CpG hypermethylation of *cellular retinol-binding protein 1* contributes to cell proliferation and migration in bladder cancer

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Abstract. We have previously reported a simple technique that combines microarray data from clinical bladder cancer (BC) specimens with those from a BC cell line (BOY) treated with a pharmacological demethylating agent [5-aza-2'-deoxycytidine (5-aza-dC)] to find candidate genes that have tumor suppressive functions. We focused on the *cellular retinol-binding protein 1* (*CRBP1*) gene that was selected by using the microarray data. As CRBP1 regulates intracellular retinoic acid (vitamin A) homeostasis, which is involved in morphogenesis, and cellular proliferation and differentiation, the loss of CRBP1 could cause tumorigenesis in BC. We hypothesized that the inactivation of the *CRBP1* gene through CpG methylation contributes to cell viability, including the migration and invasion activity of human BC cells. After the 5-aza-dC treatment, the mRNA and protein expression levels of *CRBP1* markedly increased in all BOY and T24 BC cell lines. Combined bisulfite-restriction analysis and bisulfite DNA sequencing revealed that promoter CpG hypermethylation existed in 28 out of the 65 BCs (43%) and in none of the 16 normal bladder epithelia (NBEs). Conversely, *CRBP1* mRNA expression in the BCs was significantly lower than that in the NBEs (0.63±0.11 vs. 4.92±0.80, $p<0.0001$). We found significant inhibition of cell growth ($p<0.0001$) and migration ($p<0.0001$) in the CRBP1 stable transfectants compared to the control cell line, in a cell proliferation and wound-healing assay, respectively. In conclusion, the aberrant CpG hypermethylation of the *CRBP1* gene promoter could be involved in the development of BC. We demonstrate here for the first time that the *CRBP1* gene could have a tumor suppressive function in BC.

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Introduction

Bladder cancer (BC) is among the five most common malignancies worldwide, and it is the second most common tumor of the genito-urinary tract and the second most common cause of death in patients with genito-urinary tract malignancies (1). Although 70-80% of BCs are initially classified as non-muscle invasive, ~70% of them recur. Of these recurring tumors, 10-15% proceed to muscle invasion and metastasis (2). Therefore, it is crucial to find what novel mechanisms are involved in BC invasion and metastasis.

As is often the case with other human malignancies, the transcriptional silencing of tumor suppressor genes through the methylation of CpG islands in promoter regions, is considered to be an important and early stage of carcinogenesis in human BC (3). We previously developed a simple technique that combines microarray data from clinical BC specimens with those from a BC cell line (BOY) treated with a pharmacological demethylating agent [5-aza-2'-deoxycytidine (5-aza-dC)] (4-6). In these previous studies, we identified 20 genes that were considered to be methylated in human BC (shown here in Table III). Based on that profile, we demonstrated that the *COL1A2* and *FHL1* genes have a tumor suppressive function and that they were frequently inactivated in BC due to the CpG hypermethylation of the promoter region (5,6). Thus, we believe that our method is quick and efficient in finding the methylated gene profile that is involved in cancer development. In the present study, we focused on the *cellular retinol-binding protein 1* (*CRBP1*), which was among the candidate genes.

Retinol (vitamin A) is a fat-soluble micronutrient and occurs in nature in various forms (retinoids). *In vivo*, it functions as a form of retinoic acid (RA), which has a close relationship with important cellular functions such as morphogenesis, and cellular proliferation and differentiation (7). Retinoids are bound to CRBPs (types 1, 2 and 3) in the cytoplasm. CRBP1 is widely distributed throughout the body, whereas CRBP2 and 3 demonstrate tissue-specific expression

(8). Of these three, CRBP1 is thought to be essential for vitamin A homeostasis. The importance of this gene product *in vivo* was documented in CRBP1 knockout mice, which demonstrated squamous keratinizing metaplasia (9). The loss of CRBP1 expression due to the CpG hypermethylation of its promoter region has been found in various human cancer cell lines (10). Studies have also demonstrated that the CpG hypermethylation of the gene promoter is frequently observed in various cancers such as lymphoma, gallbladder and renal cell carcinoma, as well as breast, esophageal, gastric and prostate cancers (11-17). In terms of BC, certain studies have reported the promoter hypermethylation of *CRBP1*. However, they have validated neither the expression levels of CRBP1 transcripts nor its functional role in BC development (11,18). Another study has also demonstrated that the mRNA expression levels of *CRBP1* were up-regulated in cervical cancer (19). Thus, the functional role of *CRBP1* has not been thoroughly investigated in BC and requires further study.

