Expression profile of *LIT1/KCNQ1OT1* and epigenetic status at the KvDMR1 in colorectal cancers

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The human chromosome region 11p15.5 contains a number of maternally and paternally imprinted genes, and the *LIT1/KCNQ1OT1* locus acts as an imprinting center in the proximal domain of 11p15.5. Loss of imprinting (LOI) of *LIT1* and its correlation with methylation status at a differentially methylated region, the KvDMR1, were investigated in 69 colorectal cancer tissue specimens. *LIT1* expression profiles were also examined by RNA-fluorescence *in situ* hybridization in 13 colorectal cancer cell lines. In 69 colorectal cancer tissue specimens, LOI of *LIT1* was observed in nine of the 17 (53%) informative cases. Moreover, LOI of *LIT1* was only observed in tumor samples. In the cell lines, methylation status at the KvDMR1 correlated well with *LIT1* expression profiles. Loss of expression of *LIT1* also correlated with enrichment of H3 lysine 9 (H3-K9) dimethylation and reduction of H3 lysine 4 (H3-K4) dimethylation. Thus, *LIT1* expression appears to be controlled by epigenetic modifications at the KvDMR1, although *CDKN1C* expression, which is considered to be controlled by *LIT1*, was not associated with epigenetic status at the KvDMR1 in some colorectal cancer cell lines. Therefore, these findings suggest that LOI of *LIT1* via epigenetic disruption plays an important role in colorectal carcinogenesis, but it is not necessarily associated with *CDKN1C* expression. (*Cancer Sci* 2006; 97: 1147–1154)

Genomic imprinting is an epigenetic modification that leads to the preferential or exclusive expression of a gene from one of the two parental alleles in somatic cells.[1] The imprint, such as DNA methylation and histone modification, is established as the gene passes through the parental germ line and it is reversible.[2–4] Imprinted genes play important roles in embryonic development as revealed by the highly restricted developmental potential of both androgenotes with two paternal genomes and of either gynogenotes or parthenogenotes with two maternal genomes.[5,6] Abnormal imprinting is also involved in a number of human diseases. In particular, the loss of imprinting (LOI) is one of the most frequent genetic alterations in cancers.[7] LOI is a phenomenon that involves abnormal activation of a normally silent allele. A large amount of evidence suggests that disruption of imprinting mechanisms may play a critical role in the development of cancer.[8,9]

Imprinted genes, of which more than 70 have already been identified, tend to be present as a cluster spreading over a mega base of DNA. The genes in the cluster are regulated under the control of long-range regulatory elements. This notion is corroborated by the fact that the differentially methylated regions (DMR) associated with imprinted clusters play a crucial role in maintenance of the parent-of-origin-specific gene expression pattern, which is called an imprinting control region (ICR).

The cluster on human chromosome 11p15.5 comprises two ICR. The *H19* ICR controls the imprinted gene expression of *H19* and *IGF2*,[10] whereas the KvDMR1 functions by silencing at least eight maternally expressed genes, including *CDKN1C*/*p57*^{KIP2} on the paternal allele.[11–13] An enhancer blocking assay suggests that the KvDMR1 may function as a methylation-sensitive insulator or silencer.[14–16] However, the exact mode of action of the KvDMR1 is still unknown. The KvDMR1 is located in intron 10 of *KCNQ1* and it is normally not methylated on the paternally inherited allele, but is methylated on the maternal allele. In addition, it is unmethylated and also acts as a promoter for a paternally expressed antisense RNA, *LIT1*/*KCNQ1OT1*.[17,18] More than half of all patients with Beckwith–Wiedemann syndrome (BWS) show LOI of the *LIT1* transcript, closely accompanied by a loss of methylation (LOM) of the maternal allele of the KvDMR1.[18] Moreover, LOM of the KvDMR1 strongly correlates with loss of *H3K9* dimethylation in cells derived from BWS patients.[19] We previously reported that *LIT1* LOI is observed with a high frequency in colorectal cancer patients.[20] LOM was observed in adult tumors, including colorectal cancer, although the imprinting status of *LIT1* was not examined in that report.[21] Silencing of *CDKN1C* is well correlated with epigenetic status at the KvDMR1 in BWS and esophageal cancer.[22–24]

Thus, the correlation between *LIT1* LOI and LOM in cancers has not been studied. We herein investigate *LIT1*, *IGF2*, *H19* and *CDKN1C* expression and epigenetic status at the KvDMR1 in colorectal cancer. The data provide strong evidence that LOI of *LIT1* is closely associated with epigenetic status at the KvDMR1 locus in colorectal cancer cells, suggesting that *LIT1* plays an important role in colorectal carcinogenesis.

Materials and Methods

Tissue samples and cell lines. Tumor samples and corresponding adjacent normal tissue specimens were surgically resected from 69 colorectal cancer patients with approval (#329) of the Institution Review Board at the Faculty of Medicine of Tottori University (Tottori, Japan). Tumor lesions and their adjacent non-tumoral tissue regions were removed and stored at −80°C until analysis. A part of each removed specimen was fixed in 10% formalin and embedded in paraffin wax. The sections were stained with hematoxylin–eosin and were examined histopathologically by light microscopy. Thirteen colorectal cancer cell lines were used for the present study. Of these, 10 were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS; DLD-1, HCT-15, CoLo320, SW480, CoLo205, Widr-TC, Caco-2, T84, LoVo, WiDr), two were grown in RPMI-1640 with 10% FBS (CoLo201, TCO), and one was grown in Leibovitz L-15 medium with 10% FBS (SW837). Genomic DNA and total RNA from these samples and cell lines were extracted using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN,

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USA) and RNeasy columns (Qiagen, Tokyo, Japan) according to the manufacturers' instructions.

