Clinical significance of UNC5B expression in colorectal cancer

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Received June 23, 2011; Accepted August 2, 2011

DOI: 10.3892/ijo.2011.1201

Abstract. The purpose of this study was to find a methylation-related gene that could become a biomarker or therapeutic target in colorectal carcinoma (CRC). We screened candidate genes suspected to be silenced by DNA methylation using cDNA microarray analysis. To investigate the clinical significance of one candidate gene (UNC5B), we analyzed the correlation between mRNA expression and clinicopathological features using clinical tissue samples. Moreover, methylation specific PCR analysis was performed to reveal whether the promoter region was methylated in CRC cell lines. We found 75 candidate genes that were potentially suppressed by DNA methylation in CRC. We focused on UNC5B, a possible tumor suppressor gene and regulator of apoptosis known to be inactivated in CRC. The mRNA expression analysis using tissue samples revealed that UNC5B mRNA was down-expressed in about 20% of CRC patients, and the patients with low-UNC5B-expression tumors showed a significantly higher recurrence rate after curative surgery. According to the univariate and multivariate analysis, low UNC5B expression was an independent risk factor for postoperative recurrence in stage I, II and III CRC patients. Furthermore, patients with low expression of UNC5B in tumors had significantly poorer prognosis than those with high expression of UNC5B. Although UNC5B mRNA expression was restored by the demethylation treatment in CRC cell lines, the promoter region of UNC5B was not methylated. UNC5B is a potential biomarker for the selection of patients with high risk of postoperative recurrence and worse prognosis in CRC.

Introduction

Colorectal cancer (CRC) occurs as a consequence of the successive accumulation of genetic and epigenetic alterations (1). Epigenetic alterations, such as aberrant DNA hypermethylation, are now recognized as one of the crucial mechanisms of tumor-suppressor gene inactivation (2). Recent studies have shown the methylation of multiple genes that control apoptosis, cell cycle regulation, or other cellular behaviors with advances in genome-wide methylation analysis technology (3,4).

A number of genes, including hMLH1, MGMT, and $p14^{ARF}$, are known to be frequently methylated (5,6) in CRC tumor samples, and some studies have suggested that there is a subgroup of CRC that hypermethylates a high proportion of genes, termed CIMP (CpG Island Methylator Phenotype) (7-9). Therefore, aberrant DNA hypermethylation seems to be a critical phenomenon in colorectal carcinogenesis. In addition, such aberrant hypermethylation is described as a cause rather than a consequence of colorectal carcinogenesis (10-12).

Methylation is also interesting in terms of clinical applications. Methylation of cancer-specific genes could be used as a biomarker for cancer diagnosis or a therapeutic target. There are some advantages to using the detection of methylation as a diagnostic tool (13). Because DNA is a chemically stable molecule, methylation can be detected using biopsy samples or cancer-derived free DNA in plasma. In addition, the method of detection using only one set of PCR primers is technically simple and sensitive because the sites where aberrant hypermethylation usually occur are often CpG islands in promoter regions. From a therapeutic viewpoint, demethylation could be an anticancer therapy because epigenetic events are potentially reversible, and methylated genes may be reactivated by the demethylating agents (14). Previous studies have also shown the efficacy of combination therapy that uses demethylating agents and chemotherapy drugs (15,16). Ishiguro *et al* (16) suggested that demethylating agents will function as a biosensitizer of DNA damaging agents when the apoptotic pathway is inactivated by DNA hypermethylation.

Thus, to analyze DNA methylation events that play crucial roles in the colorectal carcinogenesis may be important for clinical applications. However, none of the genes that are methylated in CRC have been clinically used as biomarkers or therapeutic targets (17). In this study, for the purpose of finding methylated genes that can be clinically useful, we screened candidate genes suspected to be silenced by DNA methylation using microarray analysis. Here we focused our study on the UNC5B gene, which was reported to be inactivated in CRC (18), and we demon-

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Key words: UNC5B, methylation, colorectal cancer

strated the clinical significance of UNC5B gene expression in CRC patients. Furthermore, we investigated the mechanism of UNC5B down-regulation in CRC tumors.