In the present study, we hypothesized that *CRBP1* was densely methylated in its promoter region and had a tumor suppressive function in BC. In order to test this, we examined the methylation status and mRNA expression in BC cell lines and clinical BC specimens by using combined bisulfite-restriction analysis (COBRA) and real-time RT-PCR. In addition, we established stable transfectants of *CRBP1* for gain-of-function studies and compared their proliferation, migration, and invasion activities with those of the control BC cell lines.

Materials and methods

Clinical samples. The tissue specimens were from 122 BC patients who underwent transurethral resection (TUR) or radical cystectomy at Kagoshima University Hospital between 2003 and 2008. We also used 25 pathologically-proven normal bladder epithelium (NBE) samples derived from organ-confined prostate cancer patients who underwent prostatectomy. The patient backgrounds and clinicopathological characteristics are summarized in Table I. Each tumor was staged and graded according to the TNM staging system (20), the Japanese Urological Association and the Japanese Society of Pathology (21). Our study was approved by the Bioethics Committee of Kagoshima University and prior written informed consent and approval were given by all patients.

Cell culture and 5-aza-dC treatment. We used two human BC cell lines. BOY was established in our laboratory from an Asian male 66-year-old patient who was diagnosed with stage III bladder cancer with lung metastasis (22) and T24 was obtained from the American Type Culture Collection. These cell lines were maintained in minimum essential medium which was supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37˚C. In order to screen for epigenetic alterations of gene methylation, these cells were treated with 1 μM of the DNA methyltransferase inhibitor, 5-aza-dC (Sigma-Aldrich, St. Louis, MO, USA). Cultured cells were harvested after seven days of exposure to 5-aza-dC, and the genomic DNA and total RNA were extracted.

Table I. Patient characteristics.

BC	
Total number	122
Median age, years (range)	75 (33-100)
Gender	
Male	80
Female	42
Stage	
Tis	1
Superficial (pTa)	21
Invasive (≥pT1)	100
Grade	
G1	11
G2	72
G3	39
Operation	
Cystectomy	16
TUR-Bt	106
Recurrence	
Recurrence (+)	61
Recurrence (-)	50
Unknown	11
Follow-up period, days (range)	513 (13-1440)
NBE	
Total number	25
Median age, years (range)	66 (58-76)
Gender	
Male	19
Female	6

Nucleic acid extraction. Genomic DNA from the BCs and NBEs was extracted with QIAamp tissue kits (Qiagen, Valencia, CA) after the microdissection of 10-μm-thick paraffin-embedded sections and this was precipitated with ethanol. Total RNA was isolated from the frozen fresh tissues and BOY cells with Isogen (Nippon Gene, Tokyo, Japan) following the manufacturer's instructions. The concentrations of DNA and RNA were determined spectrophotometrically and their integrity was verified by gel electrophoresis. The RNA quality was checked in a BioPhotometer™ (Eppendorf, Tokyo, Japan).

cDNA preparation and conventional RT-PCR. We synthesized first strand cDNA with 1 μg of total RNA using random primers and TaqMan reverse transcription reagents (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA, USA). For conventional RT-PCR, glyceraldehyde-3-phosphate 8 dehydrogenase (GAPDH) was used as the internal control. The primer sequences and RT-PCR conditions are listed in Table II. The PCR products were electrophoresed on 2% agarose gels.

Real-time quantitative RT-PCR. Gene specific PCR products were continuously measured with an Applied Biosystems 7300 Fast Real-Time PCR System following the instructions of the manufacturer. The initial PCR step was a 10-min hold at 95˚C and the 45 cycles consisted of a 15-sec denaturation step at 95˚C followed by 1-min annealing/extension at 60˚C.

Table II. Primer sequences and PCR conditions.

	Primer sequence (5'-3')	Product size (bp)	Annealing temperature, time (cycle)
Conventional RT-PCR			
CRBP1-S (sense)	TTGTGGCCAAACTGGCTCCA	320	55˚C, 30 sec (35)
CRBP1-AS (antisense)	ACACTGGAGCTTGTCTCCGT		
GAPDH-S (sense)	GAAGGTGAAGGTCGGAGTCA	255	55˚C, 30 sec (35)
GAPDH-AS (antisense)	CACTTGATTTTGGAGGGATCTC		
COBRA			
PAN-S1 (sense)	GTGGTTGTTGAGTGTGAGAAG	442	52˚C, 30 sec (40)
PAN-AS1 (antisense)	CATCTTCCAATACCCAATAAAATC		
PAN-S2 (sense)	GTTGAGTGTGAGAAGTTAATGG	391	52˚C, 30 sec (35)
PAN-AS2 (antisense)	CTAAAACCAATTAACCACAAAC		

