Table 1 *CDKN1C* expression and CpG methylation of *DMR-LIT1* and *CDKN1C* promoter

Cell line no. / *Cell line name*	#1 *TE9*	#2 *KYSE140*	#3 *YES5*	#4 *KYSE410*	#5 *KYSE510*	#6 *YES3*	#7 *KYSE960*	#8 *TE10*	#9 *HARA*	#10 *YES1*	#11 *KYSE170*	#12 *YES2*	#13 *KE3*	#14 *KYSE350*	#15 *YES4*	#16 *KE4*	#17 *YES6*
CDKN1C expression (RPA)	0.230	0.268	0.425	0.008	0.006	0.084	0.165	0.055	0.022	0.027	0.040	0.047	0.011	0.597	0.378	0.267	0.346
CDKN1C expression (RT–PCR)	0.74	0.76	1.05	0.01	0.06	0.16	0.49	0.10	0.16	0.20	0.13	0.14	0.11	1.30	1.23	0.92	1.46
DMR-LIT1 MI	52.0	52.0	53.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	34.9	100.0	100.0	0.0	100.0	71.0	100.0
Polymorphisms	hetero	hetero	hetero	NI	NI	NI	NI	NI	NI	hetero	hetero	NI	NI	NI	NI	hetero	hetero
11p15.5 copy number	2	2	2	1	1	1	1	2	2	2	2	1	2	2	1	2	2
CDKN1C promoter MI																	
Region 1/*Mbo*I	71.6	100.0	97.6	53.5	93.0	94.5	73.7	73.2	9.0	97.9	92.8	94.7	52.2	90.1	93.0	57.0	88.7
Region 1/*Eco*RV	88.1	90.4	90.6	50.2	86.9	96.5	63.8	36.8	3.1	92.7	83.9	100.0	37.9	91.8	93.0	74.5	87.3
Region 2/*Sac*II	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Region 3/*Rsa*I	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.8	0.0	0.0	0.0
Region 4/*Eco*RI	0.0	0.0	0.0	5.7	21.4	0.0	0.0	0.4	0.0	4.3	15.9	6.0	0.0	98.9	37.3	0.7	0.9
Region 5/*Hin*fI	0.0	0.0	0.0	76.3	0.0	96.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

CDKN1C expression was measured with RPA and quantitative real-time RT–PCR normalized with a housekeeping *HMBS* gene. MI of *DMR-LIT1* and *CDKN1C* promoter region 2 were analysed by Southern blotting, and MI of regions 1, 3, 4, and 5 was analysed by hot-stop COBRA. In all, 10 polymorphic loci within 11p15.5 were analysed to examine heterozygosity. Copy number of 11p15.5 was determined by polymorphic marker analyses and by measurement of Southern blotting band intensity. hetero: heterozygous for at least one of polymorphic loci; NI: not informative

(*HMBS*) (Table 1, Figure 1b). Among the cell lines, expression of *CDKN1C* varied from 0.006 to 0.597 by RPA, and from 0.01 to 1.46 by RT–PCR. Values from both experiments were closely correlated (Spearman's rank-correlation coefficient $\rho = 0.912$, $P = 0.0003$), indicating correct evaluation of *CDKN1C* expression. Second, the quantity of *DMR-LIT1* CpG methylation status was determined by Southern blotting with *Bam*HI and methylation-sensitive *Not*I (Figure 1c). Detection of only a 1.8 kb band by NotC control probe indicated complete digestion by *Not*I. The average CpG methylation index (MI) at *DMR-LIT1* obtained from two normal placentae and seven individual peripheral blood cells was 51.0±5.4% (Figure 1c and data not shown). This was consistent with the finding that *DMR-LIT1* is methylated differentially (Lee *et al*., 1999; Mitsuya *et al*., 1999; Smilinich *et al*., 1999). The MI of cancer cell lines varied from 0.0 to 100%. Since four lines unexpectedly showed only methylated bands, MI was confirmed by quantitative combined bisulfite restriction analysis (COBRA), employing the hot-stop method (Figure 1d). Next, we investigated heterozygosity of 11p15.5 using 10 polymorphic loci. The copy number of 11p15.5 was calculated by band intensity obtained from Southern blotting (Table 1). Since no informative line for a polymorphism of *CDKN1C* was found, we were not able to investigate the allelic expression of the gene. We found that 11 lines sustained two copies of 11p15.5, and the other six lines had only one copy. Among 11 cell lines harboring two copies, three (#1–3) showed normal CpG methylation of *DMR-LIT1*, indicating maintained imprinting of the *CDKN1C/LIT1* domain. The average *CDKN1C* expression of the three was 0.31±0.10 by RPA and 0.85±0.17 by RT–PCR. Of 17 lines (59%, #4–13), 10 showed lower expression of *CDKN1C* than the imprinting maintained lines (average – 2 s.d., only #7 by RPA was lower than average – 1 s.d.), whereas four (#14–17) showed the same or higher expression.

CDKN1C expression is closely correlated with CpG methylation of DMR-LIT1

To investigate whether *CDKN1C* expression was regulated by *DMR-LIT1*, an ICR of the *CDKN1C/LIT1* imprinted domain, we statistically investigated a correlation between *CDKN1C* expression and *DMR-LIT1* MI. All cell lines except three (#12–14) showed close correlation between *CDKN1C* expression by RPA and *DMR-LIT1* MI (Figure 1e and f, $\rho = 0.832$, $P = 0.0027$). Close correlation was also seen using RT–PCR data ($\rho = 0.851$, $P = 0.0022$). Cell lines #12–14 showed apparent inconsistency between the two parameters (Figure 1e). Using data from nine lines harboring two copies of 11p15.5 (#1–3, 8–11, 16, 17), *DMR-LIT1* MI still showed positive correlation with *CDKN1C* expression ($\rho = 0.831$, $P = 0.0188$ by RPA, $\rho = 0.881$, $P = 0.0127$ by RT–PCR). Thus, it was strongly suggested that *CDKN1C* expression was controlled by *DMR-LIT1* in the majority of human cancer cell lines investigated.

CDKN1C expression is not correlated with CpG methylation of its own promoter

To investigate whether *CDKN1C* expression might be correlated with CpG methylation of *CDKN1C* promoter itself, we quantified the MI of the *CDKN1C* promoter (Figure 2a, Table 1), dividing it into five regions. Regions 1, 3, 4, and 5 were analysed by hot-stop COBRA; region 2 was analysed by methylation-sensitive Southern blotting. In the three normal placentae with abundant expression of *CDKN1C*, region 1 unexpectedly showed MI varying from 15.1 to 41.2%, but the other four regions were not methylated at all (Figure 2a, data not shown). In cancer cell lines, region 1 showed CpG methylation, ranging from 3.1 to 100%. Regions 2 and 3 were mostly unmethylated in all cell lines. Few lines

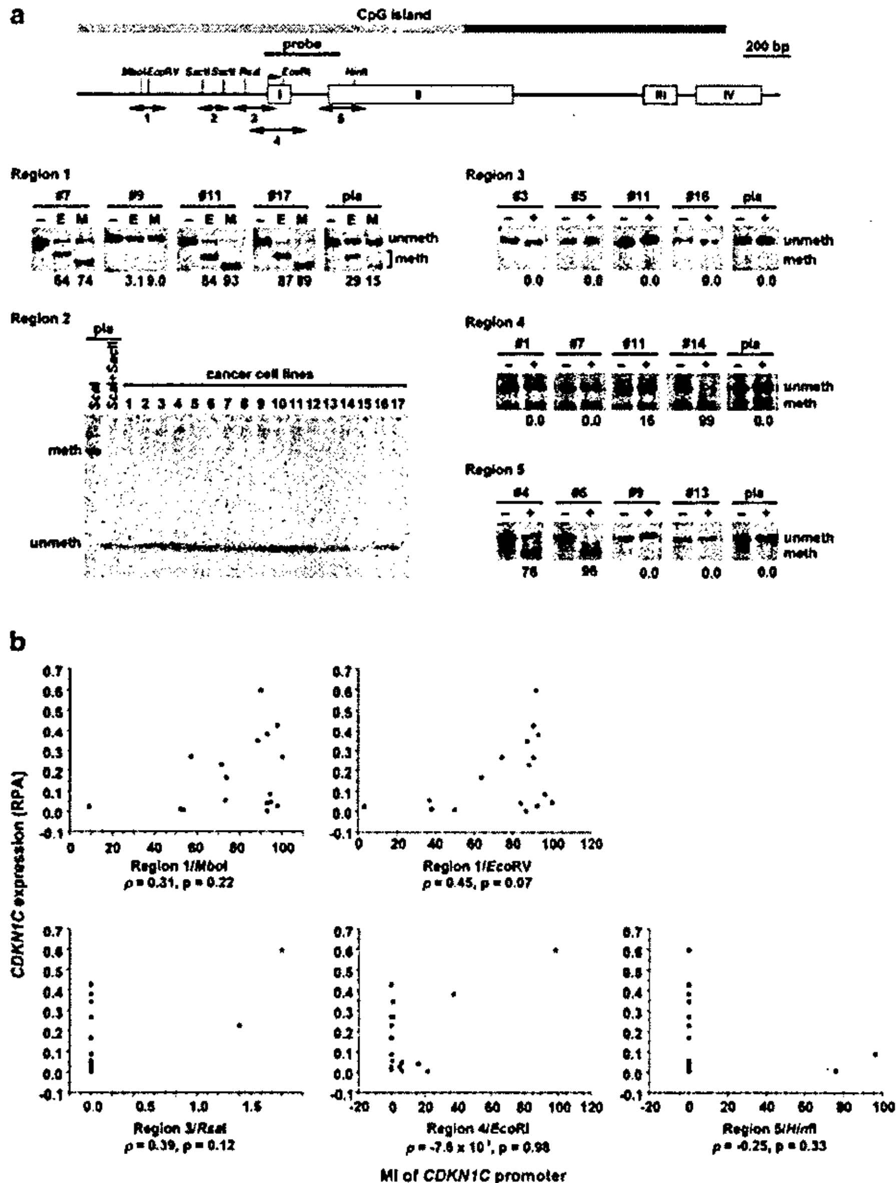

Figure 2 No correlation between *CDKN1C* expression and CpG methylation of *CDKN1C* promoter region. (**a**) Representative results of *CDKN1C* promoter methylation analyses. The genomic structure of the *CDKN1C* gene is shown in the upper panel. Arrows indicate the regions analysed for methylation status. Regions 1, 3, 4, and 5 were analysed by hot-stop COBRA. For region 1, –, E, and M indicated undigested PCR product, digested with *Mbo*I, and with *Eco*RV, respectively. For region 3, 4, and 5, – and + indicated undigested PCR product and digested with appropriate restriction enzyme shown in the map, respectively. Digested bands indicated methylation. For region 4, since there was a nonspecific band the same size as the digested band, methylation frequency was calculated by ((percentage of unmethylated band intensity without digestion - percentage of unmethylated band intensity with digestion)/ percentage of unmethylated band intensity without digestion). Hot-stop COBRA experiments were repeated three times independently. Region 2 was analysed by methylation-sensitive Southern blot with *Sca*I and *Sac*II. Methylated (meth) and unmethylated (unmeth) bands are 3.4 kb and 497 bp, respectively. Probe for region 2 Southern blot is indicated as a horizontal bar. (**b**) Plots of *CDKN1C* expression by RPA (the vertical axis) and MI of *CDKN1C* (the horizontal axis). There was no correlation between *CDKN1C* expression and methylation in any combination of cancer cell lines

showed low to high MI of either region 4 or 5. We also statistically investigated a correlation between *CDKN1C* expression and the MI of each region in *CDKN1C* promoter. In all cell lines, *CDKN1C* expression, from both RPA and RT–PCR, was not correlated with MI of any region (Figure 2b). There was still no correlation when #12–14 were excluded (data not shown).