Assessment of allele-specific expression and semiquantitative reverse transcription–polymerase chain reaction. Total RNA was treated with RNase-free DNase I (Takara, Tokyo, Japan) to remove contaminating DNA. First-strand cDNA synthesis was carried out with oligo-(dT_{15}) primer (Roche Diagnostics, Tokyo, Japan) and Superscript III reverse transcriptase (Invitrogen, Tokyo, Japan). The allelic expression analyses for *LIT1*, *IGF2* and *H19* were carried out as described previously.[20] Semi-quantitative reverse transcription–polymerase chain reaction of *CDKN1C* was carried out twice. The expression of *CDKN1C* was normalized with that of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and the signals were quantified using Scion image software.

Methylation analysis of the KvDMR1. Methylation status at the KvDMR1 locus was examined by Southern hybridization and bisulfite sequencing. For Southern hybridization, genomic DNA of colorectal cancer cell lines (5 μg) was digested with *Bam*HI and *Not*I, and separated on a 0.8% Seakem GTG agarose gel. The DNA was then transferred to Hybond-N^+ filters and hybridized with [γ-^{32}P]dCTP-labeled oligonucleotide probes. Hybridization was carried out overnight at 65°C in 5× saline-sodium phosphate-EDTA buffer (SSPE), 0.5% sodium dodecylsulfate (SDS). The filters were washed with 0.1× saline-sodium citrate buffer (SSC) and 0.1× SDS at 65°C. The probe used for analysis of the KvDMR1 was generated by polymerase chain reaction (PCR). Hybridization signals were quantified using the Scion image software package and the methylation index (MI) was thus determined where MI = intensity of methylated band/(intensity of the unmethylated band + intensity of the methylated band). For bisulfite-PCR and sequence analyses, 1 μg genomic DNA was treated with sodium bisulfite using the CpGenome DNA modification kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. The bisulfite primers were designed to amplify 22 CpG (position 68 119–68 771 of PAC U90095). To sequence the bisulfite-PCR products, fragments were purified and concentrated with a MiniElute Gel Extraction kit (Qiagen) and cloned into the pGEM-T vector using a pGEM-T Easy Vector System I (Promega, Madison, WI, USA). At least 10 independent clones were thus obtained from the colorectal cancer cell lines and they were sequenced using an ABI 3100 automated sequencer (Applied Biosystems, Foster, CA, USA).

Chromatin immunoprecipitation analysis. Polyclonal antibodies recognizing the following antigens were used in the present study: acetylated histone H3 (H3Ac), acetylated histone H4 (H4Ac), dimethylated H3 lysine 4 (H3K4diMe; Upstate Biotechnology, Charlottesville, VA, USA). In addition, we used a monoclonal antibody that recognizes dimethylated histone H3 lysine 9 (H3K9diMe)[25] and a no-antibody control sample was processed along with the others. To cross-link the DNA in chromatin to histones, 1×10^6 cells were incubated for 10 min in 1% formaldehyde at 37°C. After washing with phosphate-buffered saline (PBS) with protease inhibitor (Complete, ethylenediaminetetracetic acid (EDTA)-free; Roche Diagnostics), cells were resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, with Complete). Next the DNA was broken into 200–1000 bp fragments by sonication (UD-201; TOMY, Nerima, Tokyo, Japan). After dilution, samples containing 1×10^4 cells of the resultant solution were used as an internal control for the amount of chromatin (input). The remainder was immunoprecipitated for 16 h at 4°C using each antibody. Next, protein A- or G-agarose was used to collect the immunoprecipitated complexes with antibodies that recognize H3Ac, H4Ac, H3K4diMe or H3K9diMe. DNA in the samples was then purified by phenol–chloroform extraction, precipitated with ethanol, and resuspended in distilled water.

Fluorescence *in situ* hybridization. Fluorescence *in situ* hybridization (FISH) analysis was used to determine the *LIT1* copy number and its expression profile. The PAC probe U90095 consists of a majority of intron sequences on *KCNQ1* and can detect *LIT1* transcripts but not *KCNQ1* transcripts. DNA-FISH was carried out using standard methods. The probes were labeled with digoxigenin-11-UTP by nick translation (Roche Diagnostics). The digoxigenin signal was detected with an antidigoxigenin–rhodamine complex. At least 50 nuclei were analyzed for each cell line. RNA-FISH was carried out with several modifications, as described in a published protocol.[26] The cells were seeded in Laboratory-Tek chamber slides (Nalgene Nunc International, Rochester, NY, USA) and fixed for 20 min at room temperature with 4% paraformaldehyde. After washing with PBS, the cells were permeabilized with 0.1% pepsin in 0.01 M HCl for 10 min. The slides were post-fixed for 5 min at room temperature with 1% paraformaldehyde. They were then dehydrated through an ethanol series (70%, 90%, 100% ethanol) and air-dried at room temperature. The biotin-16-dUTP-labeled probes were dropped onto the slide, covered with parafilm and incubated at 37°C for 15 h in a humidified chamber. After hybridization, the slides were washed and incubated in 4× SSC with 1% BlockAce (Dainippon Pharmaceutical Corporation, Tokyo, Japan) containing 5 μg/mL fluorescein isothiocyanate (FITC)–avidin (Roche Diagnostics) for 1 h at 37°C. They were then washed for 5 min each with 4× SSC, 4× SSC containing 0.05% Triton-X 100, and 4× SSC. The slides were incubated in 4× SSC with 1% BlockAce containing 3 μg/mL biotinylated anti-avidin D (Vector Laboratories, Burlingame, CA, USA) for 1 h at 37°C. After washing, another layer of FITC–avidin was added for amplification. The slides were washed and mounted in antifade solution (1% diazabicyclooctane in glycerol with 10% PBS), which contained 250 ng/mL 4′,6′-diamidino-2-phenylindole and 1 mg/mL *p*-phenylenediamine. At least 100 nuclei were analyzed for each cell line. Images of DNA or RNA signals were captured using a microscope (Nikon, Tokyo, Japan) equipped with a photometric charge coupled device (CCD) camera, processed digitally, and visualized with the Argus system (Hamamatsu Photonics, Shizuoka, Japan).