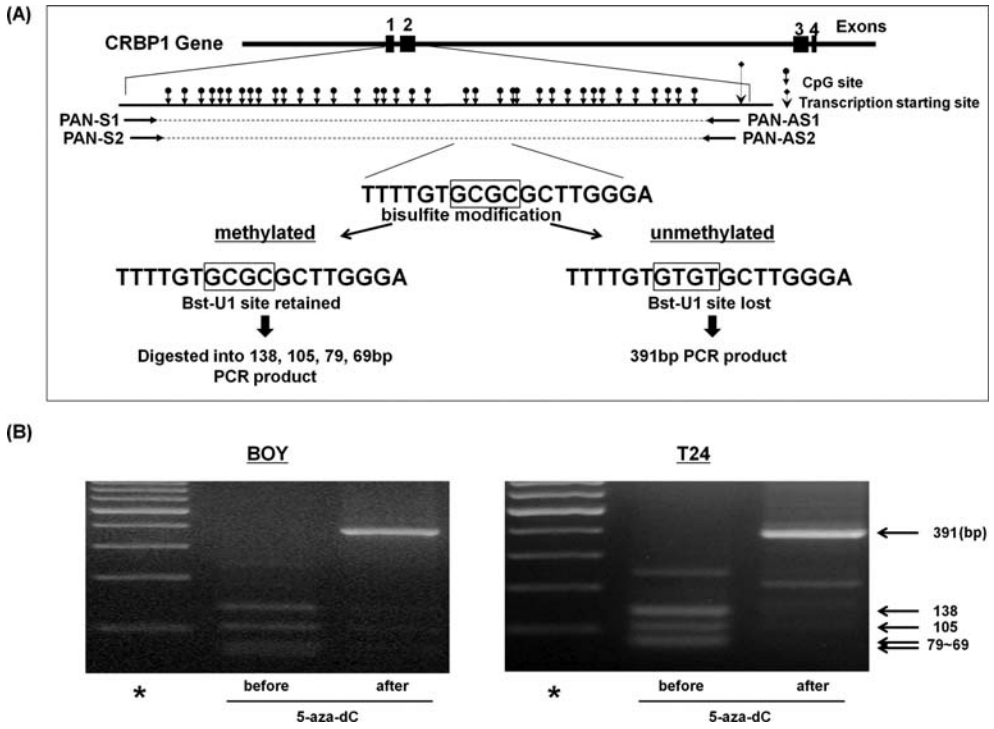

Figure 1. (A) Schematic representation of location of CpG sites and primers designed within the functional promoter of the *CRBP1* gene. Vertical arrows indicate CpG sites. Horizontal arrows indicate the positions of primers used. First, universal PCR was performed with the universal primers, PAN-S and PAN-AS, which do not carry any CpG sites. The original sequence for the target of COBRA is shown in the upper middle section. After bisulfite modification, the sequence changed into methylated (lower-left) and unmethylated patterns (lower-right). (B) Analysis of *CRBP1* promoter methylation by COBRA. *Bst*UI restriction enzyme digestion was performed to distinguish methylated and unmethylated alleles. The restriction site (CGCG) is recognized and digested by *Bst*UI when the site is methylated. Both the control BOY and T24 showed four fragments (138, 105, 79 and 69 bp), indicating that these were completely methylated. After treatment with 5-aza-dC, a 391-bp fragment appeared along with digested fragments, indicating that these were partially methylated. *100-bp DNA ladder.

All reactions were performed in triplicate and a negative control lacking cDNA was included. TaqMan® probes, primers for *CRBP1* (P/N: Hs00161252_m1, Applied Biosystems) and human *18S ribosomal RNA* (*rRNA*) (Applied Biosystems), were assay-on-demand gene expression products. The expression of *CRBP1* mRNA was normalized to the amount of human *18S rRNA* in the same cDNA using the standard curve method provided by the manufacturer.

DNA methylation analysis. In order to screen larger numbers of samples for CRBP1 promoter demethylation, we used COBRA, which produces restriction fragments from bisulfite-treated DNA only if methylation is present (Fig. 1A). A total of 65 BCs and 16 NBEs were only available for the study, as certain samples had insufficient amounts of tissue for the extraction of enough DNA for analysis. Genomic DNA (100 ng) was subjected to sodium bisulfite modification

Table III. Top 20 methylated genes in BC.