Histone modifications at DMR-LIT1 and CDKN1C promoter

To investigate histone modifications at the *DMR-LIT1* and *CDKN1C* promoter in cancer cells, we performed ChIP assay with three primer sets for each gene (Figure 3a and b). We used three cell lines – #2, #8, and #9. In these cells, *LIT1* expression was also quantified with real-time RT–PCR. The results of the control loci, *GAPD* and the chromosome 16 centromere region, indicated successful ChIP procedures (Figure 3c). All histone modifications were detected in #2. Since #2 showed normal MI at *DMR-LIT1*, acetylations and H3K4 methylation at *DMR-LIT1* were on the paternal allele and H3K9 methylation was on the maternal allele, according to our previous study (Figure 3a and d) (Higashimoto *et al.*, 2003). Cell lines #8 and #9, showing CpG unmethylation on both alleles and diminished *CDKN1C* expression, probably had loss of imprinting of *LIT1*. Acetylations and H3K4 methylation at *DMR-LIT1* were apparently higher in #8 and #9, along with the decrease of MI and increase of *LIT1* expression, than in #2 at all regions. By contrast, H3K9 methylation was undetectable in any region. In the *CDKN1C* promoter region, acetylations and H3K4 methylation were detectable in all lines. However, these were unchanged or slightly increased in #8 and #9 despite diminished *CDKN1C* expression. Furthermore, H3K9 methylation was barely detectable at *CDKN1C* promoter in any of the three lines. The results suggested that histone modifications at *CDKN1C* promoter were not associated with its expression. This was supported by ChIP on #12–14, showing association of *LIT1* expression and histone modifications and MI at *DMR-LIT1*, and no association of *CDKN1C* expression or histone modifications at *CDKN1C* promoter, although *CDKN1C* expression was inconsistent with *DMR-LIT1* MI in them (data not shown). Intriguingly, #2 showed no methylation of H3K9 in any region, although *CDKN1C* on the paternal allele is normally repressed by imprinting. Neither was H3K9 methylated in lymphoblastoid cells established from normal individual and BWS patients, in which *DMR-LIT1* MI was maintained normally (data not shown).

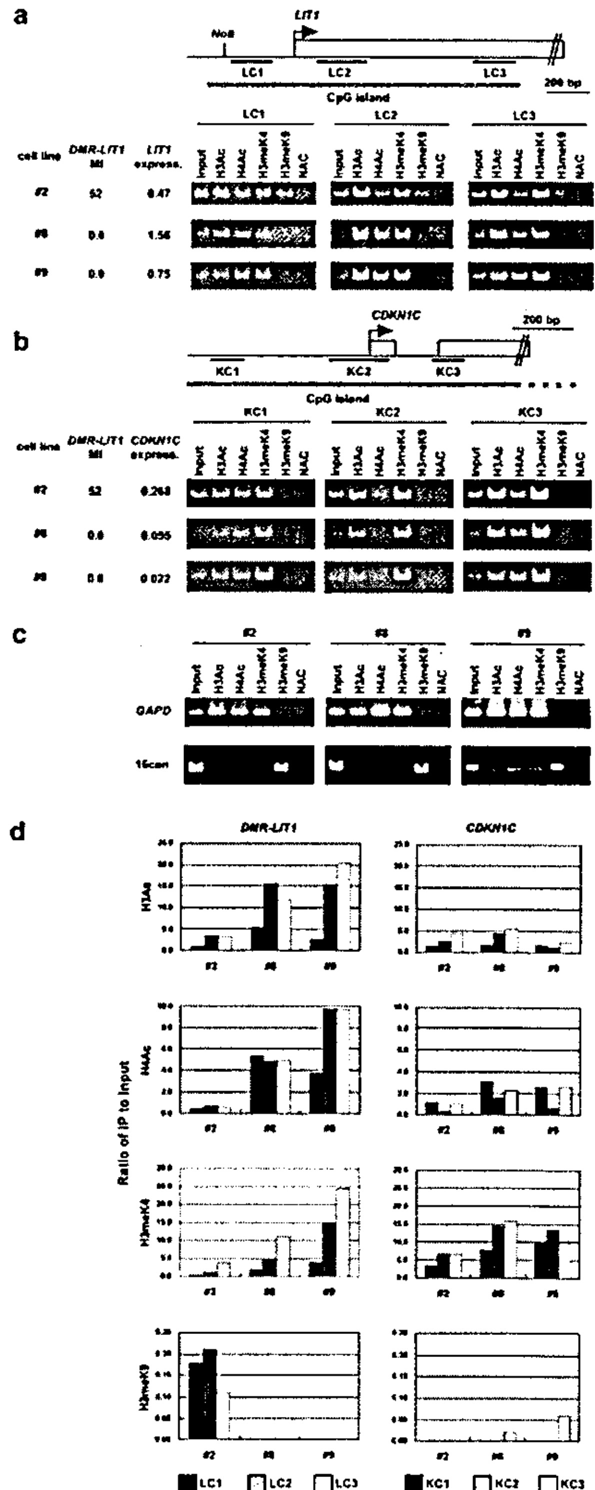

Figure 3 Histone modification status at *DMR-LIT1* and *CDKN1C* in cancer cell lines. (**a** and **b**) Representative results of ChIP-PCRs. Genomic structure, CpG island, and PCR-amplified regions of *DMR-LIT1* and *CDKN1C* are shown. #2 showed normal CpG methylation at *DMR-LIT1*. #8 and #9 showed CpG demethylation of *DMR-LIT1*, increased *LIT1* expression, and decreased *CDKN1C* expression. *LIT1* expression was measured by quantitative real-time RT-PCR and normalized with *HMBS*. Arrows indicate the transcription initiation site. The transcription initiation site of *LIT1* is putative (Oshimura M, Tottori University, personal communication). NAC: no antibody control. (**c**) Internal control of ChIP analyses. *GAPD* is a positive control for acetylations and H3K4 methylation, and centromere of chromosome 16 is for H3K9 methylation. (**d**) Histone modification status was evaluated by the ratio of PCR band intensity to input (total chromatin). ChIP was performed on each cell line at least twice independently, and PCR was at least in duplicate for each ChIP

Discussion

An important finding was that in the majority of esophageal cancer cell lines investigated, the epigenetic factor correlated with *CDKN1C* expression was the CpG methylation status of *DMR-LIT1*, not that of the *CDKN1C* promoter itself. Furthermore, H3K9 methylation of *DMR-LIT1* was closely associated with CpG methylation. Notably, approximately 36% (4/11) of lines possessing two copies of 11p15.5 showed diminished *CDKN1C* expression. These four lines showed complete or partial demethylation at *DMR-LIT1*, indicating that imprinting disruption of the *CDKN1C/LIT1* domain with loss of CpG methylation at *DMR-LIT1* leads to the reduction of *CDKN1C* expression, which would be involved to some extent in esophageal cancers. Diaz-Meyer *et al.* (2003) recently reported that in BWS fibroblast cells, silencing of *CDKN1C* is associated with hypomethylation of *DMR-LIT1*, supporting our result. Previous reports, however, described the association of hypermethylation of *CDKN1C* promoter region with gene silencing (Shin *et al.*, 2000b; Kikuchi *et al.*, 2002; Li *et al.*, 2002), but did not examine the methylation of *DMR-LIT1*. Since those analysed tumors originated from other tissues, we speculated that a tissue-specific epigenetic mechanism for *CDKN1C* expression might be involved. *CDKN1C* expression of three lines (#12–14) in this study did not correlate with the CpG methylation of *DMR-LIT1*. Although we are not able to account for it at this point, other unknown genetic or/and epigenetic alteration(s) might occur. Or these inconsistencies might originate from abnormal methylation due to cell culture artifacts.

Demethylation of *DMR-LIT1* is found in approximately half of patients with BWS, known for its high incidence of embryonal tumors. *CDKN1C* mutation is also found in a minority of BWS. Weksberg *et al.* (2001) showed a higher tumor risk in patients with demethylation of *DMR-LIT1* (5/35) than in *CDKN1C* mutation (0/5). This is supported in Lam *et al.* (1999). However, in earlier data, the tumor risk of *CDKN1C* mutation *versus* *DMR-LIT1* demethylation was the same (2/38 *versus* 6/123) (Weksberg *et al.*, 2001). In tumors without BWS, diminished expression of *CDKN1C* ranges from 35 to 100% in various tumors, including esophageal cancer (Hatada *et al.*, 1996a; Thompson *et al.*, 1996; Matsumoto *et al.*, 2000; Oya and Schulz, 2000; Schwienbacher *et al.*, 2000; Shin *et al.*, 2000a). Reasons could include demethylation of maternal *DMR-LIT1* (imprinting disruption), as mentioned here; loss of maternal allele; and other epigenetic mechanism(s).

In human and mouse, maternal *DMR-LIT1* with CpG methylation is associated with H3K9 methylation and that paternal *DMR-LIT1* is not. BWS patients with loss of CpG methylation at *DMR-LIT1* showed a loss of H3K9 methylation (Higashimoto *et al.*, 2003). These findings, in conjunction with our results, indicate that *DMR-LIT1* is an ICR for *CDKN1C/LIT1* domain and regulates the expression of neighboring imprinted genes, including *CDKN1C*, within the domain in humans as well as in mice. The findings also suggested that H3K9 methylation is involved in the maintenance of imprinting. Paternal *DMR-LIT1* without CpG and H3K9 methylation silences neighboring imprinted genes, normally active on the maternal allele. Thus, the imprinting disruption with loss of CpG and H3K9 methylation causes epigenotype change from maternal to paternal, reducing *CDKN1C* expression, and is probably involved in cancer and BWS.

It has been reported that *DMR-LIT1* are an orientation-independent silencer (Du *et al.*, 2003; Mancini-DiNardo *et al.*, 2003; Thakur *et al.*, 2003). Thakur *et al.* (2003) proposed that a repressive chromatin structure established at *DMR-LIT1* might propagate bidirectionally along the DNA to inactivate neighboring genes. Since *LIT1* is an antisense noncoding transcript, the establishment and propagation of a repressive chromatin structure might be similar to that of *Igf2r* ICR, which contains *Air*, an antisense noncoding transcript of *Igf2r* (Sleutels *et al.*, 2002). H3K9 methylation was, however, barely detectable at *CDKN1C* promoter in the cancer cell lines and undetectable in cells with normal MI, suggesting that H3K9 methylation is not involved in *CDKN1C* repression on the paternal epigenotype chromosome. Since the human *CDKN1C* sequence was also reported to be an orientation-independent silencer (Du *et al.*, 2003) and was not differentially methylated, *CDKN1C* may be synergistically repressed by the imprinting suppression and its own silencing activity on the paternal allele. In addition, methylated *DMR-LIT1* on the maternal allele may function as a repressor of the *CDKN1C* silencer, leading to maternal expression. Further studies to clarify the intricate imprinting regulation mechanism will help in understanding imprinting-associated human diseases, such as cancer and BWS.

Materials and methods

Cell lines

KYSE 140, KYSE 170, KYSE 350, KYSE 410, KYSE510, KYSE 960 (Shimada *et al.*, 1992). HARA, TE9, and TE10 were grown in Dulbecco's modified Eagle's medium with 10% FCS. KE3, KE4, YES1, YES 2, YES3, YES4, YES5, YES6, and lymphoblastoid cells established from a normal individual and BWS patients were grown in RPMI-1640 with 10% FCS. Growing cells with 70% confluence were harvested for analyses.

Quantitative analysis of gene expression

To generate probes for RPA, RT–PCR products for *CDKN1C* and *HMBS* genes were cloned into pT7Blue T-vector (Novagen). After confirming insert sequences and directions, plasmid DNAs were prepared and linearized. To make antisense riboprobes, linearized plasmid DNAs, harboring the inserts with antisense direction, were transcribed by T7 RNA polymerase with [α-^{32}P]CTP. The positive control RNAs were generated from plasmids harboring sense-directed inserts without radioisotope. Total RNAs (50 μg) were simultaneously hybridised with the two antisense riboprobes at 60°C, and then digested with RNase T1. Protected bands, 201 bp for *CDKN1C* and 255 bp for *HMBS*, were analysed by electrophoresis on denaturing gel. Band intensity was measured with

a BAS 2000 bioimaging analyzer (Fujifilm, Japan). For quantitative real-time RT–PCR, total RNA (500 ng) was treated with RNase-free DNaseI (Roche, Germany) and reverse-transcribed with ReverTra Ace reverse transcriptase (Toyobo, Japan) and random primers (Takara, Japan). Quantitative real-time RT–PCR was performed in triplicate with the FastStart DNA Master CYBR Green I on LightCycler™ system (Roche, Germany) according to the manufacturer's protocol. The expression of *CDKN1C* was normalized with that of *HMBS*.

