STAT) 1*, which acts as an effector of interferon signaling, has been listed as one of the up-regulated genes in DNMT3b4 transfectants (66). It had been reported previously that inhibition of DNA methylation in cultured human cancer cells by 5-aza-2′-deoxycytidine induces a set of genes implicated in interferon signaling primarily via over-expression of STAT1, 2 and 3 (67). DNMT3b may act to maintain the DNA methylation status of not only pericentromeric

satellite regions but also specific genes, probably in cooperation with DNMT1, in cancer cells, and this may explain why inhibition of DNMT3b activity by induction of DNMT3b4 produced a similar result to the general inhibition of DNA methylation obtained with 5-aza-2′-deoxycytidine. There is a significant correlation between the mRNA expression levels of DNMT3b4 and STAT1 in HCCs (66). Over-expression of DNMT3b4 is involved in multistage carcinogenesis not only by inducing chromosomal instability but also by affecting the expression of specific genes.

Significance of DNA hypomethylation in human cancers

In addition to the above-mentioned satellites 2 and 3, DNA hypomethylation of other tandem repeats such as NBL2 and D4Z4 and retrotransposons such as LINE-1 and Alu was frequently observed in various human cancers (68). Hypomethylation of repeated DNA sequences can disrupt the functions of neighboring genes through transcriptional interference by either sense or anti-sense transcripts (68). Activation of transposable elements can potentially lead to insertional mutagenesis (68). Although global DNA hypomethylation, i.e. reduction of the total level of 5-methylcytosine examined by high-performance liquid chromatography of DNA digested to mononucleotides, is considered to mainly reflect the above-mentioned hypomethylation of repeated DNA sequences, individual genes are also hypomethylated in human cancers. In earlier days, DNA hypomethylation of oncogenes such as *c-myc* in human cancers was frequently reported (69). However, such DNA hypomethylation affects the body or 3′ end of genes whose DNA methylation status is usually not related to gene expression. Recently, significant correlation between over-expression and DNA hypomethylation of tumor-related genes such as *maspin* (70) and *synuclein γ* (71) and cancer/testis antigens such as *melanoma and germ cell-expressed* genes (72) has been reported in human cancers.

We focused on the possibility that functional disruption of DNMT1 due to gene mutations might induce hypomethylation of repeated DNA sequences and individual genes in human cancers. Mutations of the *DNMT1* gene, including a one-base deletion resulting in deletion of the whole catalytic domain due to a premature stop codon, were detected in 7% of examined colorectal cancers (73). This was the first evidence of *DNMT1* gene mutations in human cancers. However, no stomach cancers or HCCs showed mutations in any of the 40 coding exons of the *DNMT1* gene (73). Mutational inactivation of the *DNMT1* gene may be a rare event during human carcinogenesis. Critical dietary components leading to synthesis of the methyl group donor, *S*-adenosylmethionine, include folate, vitamins B6 and B12, methionine and choline. Diets devoid of folate and choline and low in methionine are sufficient to independently induce hepatocarcinogenesis in rats (74). Non-coding RNAs such as anti-sense transcripts of individual genes may be involved in demethylation of the genes (75).

Although global DNA hypomethylation as an early event during colorectal carcinogenesis was noticed in the early days of molecular cancer research (76), the relative timing of global DNA hypomethylation has been revealed to differ between cancers derived from various organs (68). Researchers focusing on cancer epigenetics generally pay more attention to DNA hypermethylation than to DNA hypomethylation, and the clinicopathological significance of DNA hypomethylation during multistage carcinogenesis is, if anything, less well understood. DNA hypomethylation around the promoter regions of individual genes is usually correlated with global DNA hypomethylation (77), but not with DNA hypermethylation of C-type CpG islands in human cancers (78). Hypomethylation of both repeat DNA sequences and individual genes and regional DNA hypermethylation seem to be distinct consequences and are not mutually exclusive in individual patients with cancer.

Altered expression of methyl-CpG-binding proteins in human cancers

Until 1998, MeCP2 had been the only functionally defined methyl-CpG-binding protein (MBD). When MeCP2 binds to methylated CpG dinucleotide, its transcriptional repression domain recruits a co-repressor complex containing Sin 3A and histone deacetylases, resulting in compaction of the chromatin and stable repression of the target gene (79,80). Later, MBD1, MBD2, MBD3 and MBD4 were identified. MBD2 is a transcriptional repressor involved in the MeCP1 complex, identified in mammalian nuclear extracts, and represses transcription from the methylated promoter (81). MBD3 is involved in another histone deacetylase complex, Mi-2/NuRD (82). MBD4 is thought to act as a thymine DNA glycosylase, repairing G:T or G:U mismatches at CpG sites (83).

The expression level of MeCP2 mRNA in HCCs with portal vein involvement is significantly lower than that in HCCs without such involvement, suggesting that reduced expression of MeCP2 may be associated with malignant progression of HCCs (56). Reduced MBD2 mRNA expression has been observed in HCCs (56), colorectal and stomach cancers (84), suggesting that reduced MBD2 expression may be associated with a particular step in human carcinogenesis. The expression level of MBD4 mRNA in HCCs is significantly lower than that in the corresponding non-cancerous liver tissues and is significantly correlated with poorer tumor differentiation and involvement of the portal vein (56). Reduced MBD4 expression may result in frequent C–T transitions in tumor suppressor genes. Although many researchers have focused on crosstalk between DNA methylation and histone modification, abnormalities of MBDs in human cancers do not seem to have attracted much attention, and the implications of these proteins in carcinogenesis need to be further clarified.

Perspectives

Alterations of DNA methylation are associated with multistage carcinogenesis from precancerous conditions to malignant progression. Therefore, estimation of carcinogenetic risk and early diagnosis of cancers using alterations of DNA methylation as indicators are promising approaches for mass screening of clinical samples. For such purposes, non- or less invasive methodologies for detecting subtle alterations of DNA methylation have been developed for serum, urine, sputum and other body fluid samples. Moreover, recently developed array-based technology for accessing genome-wide DNA methylation status (85) will be useful for identifying the DNA methylation profile that is the optimum indicator for risk estimation and early diagnosis. Analysis of DNA methylation status in biopsy specimens and/or surgically resected materials may also become a useful tool for prognostication. Clinical trials of DNA demethylation agents are underway and many DNA demethylation agents are now being developed (86). However, global DNA hypomethylation and regional DNA hypermethylation are commonly observed during multistage carcinogenesis. In some patients, global DNA hypomethylation will have greater significance than regional DNA hypermethylation during carcinogenesis. Therefore, before using DNA demethylation agents for prevention or therapy of cancers, it will be necessary to carefully identify patients who might benefit most from this type of demethylation strategy. It may also be necessary to develop sequence-specific demethylation agents to reduce any severe side effects. In order to apply correction of DNA methylation status to practical prevention and therapy of cancers, the full picture and molecular mechanisms of DNA methylation alterations corresponding to specific carcinogenetic factors should be further clarified for each organ.

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