Results

Loss of imprinting at *LIT1*, *IGF2* and *H19* in colorectal cancer tissues. The cluster of imprinted genes on human chromosome 11p15.5 consists of two domains: *IGF2-H19* and *LIT1-CDKN1C* (Fig. 1).[27] We examined the status of genomic imprinting of the *LIT1*, *IGF2* and *H19* genes in 69 independent colorectal cancers by PCR-restriction fragment length polymorphism (RFLP) analysis (Fig. 2a–c). The allelic expression of the genes in informative, heterozygous cases is shown in Table 1 (17, 20 and 21 cases for *LIT1*, *IGF2* and *H19*, respectively). *LIT1* LOI was observed in nine of the 17 (53%) informative cases, and its LOI was observed in tumor tissues but not in adjacent histologically normal tissues. *IGF2* LOI was observed in 11 of the 20 (55%) informative cases, and all the cases showed LOI in the adjacent normal tissues. In one case, LOI was observed in the normal tissue, but not in the cancer tissue. These data were similar to those in our previous study.[20] There were only two informative cases for both *LIT1* and *IGF2* (cases 41 and 49). One (case 41) showed LOI for both genes and the other (case 49) showed LOI only for *LIT1* (Table 2). We divided colorectal cancer tissues into three differentiation types:

Table 1. Summary of allele-specific expression in 69 colorectal cancers

Gene	Informative (*n*)	Normal		Tumor		Incidence of LOI in tumor
		Imprint	LOI	Imprint	LOI	
LIT1	17	17	0	8	9	9/17 (53%)
IGF2	20	8	12	9	11	11/20 (55%)
H19	21	20	1	19	2	2/21 (9.5%)

LOI, loss of imprinting.

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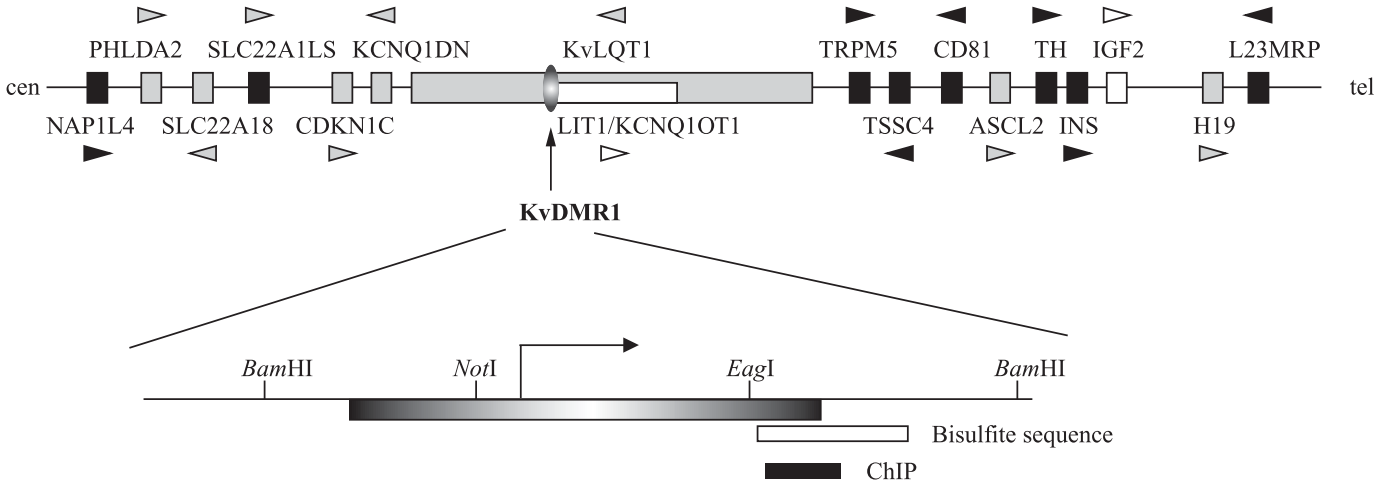

Fig. 1. Schematic representation of the imprinted cluster on human chromosome region 11p15.5. Imprinting status is indicated as follows: paternally expressed genes (white box), maternally expressed genes (gray box), biallelically expressed genes and unknown (black box). Below the map is an enlargement of the KvDMR1 region showing relative positions of sequences analyzed by bisulfite sequencing and chromatin immunoprecipitation. The putative transcription start site for *LIT1/KCNQ1OT1* is indicated by an arrow. The transcriptional direction of each gene is indicated with arrow heads.