Methylation analysis

Quantity of *DMR-LIT1* methylation status was determined by Southern blotting. Genomic DNA (3 μg) was digested with *Bam*HI and methylation-sensitive *Not*I. The blot was probed as described previously (Mitsuya *et al*., 1999). To confirm complete digestion, the blot was reprobed with NotC probe, which detected an internal control locus containing a known unmethylated *Not*I site in CpG island (Bliek *et al*., 2001; Accession number: NT_011519). *CDKN1C* promoter region 2 was also analysed by Southern blotting. Genomic DNA was digested with *Sca*I and methylation-sensitive *Sac*II, then hybridized with the probe shown in Figure 2a. Regions 1, 3, 4, and 5 were analysed by quantitative COBRA with the hot-stop method to exclude the problem of heteroduplex formation skewing the results of restriction endonuclease digestion of PCR products (Xiong and Laird, 1997; Uejima *et al*., 2000). After bisulfite-treated DNA was amplified with a number of PCR cycles sufficient to detect a product, an end-labeled primer was added, and then the PCR step was performed only once. The PCR product was digested with the appropriate restriction endonuclease (Figure 2). Methylation of *DMR-LIT1* was also examined by hot-stop COBRA with *Acc*II. Band intensity obtained from Southern blotting and hot-stop COBRA was measured with a BAS 2000 (Fujifilm, Japan). The MI was calculated by (intensity of methylated band/(intensity of methylated band + intensity of unmethylated band)).

Table 2 PCR primers and conditions

Primers	*Sequence (5′- 3′)*	*Annealing (cycles)*
RPA probe		
CDKN1C	CCTGAGAAGTCGTCGGGCGATGTC	60 (35)
	GGTTGCTGCTACATGAACGG	
HMBS	TGCCCTGGAGAAGAATGAAGTG	60 (35)
	CCCCGAATACTCCTGAACTCCA	
RT–PCR		
CDKN1C	CCTGAGAAGTCGTCGGGCGATGTC	65 (LightCycler)
	CCTCGGGGCTCTTTGGGCTCTAAA	
LIT1	CGATACTCTGTGTGCATGTTTGTGGGC	60 (LightCycler)
	CATAAGGTAGGTAAGTTTGTGTCCCTG	
HMBS	TGCCCTGGAGAAGAATGAAGTG	60 (LightCycler)
	CCCCGAATACTCCTGAACTCCA	
Southern probe		
DMR-LIT1	ACTTGAAGGAAAGCAGGCAGGCAGGAT	68 (35)
	CCAACTGGAAGTTTGAGTGGAGTCCTGTTG	
CDKN1C Region2	CCCGAGCTGGCAGCGGCGGGTCCAAGCCTC	68 (35)
	TGCTGGCTAGCTCGCTCGCTCAGGCCTGGC	
COBRA		
DMR-LIT1	GTGTTAIGGIGGTGGAGATTTTGT	55 (40)
	AACCIAAAACACIAACCAATTCTCTA	
CDKN1C		
Region 1	AGTAGGTGTGTGAGGGTTTTAG	63 (40)
	ATCTACAAACIAAAACCTCAAC	
Region 3	GTGGTGTTGTTGAAATTGAAAA	53 (40)
	CCCCCTCIATACCTACTAACTA	
Region 4	GIGTATAAAGGGGGIGTAGGIGGGT	60 (40)
	AAACCIAACICAAAAAAAACCCCIC	
Region 5	GTTTGYGTAGTTTYGGGTTATGTT	53(3), 51(4), 49(5), 47(27)
	ATTCTAATCCTCRACRTTCAACTC	
ChIP		
LIT1 LC1	CCGGTGGTGCTCAGGGACGACG	65 (33)
	ACGGATTGGCTGGGTGCGGAAAGTG	
LIT1 LC2	CCGGGGCTCCTCAGCACGAT	68 (31)
	GGAGAACCGCGCCGAAAAGC	
LIT1 LC3	CAGGGTCGAGGTCCGAGTTC	65 (31)
	CCCAATTCGGGCTTTGACTC	
CDKN1C KC1	GCTGGCAGCGGCGGGTCCAA	65 (31)
	TCCGGCCAGGCCCAACTCGA	
CDKN1C KC2	CGCCGTGGTGTTGTTGAAACTG	62 (31)
	TCGGGTTCGCCTGTCTCGTC	
CDKN1C KC3	CGTCCCTCCGCAGCACATCC	62 (31)
	TCGCGGCTCAGCTCCTCGTG	

I: inosine

Analyses of polymorphism and 11p15.5. copy number

Heterozygosity of 11p15.5 was examined using the following polymorphic loci: the microsatellites *D11S1997*, *D11S2362*, and *D11S4088*, and intragenic polymorphisms in *ORCTL2S*, *KCNQ1DN*, *CDKN1C*, *LIT1*, *tyrosine hydroxylase*, *IGF2*, and *H19* (primer sequences and PCR conditions are available on request to corresponding author). The copy number of 11p15.5 was calculated by band intensity obtained from Southern blotting. Genomic DNA (2 μg) from cancer cell lines was digested with *Bam*HI. To make the standard curve, 1, 2, 3, and 4 μg of normal genomic DNAs were also digested. The blot was probed as described previously (Mitsuya *et al.*, 1999), then the intensity of each cell line was plotted to the standard curve to determine the copy number.

ChIP

We used four kinds of antibody – antiacetylated histone H3, antiacetylated histone H4, antidimethylated histone H3K4 (Upstate Biotechnology), and monoclonal antidimethylated histone H3K9 (Nakagawachi *et al.*, 2003). Chromatin was prepared from 1.0×10^6 cells and sonicated to an average size of ~0.5 kb, then immunoprecipitated with each antibody. Protein A- and G-sepharose beads were used to collect the immunoprecipitated complex with antibodies of H3Ac, H4Ac, and H3meK4 and with monoclonal anti-H3meK9, respectively. DNA recovered from immunoprecipitated complex was subjected to PCR. To ensure quantification of PCR amplification, each reaction was initially set up at various amplification cycle numbers, and the logarithmic amplification phase was detected. As final PCR conditions, we determined 31 cycles for all regions, except for 33 cycles at LC1 region. PCR products were quantified with NIH Image 1.62, and fold enrichment in each immunoprecipitation was determined by the ratio to input DNA (total chromatin). *GAPD* and the centromere region of chromosome 16 were amplified as described previously (Higashimoto *et al.*, 2003). ChIP and PCR for each ChIP were performed at least in duplicate, respectively.

Primers and PCR conditions

Primer sequences and PCR conditions are shown in Table 2.

Statistics

Statistical analyses were performed with Spearman's rank-correlation test. $P < 0.05$ was thought to be significant.

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Gene silencing in DNA damage repair

H. Soejima, K. Joh and T. Mukai*

Division of Molecular Biology and Genetics, Department of Biomolecular Sciences, Saga University Faculty of Medicine, 5-1-1 Nabeshima, Saga 849-8501 (Japan), e-mail: mukait@post.saga-med.ac.jp

Abstract. Silencing of DNA repair genes plays a critical role in the development of the cancer because these genes, functioning normally, would prevent the accumulation of mutations leading to carcinogenesis. Epigenetic gene silencing is an alternative mechanism to genetic gene aberration, inactivating those genes in cancer. DNA methylation and histone modification are the major factors for epigenetic regulation of gene expression. Here, we describe recent advances in understanding of epigenetic silencing of DNA repair genes and their epigenetic mechanisms involving DNA methylation and histone modification.

Key words. DNA repair; cancer; DNA methylation; histone modification; heterochromatin.

Introduction

Epigenetics is defined as a heritable change in gene expression that does not involve changes in DNA sequences. Epigenetic regulation of gene expression plays a critical role in development and differentiation, X inactivation, genomic imprinting and several human diseases, including cancer [1]. Recent studies have focused on two molecular mechanisms for epigenetic gene expression, DNA methylation and histone modification. Methylation of cytosine in CpG dinucleotides is the major modification in mammals. The CpG dinucleotides are mainly localized in the CpG islands, which are short stretches of GC-rich sequences associated with promoter regions in approximately half of human genes. These islands are normally unmethylated; however, some of them are aberrantly hypermethylated in cancer cells, leading to gene silencing by transcriptional repression. On the other hand, the DNA of all eukaryotes is packed into chromatin consisting of a repetitive fundamental unit, the nucleosome, which contains a highly conserved histone octamer wrapped twice with 147 bp of DNA. Chromatin structures are divided into two types of domains, euchromatin and heterochromatin, which are generally characterized by the modification state of the histone amino termini. Euchromatin is accessible to DNA-binding factors and is transcriptionally active. In contrast, heterochromatin is inaccessible and transcriptionally inactive. The dynamic change of histone modifications is associated with alteration of chromatin structure and epigenetic gene expression [2]. Here, we review recent studies of epigenetic control of gene expression, especially focusing on DNA repair genes, their role in carcinogenesis and the silencing mechanism involving DNA methylation and histone modification in cancer.

* Corresponding author.

Epigenetic gene silencing in cancer

It is clear that gene mutations, deletions or genomic rearrangements can cause cancer. These genetic aberrations lose the function of tumor suppressor genes. Recent studies have revealed that a number of those genes are inactivated by aberrant promoter hypermethylation in human cancer and that these genetic aberrations and epigenetic promoter hypermethylation are intricately connected in cancer development from early to late stages. Promoter hypermethylation can have an effect similar to genetic aberration of the gene, leading to a loss of gene function [3–5]. For example, both mutations and promoter hypermethylation of the *VHL* gene occur in renal cancer. Several tumor suppressors, often hypermethylated in cancer, are mapped to chromosomal regions characterized by frequent loss of heterozygosity (LOH). Furthermore, in breast cancer, both genetic and epigenetic aberrations of *BRCA1* produce a similar microarray pattern of gene ex-

pression [4, 5]. Thus, epigenetic gene silencing associated with aberrant promoter hypermethylation is an alternative mechanism to genetic aberration to inactivate tumor suppressor genes. Knudson's two-hit hypothesis may account for the full inactivation of a tumor suppressor gene [6], and this is usually achieved by mutation in one allele and LOH in another allele or homozygous deletion. Recent studies have shown that aberrant promoter hypermethylation of one allele with coordination of mutation or LOH in another allele, or methylation of both alleles, would cause the same effect of two hits, leading to functional inactivation of the gene in cancer [3, 5].

Silencing of DNA repair genes

There are several DNA mismatch repair (MMR) genes – such as *MLH1, MSH2, MSH3, MSH6, PMS1* and *PMS2* – and they form complexes to recognize mismatched pairs and to repair them. Mutations in *MLH1, MSH2, PMS1* and *PMS2* have been found in human hereditary nonpolyposis colorectal cancer (HNPCC) [7]. Of these genes, *MLH1* and another DNA repair gene, *MGMT*, draw special attention with regard to aberrant promoter hypermethylation in cancer. *MLH1* is frequently methylated in sporadic colorectal, gastric and endometrial cancers with microsatellite instability [8, 9]. As a consequence of loss of MLH1 function, mutation would accumulate in the genome. *MGMT*, encoding O^6-methylguanine (O^6-mG)-DNA methyltransferase, prevents G:C to A:T transition mutation by repairing the alkylated base, O^6-mG, and is frequently methylated in a wide spectrum of cancers [9]. Loss of MGMT function also causes mutations leading to tumor induction. In fact, a correlation was found between *MGMT* methylation and transition mutations of *K-ras* and *p53* in various cancers [10, 11]. Strikingly, 71% of non-CpG transition mutations were associated with *MGMT* aberrant methylation [11]. *MLH1* and *MGMT* also frequently exhibit promoter hypermethylation in precancerous tissues [12–14], indicating that inactivation of DNA repair genes seems to be the initial event in tumor development. In other words, DNA repair systems are critically important anti-carcinogenic mechanisms, preventing genetic changes leading to malignant transformation of cells. However, in the situation where the system is overloaded and genetic abnormalities are accumulated in a cell, apoptosis can function as another anti-carcinogenic mechanism to remove precancerous cells (fig. 1) [15]. This model is supported by the results of mice deficient in *Mgmt* and *Mlh1* [16]. *Mgmt*$^{-/-}$ *Mlh1*$^{+/+}$ mice were killed by apoptotic response when even low doses of alkylating agents were administrated. In contrast, *Mgmt*$^{-/-}$, *Mlh1*$^{-/-}$ (double knockout) mice were resistant to high doses of the chemicals, although a high incidence of cancers was observed.