Materials and methods

CRC cell lines. We used eight CRC cell lines-RKO, HCT116, HCT15, HT29, SW48, SW480, LoVo, and colo320. These were purchased through the American Type Culture Collection (Manassas, VA) or Cell Resource Center for Biomedical Research, Tohoku University (Miyagi, Japan). Cells were maintained in RPMI (Sigma, Tokyo, Japan), MEM, DMEM, or McCoy's 5A medium (Gibco, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml of penicillin, 100 μg/ml of streptomycin, 10 mM of HEPES, and 1.0 mM of sodium pyruvate and incubated at 37˚C in 5% CO_2.

Clinical sample collection. We collected tissue specimens of primary CRCs and matched corresponding normal mucosa from 100 patients who had undergone surgery at Tokyo Medical and Dental University Hospital (Tokyo, Japan) between 2004 and 2006. All patients provided written informed consent, and the study was approved by the Institutional Review Boards of all institutions involved. Clinical data were obtained from the medical records of each patient, and histopathological evaluation was assessed by reference to the criteria of the TNM classification system, 6th edition. The patients were observed regularly every 3 months after surgery, and their median follow-up period was 33 months.

Microarray analysis. Oligonucleotide microarray analysis was carried out using Human Genome U133 Plus 2.0 (Affymetrix, Santa Clara, CA, USA) with 19628 probe sets. Total RNA was extracted using RNeasy Micro kit (Qiagen, Hilden, Germany) from CRC cell lines (HCT15 and HT29) and 17 tissue samples, and then total RNA was amplified. The purity and concentration of amplified total RNA was determined by an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Amplified total RNA samples were hybridized to the cDNA microarray. Candidate genes were selected when the fold changes were >1.5 and the P-value was <0.05.

mRNA expression assay. We examined UNC5B mRNA expression level in each cell line or clinical tissue sample by quantitative RT-PCR. Total RNA was extracted using an RNeasy mini kit (Qiagen, Inc.) and cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. cDNA were then amplified by PCR using a fluorescence-based real-time detection method with ABI Prism 7300 Real-Time PCR System according to the manufacturer's protocol. TaqMan Gene Expression Assays-on-demand (Assay ID Hs0090071_m1, Applied Biosystems) and TaqMan Universal PCR Master Mix (Applied Biosystems) were used for PCR amplification. UNC5B mRNA expression level in each sample was normalized to the β-actin content in each sample as an internal standard. Relative quantification of UNC5B mRNA expression was calculated by the ΔΔCt method by SDSv1.2 with RQv1.0 software (Applied Biosystems). Each experiment was performed in triplicate.

Demethylation treatment for CRC cell lines. Cells were plated in culture plates on day -2. On day 0, culture medium was removed and new medium containing 0.5 μM of 5-aza-2'-deoxycytidine (DAC) was added. The cells were treated with 0.5 μM of DAC for 72 h, according to a previous report (16). The cells were rinsed twice with FBS-free medium and collected with trypsin-ethylenediaminetetraacetic acid (EDTA) after 72 h of DAC treatment.

Methylation analysis. We studied the methylation status of the UNC5B promoter region using the methylation-specific PCR assay (MSP) (19). A CpG island in the promoter region of UNC5B gene was identified by a CpG island search program (http://cpgislands.usc.edu/). We then analyzed the methylation status of three regions in the CpG island using primers for each. Primer sequences are listed in Table I. DNA extraction from cell lines and bisulfite treatment were performed as described previously (16). PCR conditions were as follows: 95˚C for 5 min, 35 cycles of 95˚C for 30 sec, 56˚C for 30 sec, 72˚C for 30 sec, and finally 5 min at 72˚C. After amplification, PCR products were electrophoresed on 3% agarose gels.

Statistical analysis. We statistically analyzed the relationship between UNC5B mRNA expression and clinicopathological features. For the statistical analysis, UNC5B T/N expression ratio, the expression level in tumor tissue divided by that in the matched normal mucosa, was calculated. The study samples were then classified into two groups depending on the cut-off score 0.5 based on the receiver operating characteristic (ROC) curve analysis. The low expression group had a T/N ≤0.5, while the high expression group had a T/N >0.5. Differences in the frequencies between the two groups were evaluated by the χ^2 test. Survival curves were plotted according to the Kaplan-Meier method, and differences between the curves were analyzed by a log-rank test. Furthermore, univariate and multivariate analysis for risk factors of postoperative recurrence were done using the logistic regression model. Statistical analysis was performed using SPSS software (version 11.0J, SPSS inc., Chicago, IL, USA). Differences were considered statistically significant at $P<0.05$.