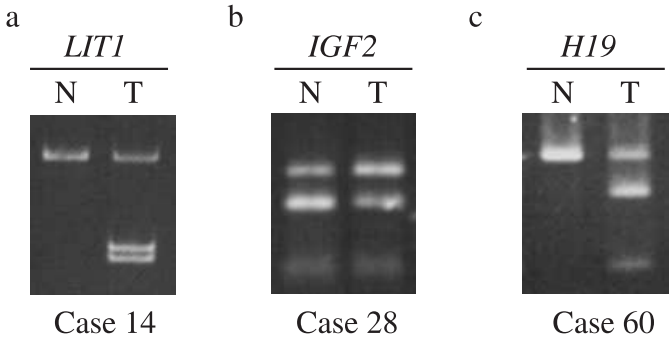

Fig. 2. Allelic expression analysis of *LIT1*, *IGF2* and *H19* in colorectal cancer tissues. Allelic expression of three genes was assessed by restriction fragment length polymorphism analysis, as described previously.[20] Representative results are shown for (a) *LIT1* loss of imprinting (LOI), (b) *IGF2* LOI and (c) *H19* LOI. N and T are normal and tumor tissues, respectively. Each number of samples is shown below the photograph.

poorly differentiated, moderately differentiated and well differentiated (Table 2). Of the nine cases with *LIT1* LOI, moderately differentiated and well differentiated were three (cases 14, 45 and 64) and six (cases 8, 34, 38, 40, 41 and 49) of these cases, respectively. Of the 11 *IGF2* LOI cases, moderately differentiated and well differentiated were six (cases 19, 28, 44, 46, 61 and 67) and four (cases 2, 6, 26 and 41) of these cases. One (case 65) was not tested. Neither *LIT1* LOI nor *IGF2* LOI were observed in the few cases that fell into the poor differentiation category. There was no significant difference between the frequencies of *IGF2* LOI and *LIT1* LOI in tumor differentiation types. No other clinicopathological differences were observed; the estimated percentage of tumor cells (~25–50%) in tumor samples, the numbers of stroma or fibroblasts and infiltrating lymphocytes. Thus, clinicopathological significance and the correlation of *IGF2* and *LIT1* LOI in colorectal carcinogenesis were still unknown. In contrast to *IGF2* and *LIT1*, we observed LOI at *H19* in only two of the 21 (9.5%) cases, and two cases (cases 8 and 60) showed LOI in cancer tissues. In one case (case 9), LOI was observed in the normal tissue, but not in the cancerous tissue.

Methylation status of the KvDMR1 in colorectal cancer. Methylation-sensitive Southern hybridization revealed that the differential methylation pattern at the KvDMR1 region was maintained in all cases (data not shown). This may be due to a high frequency of normal cells in the tumor tissues. In the present study, we showed *LIT1* LOI in 53% by expression analysis in colorectal cancer tissues. This is reasonable because these normal cells do not influence the detection of biallelic expression in cancer tissues as normal cells are monoallelic. Therefore, to clarify that the epigenetic status of the KvDMR1 plays a critical role in *LIT1* expression status, we examined methylation status at the KvDMR1 in 13 colorectal cancer cell lines (DLD-1, HCT-15, CoLo320, SW480, CoLo205, Widr-TC, Caco-2, T84, SW837, CoLo201, LoVo, WiDr, TCO; Figs 1,3a). The 6.0-kb and 4.2-kb bands represent the methylated and unmethylated alleles, respectively. Hypomethylation was observed in four cell lines (SW480, Widr-TC, Caco-2 and SW837) and hypermethylation was observed only in CoLo320. All of the other cell lines maintained normal methylation status. The MI varied from 0 to 100%. To investigate both broadly and in detail methylation status at the KvDMR1, bisulfite sequencing was carried out on three representative cell lines (CoLo320 for hypermethylation, CoLo205 for differential methylation and Widr-TC for hypomethylation). The results were consistent with methylation-sensitive Southern hybridization (Fig. 3b).

Methylation status at the KvDMR1 and *LIT1* expression profiles. The *LIT1* expression profiles were determined by DNA- and RNA-FISH in 13 colorectal cancer cell lines. First, DNA-FISH was used to analyze the copy number of *LIT1* in each cell line. At least 50 nuclei were analyzed for each cell line. Representative results of DNA-FISH are shown and summarized (Figs 4a–c,5). The analysis also revealed that the copy numbers of SW480 and Caco-2 varied more than in other cell lines, suggesting that the karyotypes of these cell lines are more unstable than those of the other cell lines. Next, to determine the expression profiles of *LIT1*, RNA-FISH was conducted. RNA-FISH detects primary transcripts in our assay, as the cells were hybridized under non-denaturing conditions and therefore cellular DNA was inaccessible. The FITC signals were detected and RNA-FISH data are shown and summarized alongside the DNA-FISH results (Figs 4d–f,5). DNA- and RNA-FISH analyses revealed that the number of DNA- or RNA-spots was variable. HCT-15, Widr-TC, SW837 and LoVo showed two DNA spots in the majority of cells. Three or more DNA spots were observed in the other cell lines. All cell

Table 2. Allelic expression profiles of *LIT1*, *IGF2* and *H19* in informative cases

Case no.	State	*LIT1* RNA	*H19* RNA	*IGF2* RNA	Differentiation type	Case no.	State	*LIT1* RNA	*H19* RNA	*IGF2* RNA	Differentiation type
	N			a/b		40	N	b			
2	T			a/b	Well		T	a/b			Well
	N	b				41	N	a		a/b	
4	T	a			Well		T	a/b		a/b	Well
	N			a/b		42	N		b		
6	T			a/b	Well		T		b		Moderately
	N	a	a			43	N		a		
8	T	a/b	a/b		Well		T		a		NT
	N		a/b			44	N			a/b	
9	T		b		Well		T			a/b	Moderately
	N	a	b			45	N	a			
14	T	a/b	b		Moderately		T	a/b			Moderately
	N		b			46	N			a	
15	T		b		Moderately		T			a/b	Moderately
	N	a				47	N		a	a/b	
18	T	a			Moderately		T		a	a	Moderately
	N			a/b		48	N			a	
19	T			a/b	Moderately		T			a	poor
	N	a				49	N	a		a	
21	T	a			Well		T	a/b		a	Well
	N		b			52	N	a			
22	T		b		Well		T	a			Moderately
	N		a	a		54	N		b		
23	T		a	a	Well		T		b		Moderately
	N		b	a/b		55	N	a	a		
26	T		b	a/b	Well		T	a	a		Moderately
	N			a/b		59	N		a		
28	T			a/b	Moderately		T		a		Moderately
	N	a				60	N		a		
29	T	a			NT		T		a/b		Well
	N		a	a		61	N			a/b	
30	T		a	a	Well		T			a/b	Moderately
	N	a				62	N		b		
31	T	a			NT		T		b		Moderately
	N		b			63	N		b		
32	T		b		Well		T		b		Well
	N			a/b		64	N	b			
33	T			a	Well		T	a/b			Moderately
	N	b	a			65	N			a/b	
34	T	a/b	a		Well		T			a/b	NT
	N		b	b		66	N	a			
35	T		b	b	Well		T	a			NT
	N			a		67	N			a/b	
37	T			a	NT		T			a/b	Moderately
	N	a				68	N		b	a	
38	T	a/b			Well		T		b	a	Moderately