It is of interest to note the expression level of both *MGMT* and *MLH1* genes in human cancer. We found that 20 and 60% of hepatocellular carcinoma lacked *MLH1* and *MGMT* expression, respectively. Ten percent of tumors lacked both expressions, correlating with an advanced pTNM stage [17, 18]. Lack of MGMT alone is a poor prognostic factor in several tumors – such as lymphoma, hepatocellular, biliary tract, gastric and breast cancers – but not in colorectal cancer [19–21]. Furthermore, lack

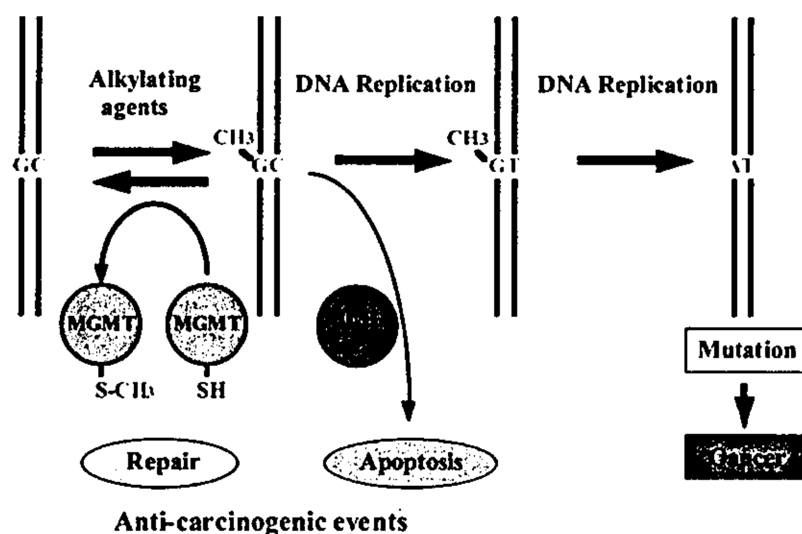

Figure 1. A model for anti-carcinogenic mechanisms. Alkylating agents produce alkylated bases such as O^6-methylguanine in DNA. This methylated guanine pairs with TTP as well as dCTP and leads to GC-to-AT transition mutation through two cycles of DNA replication. Accumulation of such mutations can cause malignant transformation of cells. This process can be prevented by two anti-carcinogenic mechanisms. MGMT protein repairs O^6-methylguanine to prevent the mutation. Cells carrying the mutagenic bases are eliminated through apoptosis, in which a protein complex, composed of MLH1 and some other mismatch repair proteins, is involved.

of both MGMT and MLH1 is much more correlated with a poor prognosis in hepatocellular and biliary tract cancers [18, 21]. On the other hand, glioma with hypermethylated *MGMT* was more sensitive to alkylating agents, suggesting *MGMT* hypermethylation as a good predictive marker for chemotherapy [22].

Epigenetic silencing mechanism: DNA methylation and histone modification

Promoter hypermethylation is associated with gene silencing, as mentioned above. There are three biologically active DNA methyltransferases (DNMTs) in mammals, DNMT1, DNMT3a and DNMT3b. DNMT1 mainly works on maintenance of methylation, whereas DNMT3a and DNMT3b work on de novo methylation. Mice targeted with each DNMT show that each protein is critical for embryonic development. $Apc^{Min/+}$ *Dnmt1*-hypomorphic mice show complete suppression of intestinal polyp formation, accompanied by reduction in frequency of CpG island methylation [23]. However, methylation of CpG islands, including $p16^{INK4a}$, is maintained in human colon cancer cells lacking *DNMT1*, though overall genomic methylation is reduced by 20% [24]. Disruption of both *DNMT1* and *DNMT3b* shows drastic reduction of genomic DNA methylation, resulting in abrogation of silencing of the $p16^{INK4a}$ gene and growth suppression, in human colon cancer cells [25].

The histone amino termini are susceptible to a variety of covalent modifications, such as acetylation, methylation, phosphorylation and ubiquitination. The most prominent among these are acetylation and methylation of lysine residues, which are modified by histone acetyltransferase (HAT) and histone methyltransferase (HMT), respectively. These play a pivotal role in chromatin assembly and epigenetic gene regulation. Acetylation of histone H3 and H4 is associated with euchromatin with increased accessibility to transcriptional factors. Methylation of histone H3 lysine 4 (H3K4) is also associated with euchromatin and correlated with transcriptional activity. In contrast, histone H3 lysine 9 (H3K9) methylation is associated with the formation of stably silenced heterochromatin [2, 26, 27]. It has been found that heterochromatin protein-1 α (HP1α) is important in establishing heterochromatin. It binds to methylated H3K9 residue and interacts with histone methyltransferases to recruit them to sites of methylated H3K9, leading to propagation of heterochromatin [2, 27].

The silencing process via DNA methylation was initially thought to be simply due to physical interference between transcription factors and their binding sequences [28]. However, it seems that DNA methylation does not lead to gene-silencing by itself. Recruitment of proteins to methylated DNA is required for the formation of heterochromatin to silence genes. Recent studies of methyl-CpG binding proteins (MBDs) have reported a gene silencing mechanism based on CpG methylation. MBDs also function as transcriptional repressors in vitro. MBDs bind to CpG nucleotides in a methylation-dependent manner and interact with a corepressor complex containing histone deacetylases (HDACs), resulting in highly deacetylated nucleosomes. A recent report showed that MBD1 interacts with histone methyltransferase, Suv39h1, and binds to HDACs via Suv39h1 [29]. MeCP2 is also associated with methyltransferase activity on H3K9 in vivo, though the identity of the H3K9 methyltransferase is unknown [30]. Thus, interaction of those proteins results in heterochromatin containing tightly compacted, highly deacetylated and highly H3K9-methylated nucleosomes (fig. 2). On the other hand, when genes with a CpG island promoter are transcriptionally active, chromatin configuration changes to a euchromatic state, in which histones are acetylated and methylated at H3K4 and the nucleosomes are sparsely and irregularly spaced, leading to high accessibility of transcriptional factors [2, 4, 5, 31].

In cancer cell lines, in which either *MGMT* or *MLH1* is silenced, the promoter region of each gene shows hypermethylation of CpG and H3K9, hypomethylation of H3K4, and hypoacetylation of H3 and H4, resulting in inactive heterochromatin [32–34]. Recently, we actually revealed the precise methylation status at each of 97 CpG sites in the *MGMT* promoter CpG island and the histone modification status in both *MGMT*-negative and -positive cancer cell lines. In addition, we also showed that MeCP2 rather than MBD1 tends to bind to methylated *MGMT* promoter [32]. The result suggests a silencing mechanism of *MGMT*, in which MeCP2 binds to CpG-methylated promoter followed by recruitment of HDAC(s) and H3K9 methyltransferase, resulting in heterochromatin. For both *MGMT* and *MLH1* genes, HDAC inhibitor, TSA, increases histone acetylation in highly DNA-methylated promoters, with little effect on DNA methylation, gene expression and H3K9 methylation. However, a DNA demethylation agent, 5Aza-dC, substantially reactivates gene expression with the same degree of increased histone acetylation and considerably decreased H3K9 methylation [33, 34, Zhao W. et al., unpublished observations]. Thus, derepression of the gene correlates with DNA demethylation and decreased H3K9 methylation rather than with increased acetylations. 5Aza-dC treatment of colorectal cancer RKO cells, in which *MLH1* is silenced with hypermethylation of DNA and H3K9, hypomethylation of H3K4 and hypoacetylation of H3 at the promoter region, leads first to promoter demethylation, second to gene derepression and finally to complete reversal of histone modifications [33]. This result suggests that DNA hypermethylation is the dominant epigenetic mechanism, rather than the repressive histone modifications, in main-

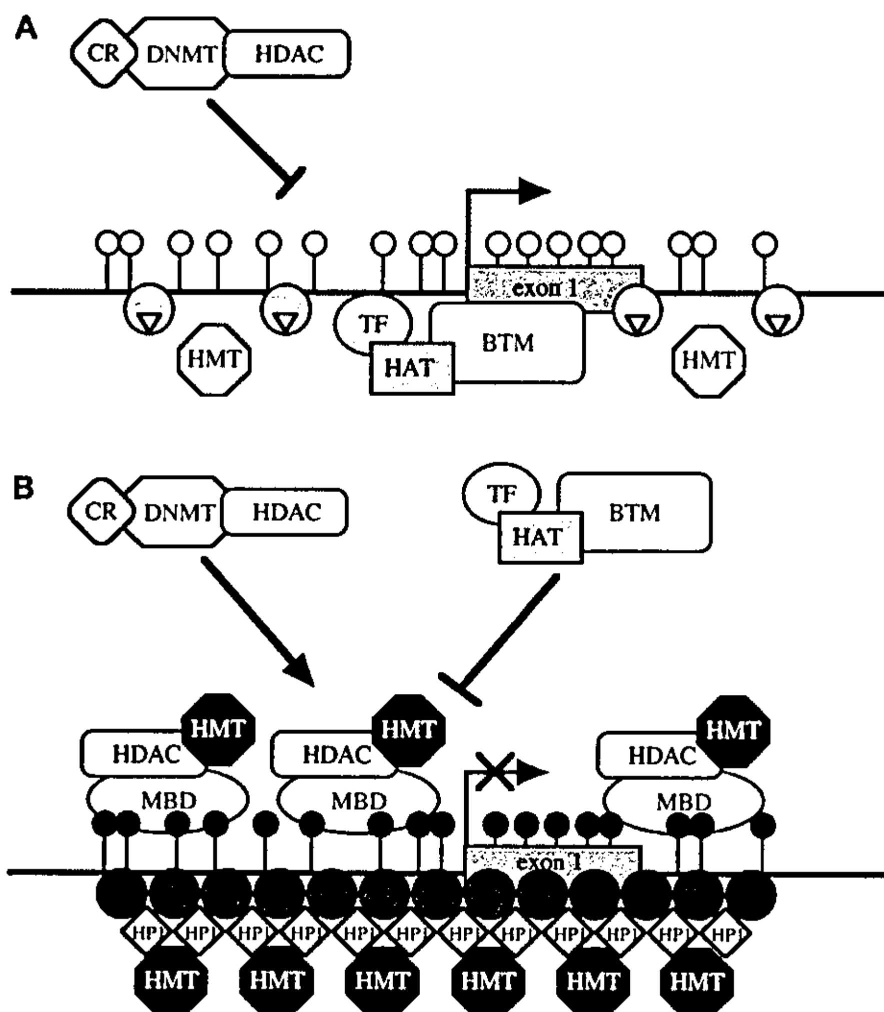

Figure 2. Aspects of the chromatin at promoter with CpG island. (*a*) Transcriptionally active euchromatin. CpGs are not methylated. The nucleosomes are distributed sparsely and irregularly, with histones acetylated and methylated at H3K4. Thus, transcriptional factors are accessible to their target sequences. (*b*) Transcriptionally inactive heterochromatin. CpGs are heavily methylated and bound by MBD protein(s), forming a complex with HDAC(s) and HMT. Histones are highly deacetylated and highly methylated at H3K9, and then the nucleosomes are tightly compacted. Finally, transcriptional factors are inaccessible to the targets, leading to gene silencing. Otherwise, DNMTs directly interact with HDACs and transcriptional corepressor. It is unknown whether all these phenomena simultaneously occur at the same locus. As for *MGMT* silencing, MeCP2 rather than MBD1 binds to methylated DNA. The identity of HMT is still unknown, and binding of HP1 is not confirmed. White lollipop, unmethylated CpG; black lollipop, methylated CpG; blue circle, acetylated histone; red circle, deacetylated histone; white triangle, methylated H3K4; black triangle, methylated H3K9; CR, transcriptional corepressor; TF, transcription factor; BTM, basic transcriptional machinery.

taining silencing of the *MLH1* gene in colorectal cancer. However, recent studies have revealed that DNMTs directly interact with HDACs and transcriptional corepressor, suggesting a contribution to heterochromatin by a mechanism other than DNA methylation [5]. In *Neurospora crassa* and *Arabidopsis thaliana*, the presence of H3K9 methylation is essential for all or a subset of DNA methylation [34, 35], though it is not clear how the H3K9 methylation directs DNA methylation. The mechanism may be more intricate in mammalian cells. Further studies must be conducted to understand the relationship between DNA methylation and histone modifications in establishment and maintenance of gene silencing.