Results

Candidate genes suppressed by DNA methylation. Seventy-five candidate genes suggested to be suppressed by DNA methylation were screened using cDNA microarray analysis. Genes which were re-expressed more than 1.5-fold after the treatment with a demethylating agent in CRC cell lines (HCT15 and HT29) and down-expressed more than 1.5-fold in cancer tissues compared to the normal mucosa were selected as the candidate genes (a list of the genes on this cDNA microarray is available from 'http://www.ncbi.nlm.nih.gov/geo' with GEO accession number GSE22598). Among these candidate genes, we focused our study on UNC5B gene because it was reported to be down-regulated in CRC (18), but the clinical significance and the mechanism of inactivation remain unclear. Fig. 1 shows the UNC5B expression levels in tumor and normal mucosa samples and the re-expression after the demethylation treatment in CRC cell lines.

Table I. Primer sequences for methylation-specific PCR.

			Nucleotide sequence (5'-3')	Tm (°C)	Product size (bp)
Region 1	M	Forward	TAAAACGTTGCGTTTAGCGTTTC	54.2	139
		Reverse	GAAACGAAACCTAAACGACCG	55.6	
	U	Forward	GGGTTTTTTAAAATGTTGTGTTTAGTGTTTT	54.4	152
		Reverse	CAACCAAAACAAAACCTAAACAACCA	54.9	
Region 2	M	Forward	GTTGGAATTTATTTCGGCGAGCGAGC	61.2	187
		Reverse	GACGCTATACCTCCGAAATTACGCG	61.2	
	U	Forward	TGGTTGGTGAATTTATTTTGGTGAGTGAGT	58.3	197
		Reverse	CTAACTCCAACACTATACCTCCAAAATTACACA	59.7	
Region 3	M	Forward	ATAGCGTCGGAGTTAGGCGAGC	61.4	142
		Reverse	GTAAAAACAACGAAAACCACGCTACG	58.0	
	U	Forward	GAGGTATAGTGTTGGAGTTAGGTGAGT	59.6	153
		Reverse	CCTTCCATAAAAACAACAAAAACCACACTACA	58.4	

Tm, melting temperature.

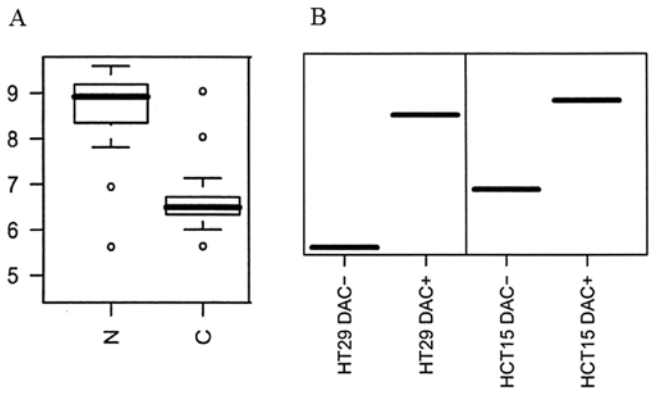

Figure 1. Expression level of UNC5B. (A) Comparison of CRC tissue (C) and normal mucosa (N). UNC5B expression was decreased by 0.26-fold in CRC tissue relative to normal mucosa (P<0.001). (B) Comparison of UNC5B mRNA levels before and after DAC treatment in two CRC cell lines. UNC5B expression levels increased 5.2- and 11.8-fold after DAC treatment in HCT15 and HT29 cells, respectively.

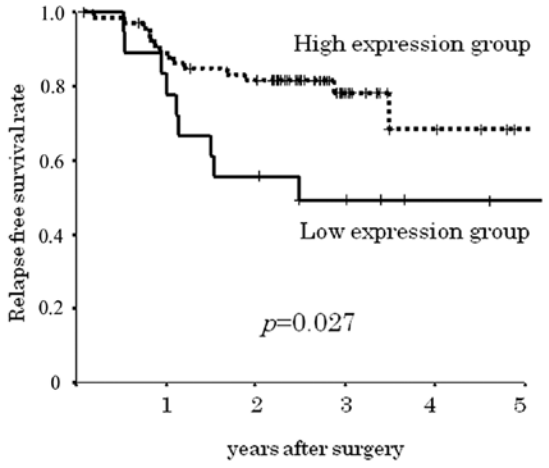

Figure 2. Relapse-free survival curves of CRC patients according to UNC5B mRNA expression. The relapse-free 5-year survival rate for patients with low (solid line) UNC5B expression and high (dotted line) UNC5B expression was 49.4 and 68.5% respectively (p=0.027).