N, normal; NT, not tested; T, tumor.

lines were divided into three groups according to their methylation status: hypermethylation, hypomethylation or differential methylation. We constructed a histogram from the FISH analyses based on methylation status. In the hypermethylation group, there was no RNA signal, although there were mainly three copies of *LIT1* (Fig. 5a). These data suggest that *LIT1* expression is repressed by hypermethylation at the KvDMR1. In the hypomethylation group, DNA signals coincided with RNA signals at each spot (Fig. 5b), indicating that *LIT1* was expressed in all alleles, although there was some spot variation. Spots were non-coincidental in the differential methylation group (Fig. 5c), indicating that silenced alleles were present. Thus, these data showed that methylation status at the KvDMR1 correlated well with *LIT1* expression profiles in colorectal cancer cell lines, as was shown in BWS studies.[18]

Histone modification status at the KvDMR1 correlated with *LIT1* expression. To investigate histone modification at the KvDMR1, we carried out a chromatin immunoprecipitation assay (ChIP) followed by PCR with CoLo320, CoLo205 and Widr-TC (cell lines in hypermethylation, differential methylation and hypomethylation, respectively). Of particular interest were modifications of histone H3 and H4 that are characteristic of transcriptionally active chromatin (H3-Ac, H4-Ac and H3K4diMe) and of transcriptionally inactive chromatin (H3K9diMe). We first searched the single nucleotide polymorphism (SNP) to separate the parent-specific allele, but unfortunately we could not find SNP in this region. Next, to compare the enrichment of these histone modifications, the ratios of immunoprecipitated DNA (IP)/input were examined (Fig. 6a). Strikingly, CoLo320 appears to repress *LIT1* expression,

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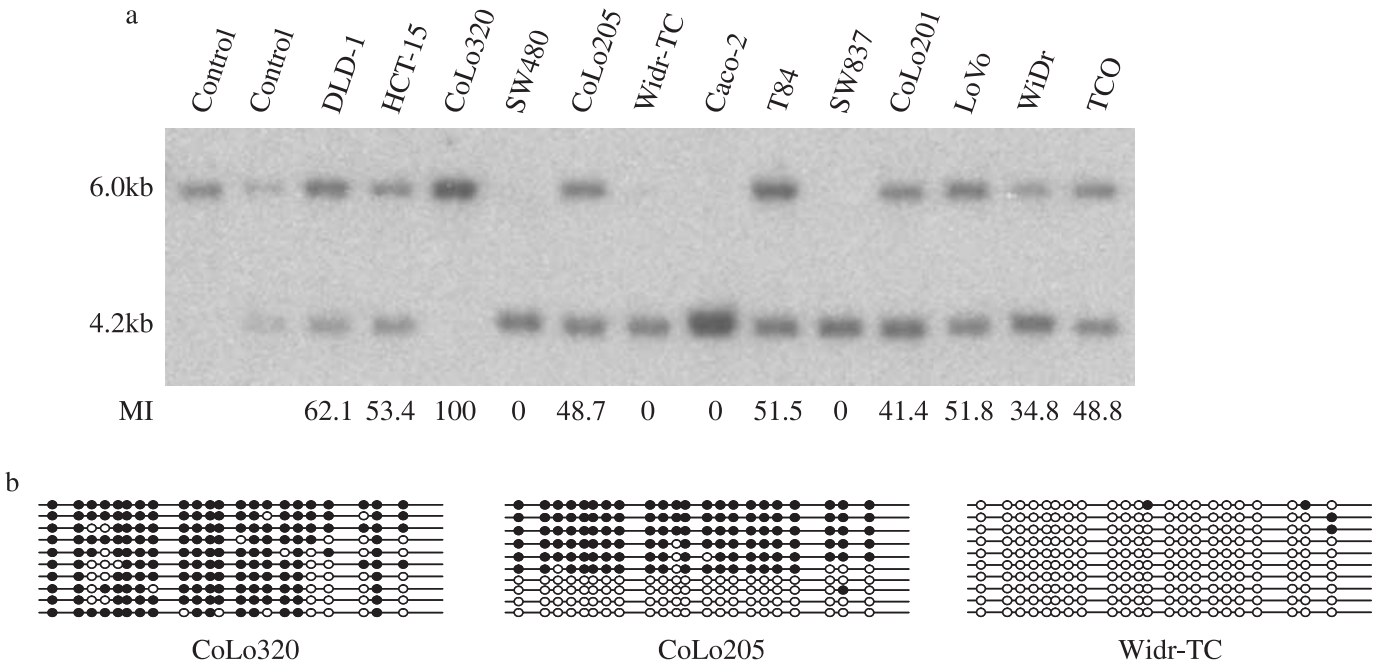