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Epigenetic inactivation of class II transactivator (CIITA) is associated with the absence of interferon-γ-induced HLA-DR expression in colorectal and gastric cancer cells

Ayumi Satoh[1], Minoru Toyota*[,1,2,3], Hideyuki Ikeda[4], Yoshikazu Morimoto[1], Kimishige Akino[1], Hiroaki Mita[2], Hiromu Suzuki[1], Yasushi Sasaki[2], Takayuki Kanaseki[4], Yukio Takamura[4], Hidenobu Soejima[5], Takeshi Urano[6], Kazuyoshi Yanagihara[7], Takao Endo[1], Yuji Hinoda[8], Masahiro Fujita[9], Masao Hosokawa[9], Noriyuki Sato[4], Takashi Tokino[2] and Kohzoh Imai[1]

[1]*First Department of Internal Medicine, Sapporo Medical University, Sapporo, Japan;* [2]*Department of Molecular Biology, Cancer Research Institute, Sapporo Medical University, Sapporo 060-8543, Japan;* [3]*PRESTO JST, Kawaguchi 332-0012, Japan;* [4]*Department of Pathology, Sapporo Medical University, Sapporo 060-8556, Japan;* [5]*Division of Molecular Biology and Genetics, Saga Medical School, Saga 849-8501, Japan;* [6]*Department of Biochemistry II, Nagoya University School of Medicine, Nagoya 466-0065, Japan;* [7]*Central Animal Laboratory, National Cancer Center Research Institute, Tokyo 104-0045, Japan;* [8]*Department of Clinical Laboratory Science, Yamaguchi University School of Medicine, Yamaguchi 755-8505, Japan;* [9]*Keiyukai Sapporo Hospital, Sapporo 003-0027, Japan*

Tightly regulated at the level of transcription, expression of MHC class II molecules varies significantly among gastrointestinal cancers. High levels of MHC class II expression are often associated with a better prognosis, which is indicative of the involvement of CD4⁺ lymphocytes in tumor suppression, but the molecular mechanism by which MHC class II expression is regulated remains unclear. In the present study, we investigated the expression of one inducible MHC class II molecule, HLA-DR, and its coactivators in a panel of colorectal and gastric cancer cell lines. Interferon-γ induced expression of HLA-DR in 14 of 20 cell lines tested; the remaining six cell lines did not express HLA-DR. Analysis of the expression of transcription factors and coactivators associated with HLA-DR revealed that the loss of CIITA expression was closely associated with the absence of HLA-DR induction. Moreover, DNA methylation of the 5′ CpG island of CIITA-PIV was detected in all cancer cells that lacked CIITA. The methylation and resultant silencing of CIITA-PIV depended on the activities of two DNA methyltransferases, DNMT1 and DNMT3B, and their genetic inactivation restored CIITA-PIV expression. It thus appears that CIITA methylation is a key mechanism that enables some gastrointestinal cancer cells to escape immune surveillance.
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*Correspondence: M Toyota, First Department of Internal Medicine, Sapporo Medical University, South 1, West 16, Chuo-ku, Sapporo 060-8543, Japan; E-mail: mtoyota@sapmed.ac.jp
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Introduction

Gastrointestinal cancer is one of the most common of human neoplasias (Neugut *et al.*, 1996; Landis *et al.*, 1999). It is noteworthy, in that regard, that because the strength of immune responses against colorectal and gastric cancers varies among patients, in many cases tumor cells to able to escape immune surveillance (Kaklamanis and Hill, 1992). In part, this reflects the cancer cells' loss of MHC molecules (Garrido and Algarra, 2001). Much attention has been paid to the role of CD8⁺ lymphocytes in antitumor immunity, as these cells are able to lyse tumor cells directly upon recognition of peptide-MHC class I complexes expressed by the tumor. However, recent evidence suggests that CD4⁺ lymphocytes also play an important role in tumor rejection (Toes *et al.*, 1999; Ossendorp *et al.*, 2000). In fact, expression of MHC class II molecules is now known to be associated with a better prognosis in colorectal cancer (Lovig *et al.*, 2002). Expression of MHC class II is induced by interferon (IFN)-γ and is tightly regulated at the level of transcription by several RFX family genes (RFX-5, RFX-AP, RFX-B and RFX-ANK) and the transactivator CIITA (Ting and Trowsdale, 2002). Little is known about the molecular mechanisms underlying the differential expression of MHC class II molecules in gastrointestinal cancers, however.

CIITA is a non-DNA binding coactivator of MHC class II molecules. Four isoforms of CIITA are transcribed from four different first exons (CIITA-PI to CIITA-PIV): promoters (P) I and III are involved in constitutive expression of CIITA in dendritic cells and B lymphocytes (Muhlethaler-Mottet *et al.*, 1997); PII has been described only in human cells, though its biological function remains unknown; and promoter PIV is involved in IFN-γ inducible gene expression and

phosphorylation of STAT1 (Muhlethaler-Mottet *et al.*, 1997).

Epigenetic mechanisms contribute to the regulation of gene expression by modifying the structure of the chromatin (Jones and Baylin, 2002). A variety of genes involved in cell cycle regulation, apoptosis and cell signaling are reportedly inactivated by DNA methylation in human cancers (Baylin *et al.*, 2001; Santini *et al.*, 2001), but little is known about the epigenetic regulation of genes involved in immune responses. In the present study, therefore, we used a panel of colorectal and gastric cancer cell lines to examine the expression of one inducible MHC class II molecule, HLA-DR, and its association with the expression of the transactivators RFX-5, RFX-AP and CIITA. We found that the absence of HLA-DR induction by IFN-γ was closely associated with the silencing of CIITA-PIV expression caused by DNA methylation in the region around the transcription start site, and with deacetylation of histone and methylation of histone H3 lysine 9. We believe these findings provide a clue to the mechanism by which colorectal and gastric cancer cells escape immune surveillance.

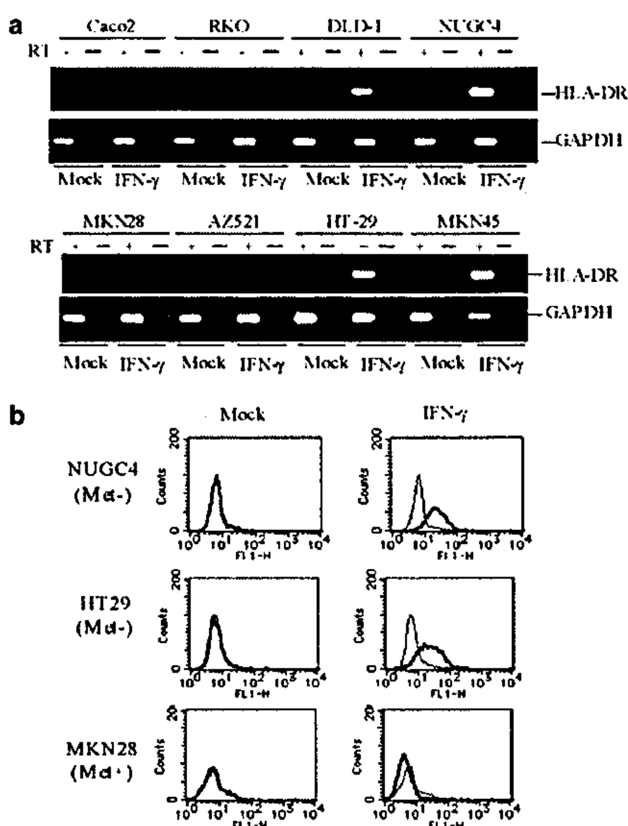

Figure 1 Expression of HLA-DR in colorectal and gastric cancer cell lines. (**a**) RT–PCR analyses of HLA-DR expression. cDNAs were prepared from cells treated with either IFN-γ or mock. Integrity of the mRNA was confirmed by amplifying GAPDH. Corresponding negative controls (amplification without reverse transcription) are shown as RT-negative. (**b**) Representative cell surface expression of HLA-DR protein in gastric cancer cell lines. Cell lines were treated for 48 h with either mock or 100 U/ml IFN-γ, after which HLA-DR expression was examined by flow cytometry

Results

IFN-γ-induced HLA-DR expression

We examined the expression of HLA-DR in colorectal and gastric cancer cell lines using RT-PCR with cDNA prepared from 20 cancer cell lines that had been incubated for 48 h with 100 U/ml IFN-γ (Figure 1a and Table 1). Whereas none of the cell lines except KatoIII expressed HLA-DR before treatment with IFN-γ, afterward HLA-DR was readily detectable in 14 cell lines (SW48, DLD-1, LoVo, HT29, Colo320, HSC39, HSC40, HSC44, HSC45, MKN7, MKN45, KatoIII, NUGC3, NUGC4); the remaining six (Caco2, RKO, HCT116, MKN28, MKN74 and AZ521) continued to show no expression at all. That cell surface expression of HLA-DR protein was well correlated with expression of HLA-DR mRNA (Figure 1b) indicates that the expression of this molecule is controlled at the level of its transcription. Both differentiated and undifferentiated adenocarcinoma cell lines tended to show an absence of HLA-DR expression; among the former only 3/7 (43%) expressed HLA-DR, while among the latter 2/5 (40%) did so. Although this was a somewhat higher frequency than was seen with the signet-ring cell type (0/6, 0%), no statistically significant difference was found in either case (Fisher's exact test, two-sided; $P = 0.25$ and 0.46).

Analysis of the STAT1 signaling pathway in colorectal and gastric cancer cells

STAT1 signaling is reportedly altered in a subset of gastric cancer cells (Abril *et al.*, 1998). We therefore explored the possibility that impairment of the signaling pathway leading to STAT1 activation is associated with the loss of HLA-DR from gastrointestinal cancer cells by evaluating STAT1 phosphorylation in several cell lines. After treatment with IFN-γ, the cells were labeled with antiphospho-STAT1 antibody (Figure 2a, b). The majority of cells showed strong nuclear accumulation of the antibody, regardless of whether or not HLA-DR was expressed. We then examined expression of STAT1 in both HLA-DR-positive and HLA-DR-negative cell lines (Figure 2c) and found STAT1 protein to be expressed in all cell lines tested. Finally, we examined the expression of IRF-1, a downstream target gene of STAT1 in both HLA-DR-positive and HLA-DR-negative cell lines (Figure 2d) and detected its expression in all cell lines examined. Apparently, impaired STAT1 activation was not responsible for the loss of HLA-DR induction by IFN-γ.