Relationship between UNC5B mRNA expression and clinicopathological features. We analyzed the association between UNC5B mRNA expression level and clinicopathological features, including age, gender, tumor location, histological type, depth of tumor invasion, lymph node metastasis, lymphatic or venous permeation, distant metastasis, TNM stage, and recurrence rate after surgery. We found that, in 20 out of 100 patients, UNC5B expression in cancer tissue was decreased by at least 2-fold relative to expression in the matched normal mucosa. The relationships between UNC5B expression and clinicopathological factors are summarized in Table II. A significant relationship was detected between low UNC5B expression and high recurrence rate in 86 colorectal cancers without distant metastases (P=0.011, χ^2 test).

We then investigated whether UNC5B expression could be a predictor of tumor recurrence after surgery. The analysis of risk factors for postoperative recurrence in stage I, II and III patients are shown in Table III. According to a univariate analysis, lymph node metastasis (P=0.001) and low UNC5B expression (P=0.011) were possible risk factors for tumor recurrence after surgery. A multivariate analysis also showed that low UNC5B expression was an independent risk factor for tumor recurrence after surgery (P=0.015). Furthermore, we found low UNC5B expression was an independent risk factor in stage II and III patients (P=0.042). Fig. 2 presents the prognosis of the patients according to UNC5B expression. The patients with low expression of UNC5B in tumors had significantly shorter relapse-free survival than those with high expression of UNC5B (P=0.027).

Table II. Relationship between UNC5B expression and clinicopathological factors.

Clinicopathological factors	Low expression (n=20) No.	%	High expression (n=80) No.	%	P-value
Age at surgery					
<Median (n=67)	9	45.0	40	50.0	
≥Median (n=67)	11	55.0	40	50.0	0.689
Gender					
Male	12	60.0	52	65.0	
Female	8	40.0	28	35.0	0.677
Location					
Colon	18	90.0	62	77.5	
Rectum	2	10.0	18	22.5	0.176
Histological type					
Differentiated type[a]	7	35.0	28	35.0	
Undifferentiated type[b]	13	65.0	52	65.0	1.000
Tumor depth of invasion					
T=1,2	4	20.0	18	22.5	
T=3,4	16	80.0	62	77.5	0.809
Lymph node metatasis					
Absent	9	45.0	43	53.8	
Present	11	55.0	35	43.8	
Unknown	0	-	2	2.5	0.418[c]
Distant metastasis					
Absent	17	85.0	69	86.3	
Present	3	15.0	11	13.8	0.885
Lymphatic permeation					
Absent	5	25.0	20	25.0	
Present	15	75.0	59	73.8	
Unknown	0	-	1	1.3	0.977[c]
Venous permeation					
Absent	4	20.0	9	11.3	
Present	16	80.0	70	87.5	
Unknown	0	-	1	1.3	0.309[c]
TNM stage					
I, II	9	45.0	39	48.8	
III, IV	11	55.0	40	50.0	
Unknown	0	-	1	1.3	0.569[c]
Recurrence after surgery					
-	9	45.0	55	68.8	
+	9	45.0	13	16.3	
Stage IV	2	10.0	12	15.0	0.011[d]

[a]Differentiated type includes well differentiated adenocarcinoma. [b]Undifferentiated type includes moderately, poorly differentiated, and mucinous adenocarcinoma. [c]Unknown is excluded from the χ^2 test. [d]Patients in stage IV are excluded from the χ^2 test.