Fig. 3. Analysis of methylation status at the KvDMR1. (a) A 6.0-kb *Bam*HI fragment encompassing the KvDMR1 was digested with *Not*I, resulting in a 4.2 kb fragment. A control (left side) was digested with *Bam*HI alone and only a 6.2-kb fragment was observed. The experimental Southern blot analysis differentiates between methylated (6.0 kb) and unmethylated status (4.2 kb). Densitometry analysis of the bands was calculated using the Scion image software package. The relative ratio of the methylated band was indicated as a methylation index (MI) value. (b) Bisulfite sequencing was carried out on a region containing 22 CpG located 3′ of the second *Eag*I site at the KvDMR1 (see Fig. 1, an open square is indicated for the sequencing region). We analyzed three representative cell lines (CoLo320, CoLo205 and Widr-TC), which contained only the methylated band, both bands and only the unmethylated band, respectively. Each line represents the result for a single cloned DNA molecule. Black circles represent methylated CpG, whereas white circles indicate unmethylated CpG.

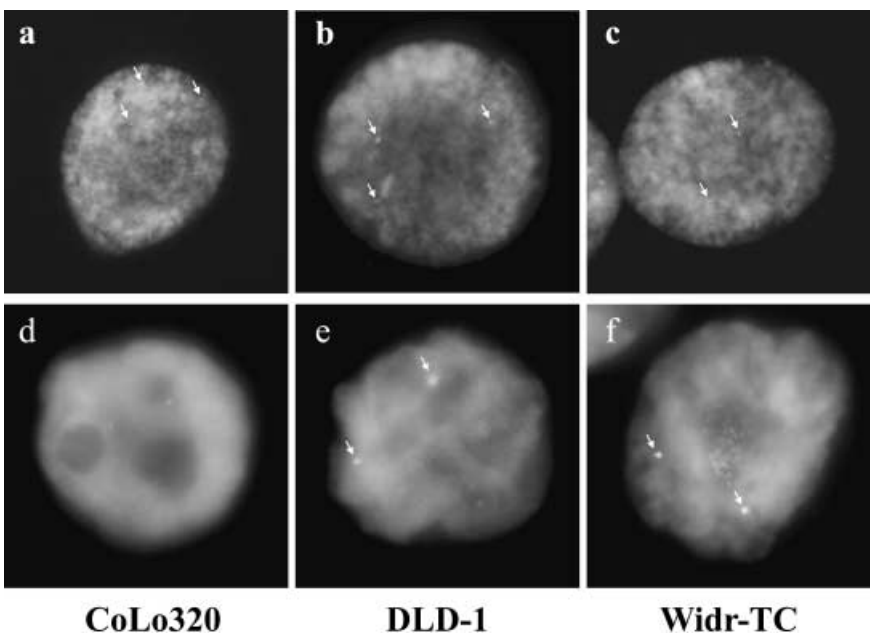

Fig. 4. Fluorescence *in situ* hybridization (FISH) analysis of *LIT1* in 13 colorectal cancer cell lines. DNA- and RNA-FISH were carried out in 13 colorectal cancer cell lines. Shown are photomicrographs of (a–c) DNA-FISH and (d–f) RNA-FISH for the representative cell lines CoLo320, DLD-1 and Widr-TC. Red signals, DNA; green signals, RNA. These signals are indicated with an arrow.

as we observed more robust hypermethylation of the KvDMR1, decreased H3-Ac and H3-K4 dimethylation levels and increased H3-K9 dimethylation in CoLo320 compared with the other two cell lines. Thus, these data suggest that a repressive chromatin structure exists at KvDMR1 in CoLo320, which is consistent with *LIT1* silencing. CoLo205 maintaining *LIT1* imprinting increases H3-Ac and H3-K4 dimethylation levels and heavily decreases H3-K9 dimethylation in CoLo205. Moreover, Widr-TC, as we observed with *LIT1* LOI, showed the greatest increase in transcriptionally active chromatin among the three cell lines and H3-K9 dimethylation was not detectable, consistent with *LIT1* LOI and active chromatin structure. Thus, histone modification was linked to DNA methylation status at the KvDMR1 and the expression profiles of *LIT1*.

Expression of *CDKN1C* in cell lines. To investigate whether *CDKN1C* expression is regulated by the KvDMR1, we examined the correlation between *CDKN1C* expression and methylation status