Expression of transcription factors and coactivators of MHC class II

To determine the extent to which loss of HLA-DR expression was related to a change in the gene's transcription, we next examined the expression of RFX5, RFXAP and CIITA, three transcription factors

Table 1 Expression of HLA-DR mRNA and methylation of CIITA-PIV in colorectal and gastric cancer cell lines

Cell lines	*Type*	*HLA-DR*		*CIITA-PIV*		*Methylation*
		Mock	*IFN-γ*	*Mock*	*IFN-γ*	*CIITA-PIV*
Colorectal						
Caco2	Differentiated	None	None	None	None	Methylated
RKO	Pooly differentiated	None	None	None	None	Methylated
SW48	Differentiated	None	Positive	None	Positive	Unmethylated
HCT116	Pooly differentiated	None	None	None	None	Methylated
DLD1	Pooly differentiated	None	Positive	None	Positive	Unmethylated
LoVo	Differentiated	None	Positive	None	Positive	Unmethylated
HT29	Differentiated	None	Positive	None	Positive	Unmethylated
Colo320	neural	None	Positive	None	Positive	Unmethylated
Gastric						
HSC39	signet-ring cell	None	Positive	None	Positive	Unmethylated
HSC40	signet-ring cell	None	Positive	None	Positive	Unmethylated
HSC41	signet-ring cell	ND	ND	ND	ND	Unmethylated
HSC42	Differentiated	ND	ND	ND	ND	Methylated
HSC43	signet-ring cell	ND	ND	ND	ND	Unmethylated
HSC44	signet-ring cell	None	Positive	None	Positive	Unmethylated
HSC45	signet-ring cell	None	Positive	None	Positive	Unmethylated
MKN7	Differentiated	None	Positive	None	Positive	Unmethylated
MKN28	Differentiated	None	None	None	None	Methylated
MKN45	Pooly differentiated	None	Positive	None	Positive	Unmethylated
MKN74	Differentiated	None	None	None	None	Methylated
KatoIII	signet-ring cell	Positive	Positive	Positive	Positive	Unmethylated
AZ521	Uncertain	None	None	None	None	Methylated
NUGC3	Pooly differentiated	None	Positive	None	Positive	Unmethylated
NUGC4	signet-ring cell	None	Positive	None	Positive	Unmethylated

Positive: expression positive, None: no expression, ND: not determined

known to be involved in regulating IFN-γ-induced HLA-DR expression (Figure 3a, b). Expression of both RFX and RFXAP was readily detectable in all cell lines tested. Moreover, when the expression levels of three CIITA isoforms were separately examined using primers that specifically amplified each isoform (Figure 3a), we found that expression of CIITA-PI and PIII was always very weak or absent (data not shown), but that expression of CIITA-PIV was well correlated with HLA-DR expression. To confirm the extent to which CIITA-PIV expression affects HLA-DR expression, we introduced CIITA-PIV into RKO cells and found that the exogenous molecule restored expression of HLA-DR, even in the absence of IFN-γ (Figure 3c and d). CIITA-PIV thus appears to be a critical regulator of HLA-DR expression.

CpG island methylation of CIITA-PIV

Using Blast (http://www.ncbi.nlm.nih.gov/BLAST/) and CpG island Searcher (http://www.uscnorris.com/cpgislands/), we found that the 5′ region of CIITA-PIV contains a CpG island that spans 500 bp (CpG:GpC = 0.65, GC% = 55%) (Figure 4a). Then using four sets of primers, we carried out a combined bisulfite restriction analysis (COBRA) of the entire CpG island of CIITA-PIV. Although methylation of the edges of the CpG island (regions 1 and 4) was detected in virtually all cell lines tested, methylation of the region around exon 1 of CIITA-PIV (regions 2 and 3) was detected only in cell lines where IFN-γ-induced expression of CIITA-PIV mRNA was absent (Figure 4b). Furthermore, bisulfite-sequencing confirmed that in cell lines methylated in regions 2 and 3, virtually all available CpG sites were methylated (Figure 5). Methylation of CIITA-PIV was also detected in five of 32 (16%) primary gastric cancers, indicating that this epigenetic change is not a cell line-specific phenomenon (Figure 4c).

When we correlated CIITA-PIV expression with the methylation pattern determined by COBRA, we found that whereas methylation of regions 1 and 4 had no effect on gene expression, there was a strong correlation between methylation of regions 2 and 3 and expression (Figure 6). Indeed, IFN-γ induced expression of CIITA in all cell lines that lacked methylation in regions 2 and 3, but in none of the cell lines methylated in those regions.

Role of histone modification in the silencing of CIITA gene expression

To determine the histone acetylation status among the methylated CIITA genes, we carried out chromatin immunoprecipitation (ChIP) assays in eight cancer cell lines, with or without CIITA methylation, using primers that amplify selected regions of the CIITA-PIV CpG island (Figure 7a–c). Little acetylation of histone H3 was noted among the cell lines treated with mock. After treatment with IFN-γ, however, histone acetylation was induced in HT29 cells, but remarkably suppressed in other six cell lines that showed CIITA-PIV methylation.

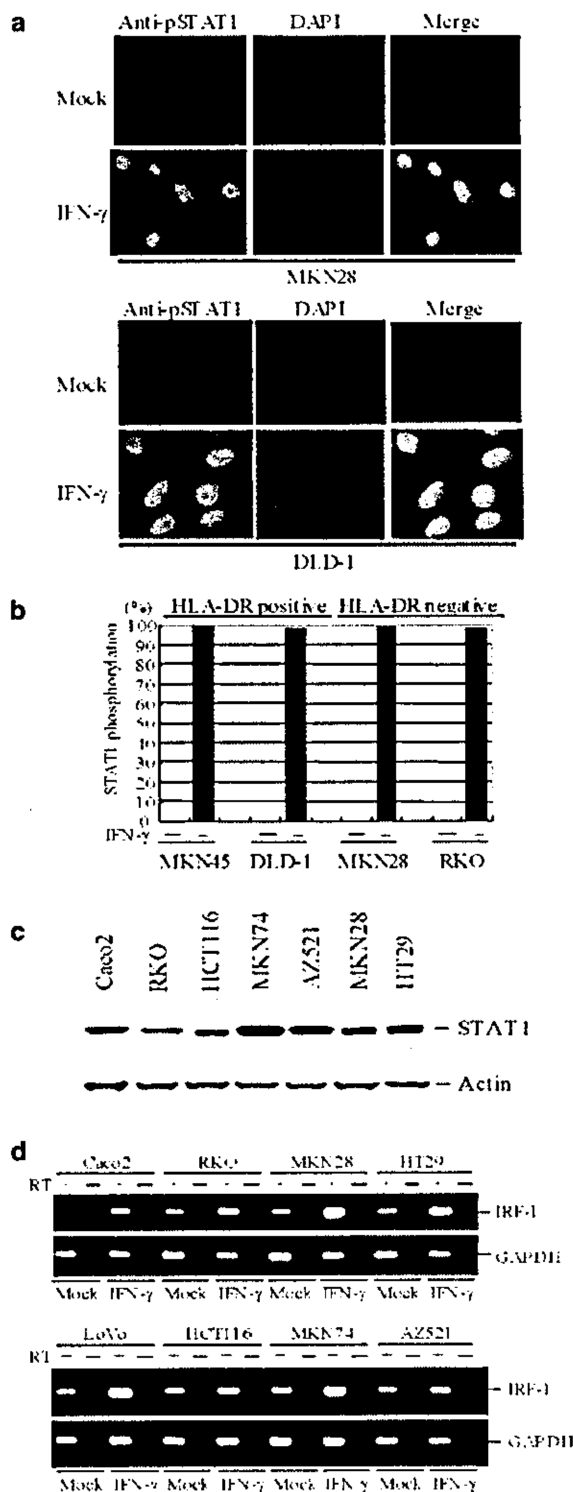

Figure 2 IFN-γ signaling pathway in colorectal and gastric cancer cell lines. (**a**) IFN-γ-induced phosphorylation of STAT1. Colorectal and gastric cancer cell lines were treated for 15 min with IFN-γ, after which STAT1 phosphorylation was examined immunohistochemically using antiphospho-STAT1 antibody. The nucleus was visualized by staining with DAPI. (**b**) Percentages of cells positive for phospho-STAT1 were determined by fluorescence microscopy. (**c**) Western blot analysis of STAT1 in colorectal and gastric cancer cell lines. (**d**) RT-PCR analysis of IRF-1 expression in colorectal and gastric cancer cells; cDNA were prepared from cells treated for 48 h with either mock or 100 U/ml IFN-γ

When ChIP assays were carried out using anti-acetyl histone H3 antiserum and anti-dimethyl histone H3 lysine 9 (H3K9) antibody, acetylation of histone H3 was readily detectable after treatment with IFN-γ in HT29 and MKN45 cells, which also express CIITA-PIV (Figure 7c, d). By contrast, acetylation of histone H3 remained low in MKN28 cells, which do not express CIITA-PIV. Because methylation of histone H3K9 was recently shown to be correlated with gene silencing (Fahrner *et al.*, 2002; Nguyen *et al.*, 2002; Kondo *et al.*, 2003), we also carried out ChIP analysis using anti-dimethyl-histone H3K9 antibody and found that the level of histone methylation is higher in MKN28 cells than in MKN45 or HT29 cells. After treatment with IFN-γ, histone methylation remained high in MKN28 cells, but declined in MKN45 and HT29 cells. In sum, the overall acetylation of histone H3 was directly related to CIITA-PIV gene expression, while methylation of histone H3K9 was inversely related to the expression.

Role of DNA methyltransferase in silencing CIITA-PIV gene expression

The role of DNA methyltransferases in silencing CIITA-PIV gene expression was evaluated by using COBRA to examine the methylation status of the 5′ region of CIITA-PIV in DNMT1−/−, DNMT3B−/− and DNMT1−/−DNMT3b−/− (DKO) cells, which were established from the HCT116 colorectal cancer cell line (Rhee *et al.*, 2002). Only slight demethylation was observed in DNMT1−/− cells, and no demethylation at all was seen in DNMT3b−/− cells (Figure 8a). On the other hand, significant demethylation was observed in DKO cells, indicating that disruption of DNMT1 and DNMT3B leads to demethylation of CIITA-PIV (Figure 8a). Bisulfite-sequencing confirmed that significant demethylation of the entire CpG island occurred only in DKO cells (Figure 8b). Moreover, this demethylation led to restoration of expression of two genes, HPP1/TPEF and COL1A2, which had previously been shown to be silenced in colorectal cancer (Figure 8c), as had p16INK4A, TIMP3 and CHFR (Liang *et al.*, 2000; Rhee *et al.*, 2002; Sengupta *et al.*, 2003; Toyota *et al.*, 2003). Still, CIITA-PIV gene expression was induced only after IFN-γ was added (Figure 8c), which indicates that, even in the absence of CpG methylation, transcription factor stimulation is necessary for CIITA-PIV gene expression.

The fact that HLA-DR was induced when IFN-γ was added to DKO cells is indicative of the key role played by epigenetic control of CIITA-PIV in the expression of HLA-DR. That role was confirmed by our finding that expression of CIITA-PIV and HLA-DR in two colorectal cancer cell lines was restored only when cells were treated with both 5-aza-dC, a methyltransferase inhibitor, and IFN-γ (Figure 8d).