Methylation status and gene expression of UNC5B in CRC cell lines. We investigated the association between methylation status and gene expression in CRC cell lines to determine whether the down-regulation of UNC5B expression was related to hypermethylation of the promoter as suggested by the microarray analysis. The mRNA expression levels of UNC5B in RKO, HCT116, HCT15, and HT29 cells were lower relative to those in SW48, SW480 and colo320 (Fig. 3A). The expression levels in the cell lines with low UNC5B mRNA expression were increased significantly by the demethylation treatment (Fig. 3B), suggesting that the expression of UNC5B might be regulated by the UNC5B promoter hypermethylation. To analyze the methylation status of the UNC5B promoter region, we identified a 2405-bp CpG island around the transcription start site (Fig. 4A), and methylation-specific PCR (MSP) was then performed in each cell line. The UNC5B

Table III. Analysis of risk factor of postoperative recurrence in 86 stage I, II and III CRC patients.

	Univariate analysis			Multivariate analysis	
Clinicopathological factors	Recurrence (+) (n=22)	Recurrence (-) (n=64)	P-value	OR (95% CI)	P-value
Age at surgery					
<Median (n=67)	12	31	0.621	-	-
≥Median (n=67)	10	33			
Gender					
Male	16	40	0.385	-	-
Female	6	24			
Location					
Colon	16	52	0.383	-	-
Rectum	6	12			
Histological type					
Differentiated type	12	20	0.051	-	-
Undifferentiated type	10	44			
Tumor depth					
T=1,2	3	19	0.137	-	-
T=3,4	19	45			
Lymph node metastasis					
Absent	6	42			
Present	16	21	0.001[a]	5.641 (1.818-17.505)	0.003[b]
Unknown	0	1			
Lymphatic permeation					
Absent	3	21			
Present	19	42	0.077[a]	-	-
Unknown	0	1			
Venous permeation					
Absent	1	10			
Present	21	53	0.275[a]	-	-
Unknown	0	1			
UNC5B expression					
Low	9	9	0.011	4.505 (1.339-15.15)	0.015[b]
High	13	55			

[a]Unknown is excluded. [b]Logistic regression model.

promoter region was not methylated in any of the CRC cell lines studied (Fig. 4B).

Discussion

UNC5B, a transmembrane receptor, is proposed to regulate cell fate as a 'dependence receptor' (18). Dependence receptor induces apoptosis in the absence of ligand, whereas the presence of the ligand blocks the proapoptotic activity, and this mechanism is suggested to be involved in tumor suppression (20,21). Hence, the cancer cells that lose receptor expression cannot induce apoptosis, and the loss of apoptotic signaling is thought to be a selective advantage for tumor progression (20,22,23). Indeed, several studies have shown the down-expression of dependence receptors in various human cancers (18,20,24). DCC (deleted in colorectal cancer), one of the dependence receptors, has been reported to be inactivated in more than 70% of colorectal cancer due to the 18q LOH (loss of heterozygosity) (25,26). UNC5B has also been reported to be down-expressed in 27% of the colorectal tumors (18), but the precise clinical significance and mechanism of UNC5B down-expression is not understood.

In this study, the down-expression of UNC5B in cancer tissues compared with corresponding normal mucosa was observed in about 20% of patients, and these patients experienced significantly higher rates of tumor recurrence after surgery. We propose