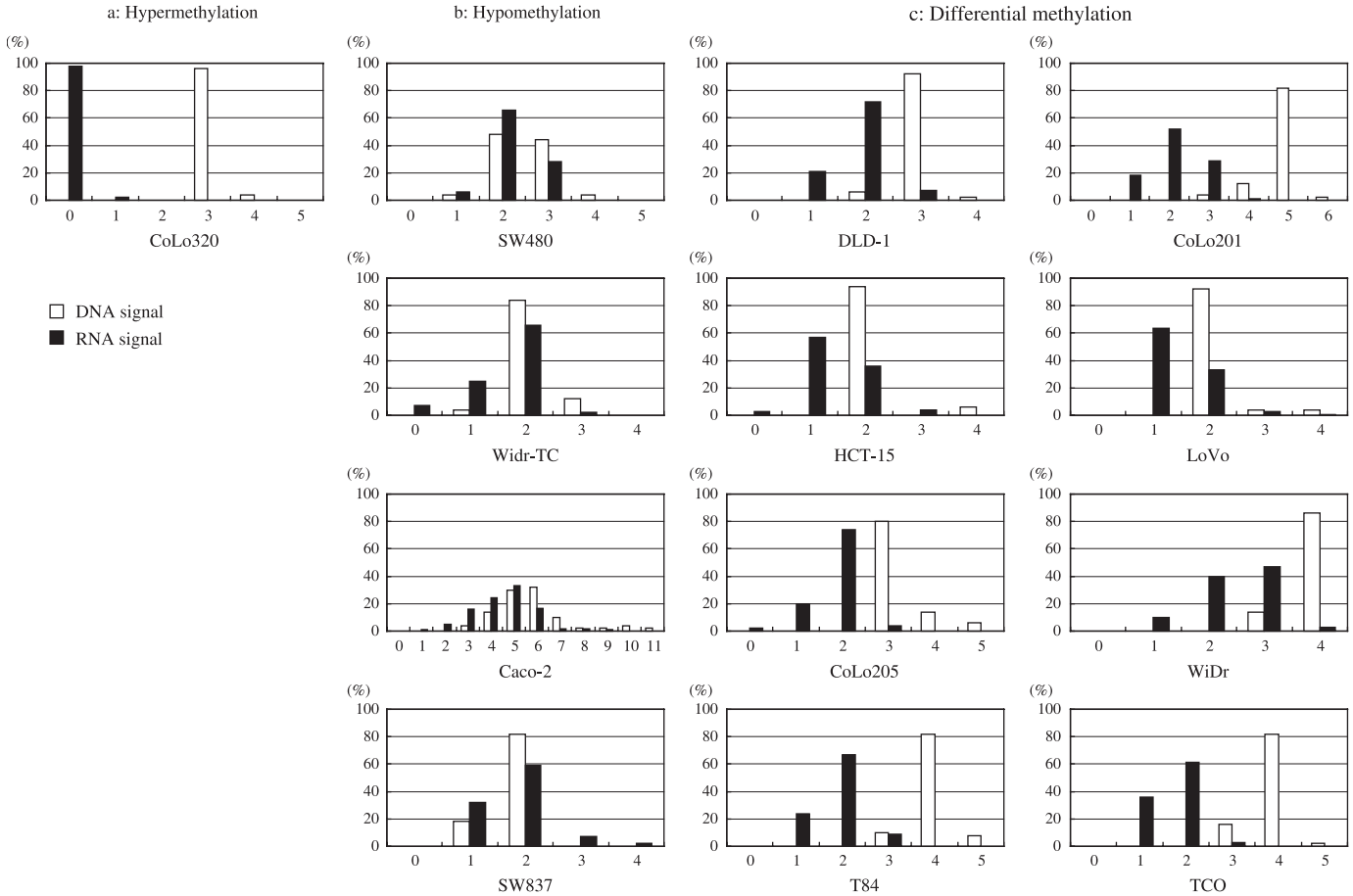

Fig. 5. Correlation between *LIT1* expression and methylation status at the KvDMR1. Histograms of the DNA- and RNA-fluorescence *in situ* hybridization (FISH) analyses were divided according to methylation status. A representative case is shown for each group. (a) Hypermethylation, (b) hypomethylation and (c) differential methylation. (a) No RNA signals were detected in CoLo320, indicating a lack of detectable *LIT1* expression. (b) The RNA and DNA signals were detected in each numbered spot and the DNA and RNA signals coincided at each spot. (c) In the differential group, the peaks of RNA and DNA signals are indicated by numbered spots. White bar, DNA; black bar, RNA.

at the KvDMR1 (Fig. 6b). Statistical analysis was carried out for all cell lines. Four cell lines (SW480, Widr-TC, Caco-2 and SW837) showed differential levels of *CDKN1C* even with hypomethylation at the KvDMR1. However, differentially methylated cell lines showed very different *CDKN1C* expression levels. Thus, there was no correlation between *CDKN1C* and methylation status at the KvDMR1 (correlation factor = 0.02303).

Discussion

The cluster of imprinted genes on human chromosome 11p15.5 consists of two domains: *IGF2-H19* and *LIT1-CDKN1C*.[27] LOI of *IGF2* has been observed in 10% of the lymphocytes from normal individuals.[28] In addition, *IGF2* LOI is a significant risk factor for human colorectal carcinogenesis and is thought to promote tumorigenesis by inhibiting apoptosis.[29] *Igf2* LOI with $Apc^{+/Min}$ mice showed a shift toward less differentiation and an increase in tumor initiation.[30] The present findings showed that *IGF2* and *LIT1* LOI were observed at a high frequency in colorectal cancer. A concurrent and high frequency of *IGF2* LOI was observed in tumor and adjacent normal tissues, indicating that *IGF2* LOI occur at an early stage in cancer development. This idea is consist with a previous report.[30] However, *LIT1* LOI was observed only in tumor tissues, suggesting that *LIT1* LOI takes advantage of cancer progression to activate or inactivate a target sequence. This idea supports a recent study showing global LOI in *Dnmt1* conditional knockout cells.[31] The study concluded that imprinted loci other than *H19* ICR and *Igf2r* are primarily responsible for the altered growth characteristics and transformed phenotype of cells with LOI, although *H19* ICR has been shown to be highly susceptible to *de novo* methylation during cancer progression.[31] Thus, the KvDMR1 may be primarily responsible for the altered growth characteristics and transformed phenotype of cells with LOI, and our data suggest that *LIT1* LOI and LOM at the KvDMR1 may therefore be associated with colorectal cancer tumorigenesis in a manner that differs from what has been proposed for *IGF2* LOI.