Finally, we examined the extent to which methylation of CIITA-PIV is correlated with absence of HLA-DR expression in 10 primary gastric cancers. Immunohistochemical staining showed that of the three gastric cancers with CIITA methylation none expressed

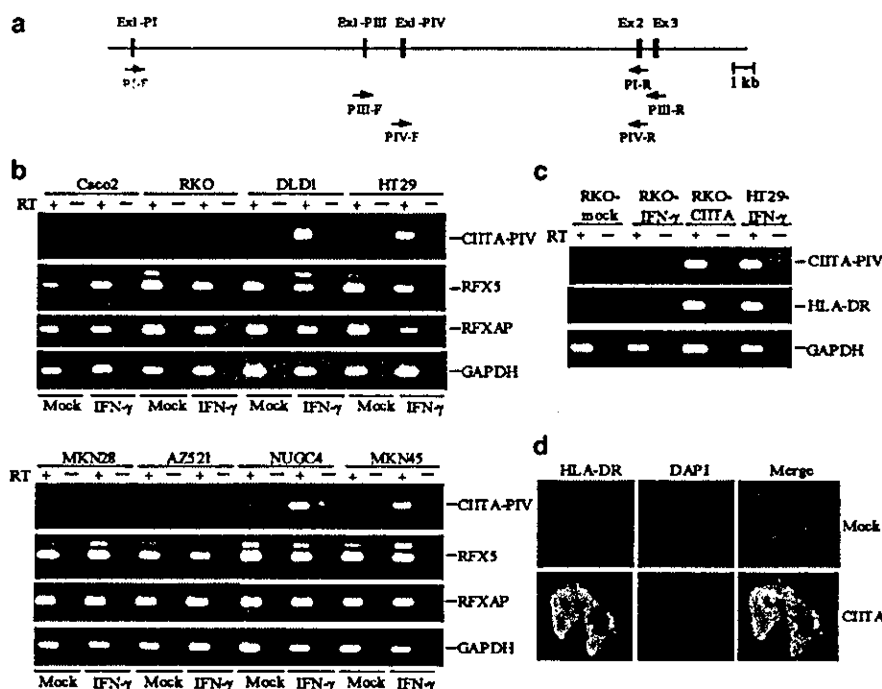

Figure 3 Expression of HLA-DR is associated with expression of CIITA-PIV in colorectal and gastric cancer cells. (a) Schematic representation of the structure of the CIITA gene. Three sets of primers (PI-F/R, PIII-F/R and PIV-F/R) were used to separately amplify the three CIITA isoforms. The location of the primers are indicated by the arrow. (b) Expression of CIITA-PIV, RFX5 and RFXAP in colorectal and gastric cancer cells. cDNA were prepared from cells treated with either IFN-γ or mock; the integrity of mRNA was determined by amplifying GAPDH. The cell lines used are shown on the top. (c) Induction of HLA-DR by exogenous CIITA-PVI: RT–PCR analysis of CIITA-PIV and HLA-DR mRNA expression. (d) Immunofluorescence images showing expression of HLA-DR protein with (CIITA) and without (mock) introduction of exogenous CIITA-PVI. RKO cells, which do not express CIITA, were transfected with CIITA-PIV, after which expression of HLA-DR protein was analysed by immunofluorescence microscopy. The nuclei were stained with DAPI

HLA-DR (Figure 9). By contrast, of the seven gastric cancers without CIITA methylation, three strongly expressed HLA-DR, two weakly expressed it and two showed no expression. Thus, methylation of CIITA appears also to play a role in the absence of HLA-DR in primary tumors.

Discussion

For unknown reasons, gastrointestinal cancer cells often do not express MHC class II molecules (e.g., HLA-DR), which are associated with T-cell-mediated antitumor immune responses (McDougall *et al.*, 1990; Lovig *et al.*, 2002). In that regard, the prognoses of colorectal cancers strongly correlate with the infiltration of CD4+ lymphocytes, which recognize tumor antigen presented with MHC class II molecules (Andersen *et al.*, 1993; Lovig *et al.*, 2002), so that colorectal cancers that lack HLA-DR have worse prognoses than those that do (Lovig *et al.*, 2002). For these reasons, understanding the molecular mechanisms regulating expression of MHC class II molecules may represent an important step toward developing efficient immunotherapies with which to treat gastrointestinal cancers. Indeed, several reports suggest that forced expression of MHC in cancer cells leads to loss of tumorigenicity (Armstrong *et al.*, 1997, 1998). In the present study, we found that aberrant methylation of CIITA-PIV silences IFN-γ-induced HLA-DR expression in colorectal and gastric cancer cells. Conversely, Meazza *et al.* reported that introduction of CIITA leads to upregulation HLA-DR expression and T-cell activation in mammary adenocarcinomas, which in turn leads to rejection of the tumor cells (Meazza *et al.*, 2003). Obviously, further study will be necessary to clarify whether *in vivo* epigenetic restoration of CIITA induces activation of immune surveillance, but the fact that the loss of HLA-DR is caused by epigenetic inactivation of CIITA suggests restoration of CIITA expression may be a useful therapeutic target. Furthermore, the fact that methylation of CIITA occurs less frequently than the loss of HLA-DR indicates that other mechanisms (e.g., altered histone modification) also contribute to the silencing of MHC class II molecules.

Expression of HLA-DR is controlled by mediators downstream of IFN-γ, including JAK/STAT and transactivators such as CIITA, RFX5, RFXAP and NF-Y (Ting and Trowsdale, 2002). The absence of MHC class II could thus be the result of a variety of defects in the transcription machinery. For instance, genetic defects in CIITA and RFX cause severe immunodeficiency called bare lymphocyte syndrome (BLS); impairment of STAT1 has been observed in a subset of gastric cancers (Abril *et al.*, 1998); and IRF-1

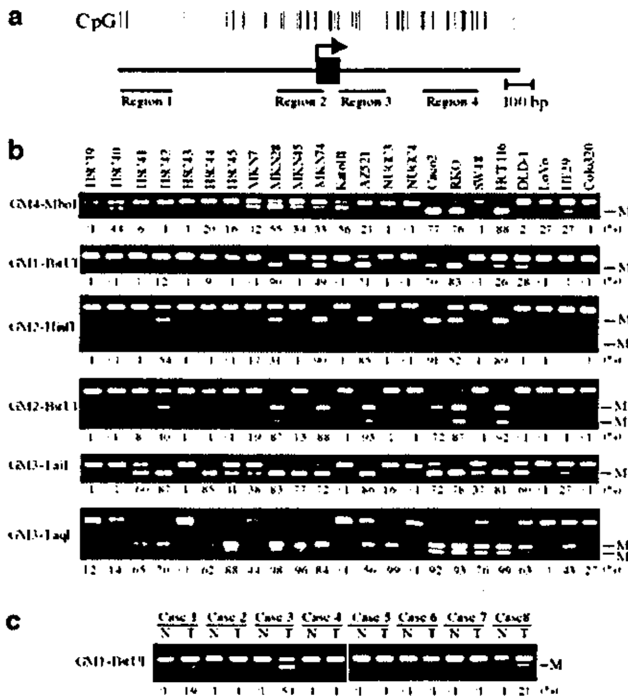

Figure 4 Analysis of CIITA-PIV methylation. (a) The 5′ CpG island of CIITA-PIV. CpG sites are shown as vertical bars; exon 1 is shown as a solid box; the regions analysed are shown by the horizontal lines. (b) COBRA of CIITA-PIV in a panel of colorectal and gastric cancer cell lines. Methylation of the four indicated regions in the CIITA-PIV CpG island was examined using bisulfite-PCR with the appropriate primers. The regions and restriction enzymes used are shown on the left. The degrees of methylation were calculated by densitometry and are shown as percentages below the gels; M, methylated alleles. (c) Methylation of CIITA-PIV in primary gastric cancers. Methylation of region 2 was examined by COBRA

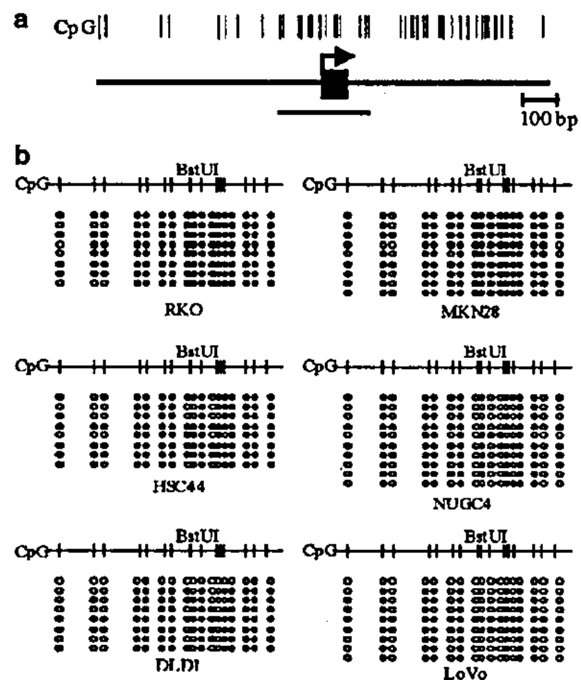

Figure 5 Bisulfite-sequencing of the 5′ region of CIITA-PIV. (a) The region analysed is shown by the horizontal bar. (b) Schematic representation of the methylation of CpG sites. PCR products were cloned into pCR4 using a TOPO-TA cloning kit; at least eight clones were sequenced for each cell line. Open and closed circles represent unmethylated and methylated CpG dinucleotides, respectively. The cell lines examined are shown below. CpG sites and a BstUI site used for COBRA are shown on the top

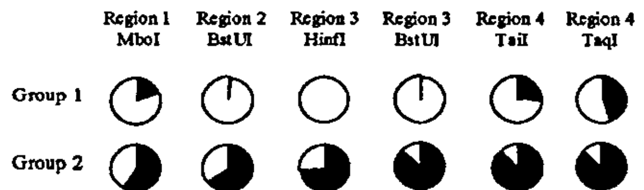

Figure 6 Correlation between methylation of the CIITA-PIV CpG island and induction by IFN-γ. The percentages of methylated alleles were determined by bisulfite-PCR as in Figure 4; the average of the methylated alleles at each site is shown in black within the circles. The 14 cell lines belonging to Group 1 (SW48, DLD-1, LoVo, GT29, Colo320, HSC39, HSC40, HSC44, HSC45, MKN7, MKN45, KatoIII, NUGC3, NUGC4) expressed CIITA-PIV after treatment with IFN-γ; the six cell lines belonging to Group 2 (Caco2, RKO, HCT116, MKN28, MKN74, AZ521) did not express the gene

knockout mice do not express CIITA. Likewise, overexpression of c-*myc* and HASH1 represses CIITA expression in neuroblastoma cells (Yazawa *et al.*, 2002). In the present study, however, we confirmed that the inability of IFN-γ to induce HLA-DR expression in some cancers was not the result of defects in either STAT1 activation or IRF-1 expression.

CpG dinucleotides are sparsely distributed in the 5′ region of HLA-DR, and so do not satisfy the criteria for a CpG island. Consequently, methylation of HLA-DR may not be the cause of the gene silencing. In fact, we found that introduction of exogenous CIITA into cells that did not express the molecule endogenously induced expression of HLA-DR, even in the absence of IFN-γ (Figure 3b, c). This confirms that the HLA-DR promoter is functional, and that the loss of HLA-DR expression is not due to a general defect in the IFN-γ signaling pathway. It also shows that suppression of CIITA is exclusively responsible for the loss of HLA-DR expression in colorectal and gastric cancer cells.

Expression of CIITA is controlled by four distinct promoters that differ only in their N-termini (Muhlethaler-Mottet *et al.*, 1997). PI and PIII play a role in constitutive expression in B- and dendritic cells (Lennon *et al.*, 1997; Muhlethaler-Mottet *et al.*, 1997), while PIV is activated by IFN-γ in various cell types, including human endothelial cells and fibroblasts (Muhlethaler-Mottet *et al.*, 1997) and murine astrocytes (Waldburger *et al.*, 2001). In the current study, we found that colorectal and gastric cancer cells respond to IFN-γ, but that CIITA-PIV is expressed only if the promoter region is not methylated.