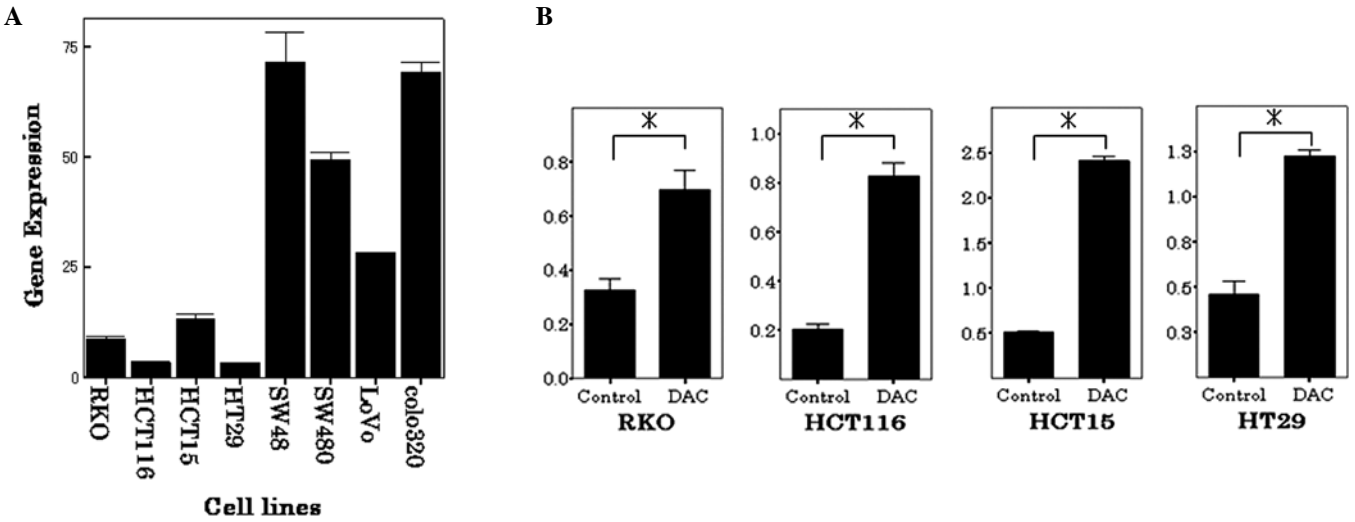

Figure 3. (A) UNC5B mRNA expression in eight CRC cell lines was assayed using real-time quantitative PCR. To normalize UNC5B expression, β-actin was used as internal control. Each expression level is shown as mean + SD. (B) Changes in UNC5B mRNA expression after the demethylation treatment. Expression levels are shown as the mean + SD. *p<0.01 (paired t-test). DAC, 5-aza-2'-deoxycytidine.

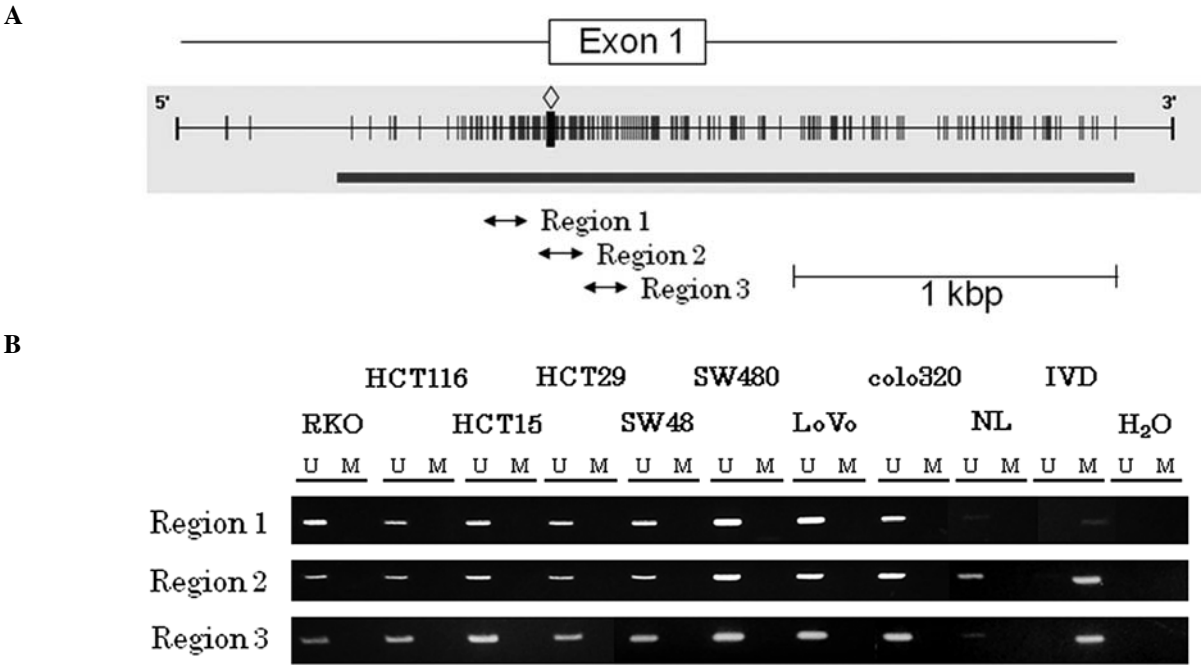

Figure 4. (A) The 5' CpG island of UNC5B: CpG sites are shown as vertical bars. The diamond on the top of the vertical bar indicates the translation start site. CpG island is shown as a solid box; the regions assayed by MSP to amplify nucleotides are shown by double headed arrows. (B) Methylation analysis of UNC5B in a panel of colorectal cancer cell lines. *In vitro* methylated DNA (IVD) and normal human peripheral lymphocytes (NL) served as the positive and negative methylation controls, respectively. Lane M, methylated DNA; lane U, unmethylated DNA; H_2O, no DNA added.