KvDMR1 is thought to be an imprinting center at the *LIT1-CDKN1C* domain and has been shown to have a bidirectional 'silencer' or 'insulator' activity.[14–16] A number of studies have shown that LOM of the KvDMR1 is associated with *LIT1* LOI in BWS patients.[17,18] Another report showed that LOM at KvDMR1 was observed in adult tumors.[21] Our results in colorectal cancer cell lines suggest that *LIT1* expression is controlled by epigenetic status at the KvDMR1. A ChIP assay showed that H3-Ac and H3-K4 dimethylation increased and H3-K9 dimethylation decreased, consistent with the *LIT1* expression profile in three cell lines. In particular, H3-K9 dimethylation was heavily decreased in CoLo205, suggesting that H3-K9 dimethylation was correlated strongly with *LIT1* expression. However, H4-Ac of Widr-TC with *LIT1* LOI was increased a little more than in the other two cell lines, suggesting that H4-Ac is less responsible

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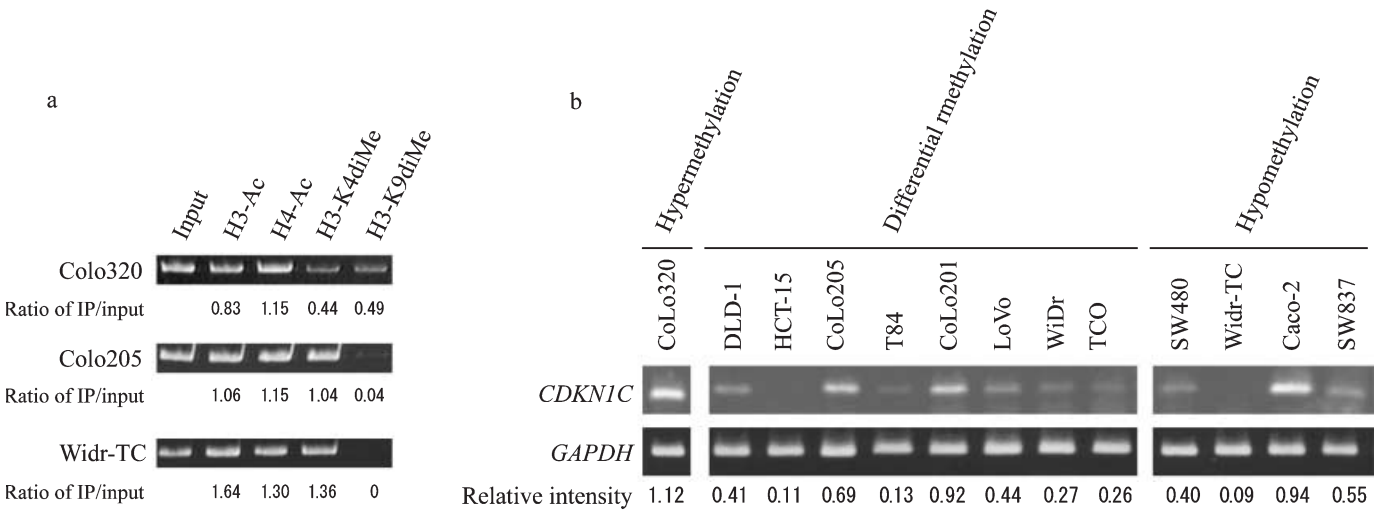

Fig. 6. Histone modification status at the KvDMR1. Representative results from chromatin immunoprecipitation assay polymerase chain reactions of cell lines are shown. CoLo320 showed hypermethylation, CoLo205 showed differential methylation and Widr-TC showed hypomethylation. The ratios of immunoprecipitated DNA (IP)/input are shown. (b) Correlation between *CDKN1C* expression and methylation status at the KvDMR1. *CDKN1C* expression was determined by semiquantitative reverse transcription–polymerase chain reaction intensity relative to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), as relative intensity at the bottom of the figure.

for *LIT1* expression status than other histone modifications. Thus, histone modifications, as well as DNA methylation, are important for the regulation of *LIT1* expression to form active or repressive chromatin structure, similar to esophageal cancer cell lines.[24] The KvDMR1 is also thought to be a *LIT1* promoter. *LIT1* is a non-coding RNA, like *Xist*, *Tsix* and *Air*. The *Xist* gene has been well characterized. Xist RNA transcribed from X inactivation center coats the X-chromosome to inactivate gene expression, which is followed by sequential epigenetic modification.[32,33] Study of the truncated *Air* gene showed deregulation of gene expression in the proximal region.[34] Previous study of an episome-based vector system has pointed to the possibility that the production of *LIT1* RNA plays a critical role in the bidirectional spreading of inactive chromatin structures.[35] A recent study *in vivo* showed that premature termination of the *LIT1* transcript leads to LOI in the proximal region. This indicates that elongation of the *LIT1* transcript is needed for genomic imprinting in neighboring genes.[36] Moreover, an *in vitro* study showed that repressive chromatin-specific histone modifications depend on the length of *LIT1* transcript.[37]

There are at least three silencing mechanisms for *CDKN1C*: (1) DNA hypermethylation at its own promoter region;[38] (2) repressive chromatin structure (histone modifications) at its own promoter;[23] and (3) changes in epigenetic status at the KvDMR1.[24] Soejima *et al*. reported that *CDKN1C* expression was associated with methylation status at the KvDMR1 in 14 of 17 esophageal cancer cell lines but, surprisingly, there was no association in the other three cell lines.[24] In contrast, we found that LOM at the KvDMR1 is not necessarily associated with *CDKN1C* expression in the present study. Other than epigenetic status at the KvDMR1, the repressive chromatin structure mechanism provides a way to explain our observation that *CDKN1C* expression was low. However, the results observed for some cells lines (such as Caco-2 and SW837 of the colorectal cancer lines and the #14 esophageal cancer line) in which hypomethylation at the KvDMR1 and high levels of expression of *CDKN1C* were observed may not be explained with the three proposed regulation mechanisms. Taken together, the results from colorectal and esophageal cancer cell lines suggest that there may be another regulation mechanism of *CDKN1C* expression yet to be defined. Disruption of this hypothetical regulation mechanism caused by chromosome rearrangement, which disrupts regulatory domains including KvDMR1, could explain the aberrant expression of *CDKN1C*.

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