The silencing of CIITA-PIV gene expression by DNA methylation has also been observed in cell lines derived from trophoblastic and developmental tumors (Morris *et al.*, 2000; van den Elsen *et al.*, 2001). On the other

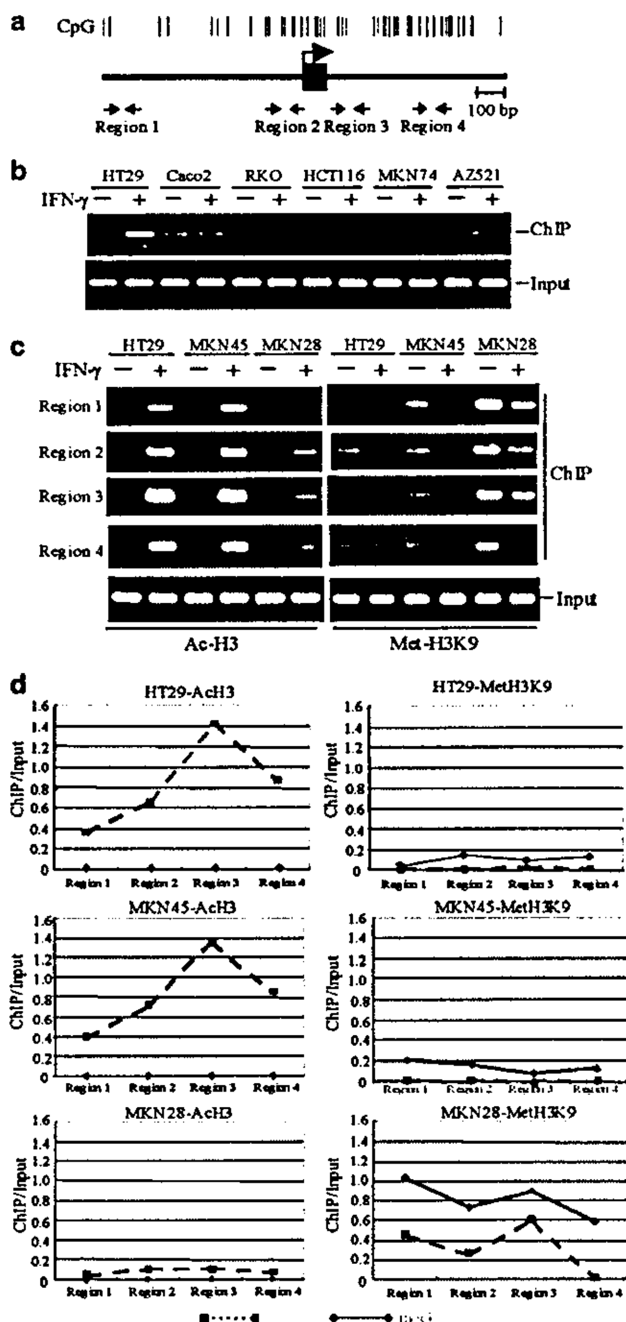

Figure 7 The role of histone modification in silencing CIITA gene expression. (**a**) The regions examined in ChIP assays: the locations of the primers are indicated by the arrows. (**b**) Acetylation of histone H3 in cancer cell lines with and without expression of CIITA. ChIP assays were carried out using DNA prepared from cells treated with mock (−) or IFN-γ (+). The cell lines used are shown on the top. As an internal control, input DNA was used as a template in some cases. (**c**) Acetylation and methylation status of histone H3 in the 5′ region of CIITA-PIV. ChIP-PCR was carried out using four sets of primers that covered the 5′ region of CIITA-PIV. The cells were treated with IFN-γ (+) or mock (−), after which the DNA and chromatin were cross-linked using formaldehyde. The antibodies used for ChIP analysis are shown below the column; the cell lines used are shown at the top. (**d**) Quantitative analysis of histone H3 acetylation and histone H3K9 methylation. Solid lines show the signal intensity ratio between ChIP-PCR and the CIITA-PIV input. The cell lines and antibodies used for ChIP analysis are shown above

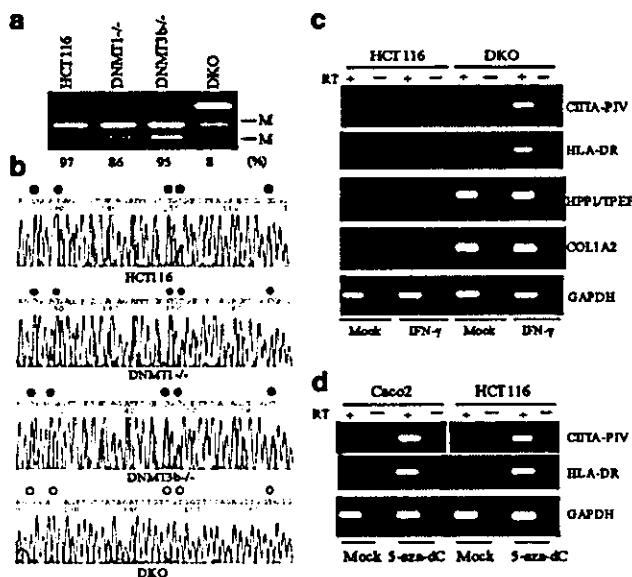

Figure 8 The role of DNMT1 and DNMT3B in the silencing of CIITA-PIV gene expression. (**a**) Analysis of CIITA methylation in DNMT1−/−, DNMT3B−/− and DNMT1−/−DNMT3B−/− (DKO) cells. The methylation status of CIITA was examined using primers CIITAGM1 and CIITAseqR. PCR products were digested with BstUI and then electrophoresed; M, methylated alleles. Percentages of methylated alleles are shown below the column. (**b**) Bisulfite-sequencing of CIITA-PIV. Bisulfite-PCR was carried out using primers GM1F and SeqR, after which direct sequencing was carried out to examine methylation. The cell lines used are shown below; closed circles, methylated cytosines; open circles, unmethylated cytosines. (**c**) Expression of CIITA-PIV, HLA-DR, HPP1/TPEF and COL1A in HCT116 and DKO cells. Expression of CIITA-PIV was examined with (IFN-γ) and without (mock) IFN-γ treatment. (**d**) Effect of 5-aza-dC on expression of CIITA-PIV and HLA-DR. Caco2 and HCT116 cells were treated with mock or 1 μM 5-aza-dC for 96 h and then treated with 100 U of IFN-γ for 48 h

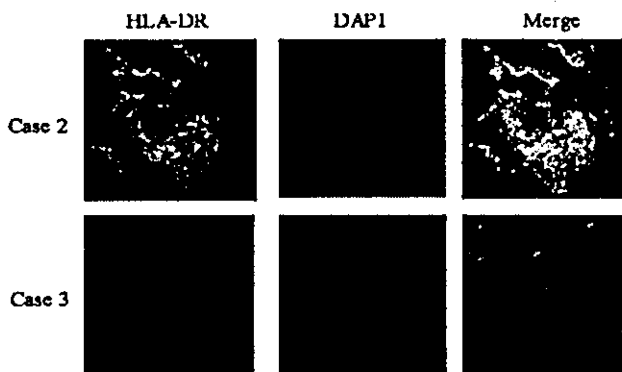

Figure 9 Immunohistochemical analysis of HLA-DR in primary gastric cancers. Representative images show immunofluorescent staining of HLA-DR in primary gastric cancers. Tissue sections were stained with anti-HLA-DR (green); nuclei were stained with DAPI (blue). The cases examined are indicated on the left

hand, the silencing of CIITA-PIV in head and neck cancers, neuroblastoma and small cell lung cancer is apparently caused by other mechanisms (Yazawa *et al.*, 2002; Croce *et al.*, 2003; Kanaseki *et al.*, 2003).

CIITA-PIV has a small CpG island that spans about 500 bp (CpG : GpC = 0.65, GC% = 55%). Until now, analysis of CIITA-PIV methylation has been limited to the region around exon 1; however, we used semiquantitative methylation analysis to examine the entire CIITA-PIV CpG island. We found that the edge of the island was methylated regardless of gene expression, but that the region around the transcription start site of CIITA-PIV was methylated exclusively in cell lines that do not express CIITA. The reason DNA methylation at the edge of CpG island does not affect gene expression is unknown, though the fact that cell lines showing methylation around the transcription start site were also more densely methylated at the edge of CpG island suggests the edge may serve as a trigger for methylation.

How DNA methylation silences cytokine-induced gene expression is also not fully understood. Recently, however, deacetylation of histone was shown to be involved in gene silencing (Jones and Baylin, 2002). To investigate the role of histone modification in the silencing of CIITA-PIV gene expression, we carried out ChIP assays using primers that covered the entire 5′ region of CIITA-PIV. The results showed that the silencing of CIITA-PIV expression in colorectal and gastric cancer cells is closely associated with deacetylation of histone in the 5′ region of the gene. Treatment with IFN-γ induced significant acetylation of histone in unmethylated cell lines but not in methylated ones, which suggests that the structure of the chromatin in the promoter region of methylated genes preferentially recruits histone deacetylase over histone acetyltransferase. In addition, recent reports suggest that histone methylation also contributes to gene silencing (Nguyen *et al.*, 2002; Fahrner *et al.*, 2002; Kondo *et al.*, 2003), and we were able to show for the first time that histone methylation plays a role in silencing CIITA-PIV gene expression. That a small amount of methylation of histone H3K9 was observed before treatment with IFN-γ, even in unmethylated cell lines, suggests histone methylation is regulated by both DNA methylation-dependent and -independent mechanisms. Overall, there appears to be an inverse relationship between histone acetylation and histone methylation.

DNA methylation is regulated by three DNA methyltransferases, DNMT1, DNMT3A and DNMT3B, and DNA methylation is reduced by greater than 95% globally in colorectal cancer cells that lack both DNMT1 and DNMT3B (DKO cells) (Rhee *et al.*, 2002). These changes result in the loss of insulin-like growth factor II (IGF2) imprinting and restoration of p16INK4A, TIMP3 and other cancer-related genes, including CHFR, HPP1/TPEF and COL1A (Liang *et al.*, 2000; Rhee *et al.*, 2002; Sengupta *et al.*, 2003; Toyota *et al.*, 2003). Thus, DNMT1 and DNMT3b play critical roles in the gene silencing associated with gastrointestinal cancer. Consistent with those results, significant demethylation of CIITA-PIV was observed in DKO cells but not in DNMT1−/− or DNMT3B−/− cells, which indicates that both DNMT1 and DNMT3B catalyse methylation of the 5′ CpG island of CIITA-PIV. Although DKO cells showed demethylation, expression of CIITA-PIV was observed only when IFN-γ was added, which means that in addition to an unmethylated promoter, expression of CIITA-PIV also requires stimulation by transcription factors.

In summary, we have shown that epigenetic silencing of CIITA-PIV is responsible for the loss of IFN-γ-induced HLA-DR expression in colorectal and gastric cancer cells. It is plausible that reactivation of CIITA may lead to immune system activation, making epigenetic activation of CIITA-PIV a potential target of immune therapy. Further study should clarify whether restoration of CIITA-PIV expression in cancer cells will contribute to the upregulation of antitumor immune responses.

Materials and methods

Cell lines and specimens

In all, 15 gastric cancer and eight colon cancer cell lines were used for methylation analysis. Of these, eight gastric cancer cell lines (MKN7, MKN28, MKN45, MKN74, KatoIII, AZ521, NUGC3 and NUGC4) and eight colon cancer cell lines (Caco2, RKO, SW48, HCT116, DLD1, LoVo, HT29 and Colo320) were obtained from either the American Type Culture Collection (Manassas, VA, USA) or the Japanese Collection of Research Bioresources (Tokyo, Japan). Seven gastric cancer cell lines (HSC39, HSC40, HSC41, HSC42, HSC43, HSC44 and HSC45) were kindly provided by Dr Yanagihara of the National Cancer Center Research Institute. All cell lines were cultured in appropriate media. In addition, 32 primary gastric cancer specimens and corresponding normal tissues were acquired from the Department of Surgery, Sapporo Keiyukai Hospital after obtaining informed consent from each patient.

For experimentation, cells were treated for 48 h with 100 U/ml of IFN-γ kindly provided by Shionogi Pharmaceutical (Osaka, Japan) and then harvested. The DNA was then extracted using the phenol/chloroform extraction method, while total RNA was extracted using ISOGEN (Nippon Gene, Japan) according to the manufacturer's instructions. DNMT1−/−, DNMT3b−/− and DNMT1−/−DNMT3b−/− (DKO) cells were kindly provided by Dr Kornel E Schuebel of the Johns Hopkins Medical Institutions and were described previously (Rhee *et al.*, 2000, 2002).

Combined bisulfite restriction analysis (COBRA)

COBRA, a semiquantitative analysis of DNA methylation, was carried out as described previously (Xiong and Laird, 1997). Initially, genomic DNA was treated with sodium bisulfite (SIGMA) as described previously (Clark *et al.*, 1994). Briefly, 2 μg of DNA were denatured for 10 min at 37°C in 2 M NaOH, after which 30 μl of 10 mM hydroquinone (Sigma Chemical Co) and 520 μl of 3 M sodium bisulfite (pH 5.0) were added, and the resultant mixture was incubated for 16 h at 50°C. The modified DNA was then purified using a Wizard DNA Purification System (Promega, Madison, WI, USA), after which it was again treated with NaOH and precipitated. Finally, the DNA precipitate was resuspended in 20 μl of TE buffer and stored at −20°C until use.

For COBRA, PCR was performed in a volume of 50 μl containing 1 × PCR buffer (67 mM Tris-HCl, pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 6.7 mM $MgCl_2$, and 10 mM beta-mercaptoethanol)