the following model to explain this correlation. It is reported that the alternative UNC5B ligand, netrin-1, does not exist in the blood (27); hence, the tumor cells expressing UNC5B will undergo apoptotic signaling and apoptosis when they migrate to the blood. In contrast, tumor cells that do not express UNC5B cannot induce the apoptotic signaling in the blood. These tumor cells that do not express UNC5B will circulate and survive in the blood and successfully re-colonize the primary organ or a distant site after the primary tumor was surgically removed. This hypothesis can explain the correlations between low UNC5B expression and high tumor recurrence rates and poor prognosis. Our result suggests that low UNC5B expression may be a useful clinical biomarker for predicting recurrence after surgery in Stage II and Stage III CRC. Recent studies have shown the efficacy of the adjuvant chemotherapy to prevent the recurrence after the curative resection for the patients with high recurrent

risk in CRC (28-30). UNC5B may be useful for the identification of patients that may benefit from adjuvant chemotherapy. Further validation or prospective studies are needed to assess the clinical utility of UNC5B as a biomarker.

The mechanism responsible for the loss of UNC5B expression also remains unclear. In this study, UNC5B was detected as one of the candidate genes in which mRNA expression levels were increased after the demethylation treatment, suggesting that the down-expression of UNC5B was due to the promoter hypermethylation. However, we could not detect the hypermethylation of UNC5B promoter region in any of the CRC cell lines. One possible explanation for this unexpected result is that the expression levels of UNC5B were elevated by the cytotoxicity of the demethylating agent because it is known that the high concentrations of demethylating agents may activate the genes that induce apoptosis or growth arrest in response to the cellular stress (31). However, in our demethylation experiment, UNC5B expression levels were not elevated in some CRC cell lines (data not shown), suggesting that the increase of UNC5B expression after the demethylation treatment was not necessarily induced by the drug cytotoxicity. Another possible explanation is that a regulator of UNC5B expression, such as transcription factor, was methylated. We hypothesized that UNC5B might be down-expressed as a result of methylation-induced inactivation of a regulator. In our demethylation experiment, UNC5B expression may have been restored because the regulator was reactivated by demethylation. However, there is no molecule known to regulate UNC5B expression in this manner. Tanikawa *et al* identified p53-binding sites within UNC5B genomic sequence and reported that UNC5B would be one of the p53 target genes that mediate p53-dependent apoptosis (32-34). They suggested that the transcription of UNC5B could be activated by various cellular stresses in a p53-dependent manner. In this study, we could not observe the correlation between p53 status and mRNA expression levels in CRC cell lines and tissue samples (data not shown), and these observations implied that a p53-independent mechanism of UNC5B expression exists. Interestingly, Suzuki *et al* (35) have recently reported that p53 target genes were frequently regulated by methylation in several malignancies, and they also suggested that p53 target genes might be regulated by methylation without p53 mutation. This theory is consistent with the idea that UNC5B, which is a p53 target gene, may be regulated by a methylation-related mechanism. If methylation-related mechanisms contributed to the inactivation of UNC5B, demethylation treatment could be a therapeutic strategy. Recent studies have shown the clinical efficacy of the demethylating agents in the treatment of patients with hematological malignancies (36,37), and the use of demethylating agents is now being tested for the treatment of solid tumors. Further investigations will be required to reveal the mechanism of UNC5B inactivation in CRC.

In conclusion, our study revealed the clinical significance of UNC5B mRNA expression in CRC. Patients with tumors expressing low levels of UNC5B mRNA experienced significantly higher rates of tumor recurrence after the curative surgery and worse prognoses. Hence, UNC5B could be used as a biomarker to predict the postoperative recurrence. Furthermore, if methylation, an epigenetic mechanism, contributed to inactivation of UNC5B, demethylation treatment could be a therapeutic strategy in the future.

Acknowledgements

We thank Y. Takagi and M. Itoda for their excellent technical assistance.

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