No.	Symbol	Gene name	Accession	UniGene	Location	Function	Fold changes	CpG island
1	*COL1A2*	Alpha 2 type 1 collagen	NM_000089	Hs.489142	7q22.1	Skeletal development	6.01	Yes
2	*COL1A1*	Alpha 1 type 1 collagen	NM_000088	Hs.172928	17q21.33	Skeletal development	4.03	Yes
3	*AEBP1*	Adipocyte enhancer binding protein 1	NM_001129	Hs.439463	7p13	Cell adhesion	3.82	Yes
4	*RBP1*	Retinol-binding protein 1	NM_002899	Hs.529571	3q23	Retinol metabolic process	3.50	Yes
5	*CLU*	Clusterin	NM_001831	Hs.436657	8p21-p12	Anti-apoptosis	3.09	Yes
6	*CKB*	Creatine kinase, brain	NM_001823	Hs.173724	14q32	Creatine kinase activity	2.75	Yes
7	*MGLL*	Monoglyceride lipase	NM_007283	Hs.277035	3q21.3	Hydorase activity	2.73	No
8	*FHL1*	Four and a half lim domains 1	NM_001449	Hs.435369	Xq26	Cell differentiation	2.48	Yes
9	*COX7A1*	Cytochrome c oxidase subunit viia polypeptide 1, muscle	NM_001864_	Hs.421621	19q13.1	Electron carrier activity	2.28	No
10	*NRIP1*	Receptor interacting protein	NM_003489	Hs.155017	21q11.2	Receptor binding	2.21	No
11	*PDLIM3*	Alpha-actinin-2-associated lim protein	NM_014476	Hs.85862	4q35	Metal ion binding	2.20	Yes
12	*C6orf37*	Hypothetical protein flj20037	NM_017633	Hs.10784	6q14	-	2.03	Yes
13	-	Bcr-abl mrna 5' fragment clone 3c	X14675_1	-	-	Signal transduction	1.96	Unknown
14	*NICAL*	Hypothetical protein flj11937	NM_022765	Hs.33476	6q21	Signal transduction	1.95	Yes
15	-	Immunoglobulin m heavy chain	AF267819_1	-	-	-	1.95	Unknown
16	*CKIP-1*	Ck2 interacting protein 1	NM_016274	Hs.438824	1q21.3	Negative regulation of cell cycle	1.89	No
17	*RGS2*	Regulator of g-protein signalling 2	NM_002923	Hs.78944	1q31	Negative regulation of signal transduction	1.87	Yes
18	*CAV1*	Caveolin 1	NM_001753	Hs.74034	7q31.1	Cholesterol binding	1.80	Yes
19	*HYAL4*	Hyaluronoglucosaminidase 4	NM_012269	Hs.28673	7q31.3	Hydrolase activity, acting on glycosyl bonds	1.80	No
20	*HBA1*	Alpha one globin	NM_000558	Hs.449630	16p13.3	Oxygen transport	1.76	Yes
21	*DF*	Adipsin/complement factor d precursor	NM_001928	Hs.155597	19p13.3	Proteolysis	1.75	No
22	*VWF*	Von willebrand factor	NM_000552	Hs.440848	12p13.3	Cell-substrate adhesion	1.74	No
23	*IGFBP6*	Insulin-like growth factor binding protein 6	NM_002178	Hs.274313	IGFBP6	Negative regulation of cell proliferation	1.67	Yes
24	*CFL2*	Ensembl genscan prediction	NM_021914	Hs.180141	14q12	Actin binding	1.65	Yes

using the EpiTect Bisulfite Kit (Qiagen). DNA modified with sodium bisulfite was selectively amplified by first-round PCR with the primers Pan-sense 1 (PAN-S1) and Pan-anti-sense 2 (PAN-AS1), followed by second-round PCR with the primers (PAN-S2 and PAN-AS2), of which neither primer set carried any CpG sites (Fig. 1A). The primer sequences and PCR conditions are summarized in Table II. For COBRA, an equal amount of sodium bisulfite PCR product was digested with *Bst*UI (New England Biolabs, Hitchin, UK) at 60˚C for 2 h. Digested fragments were visualized by the ethidium bromide staining of DNA, and were electrophoresed through a 2% agarose gel. Under these PCR conditions, we obtained DNA fragments of 391 bp, which were cleaved into fragments of 138, 105, 79 and 69 bp by digestion with *Bst*U1 when CpG was methylated. CpGenome™ Universal Methylated DNA and CpGenome™ Universal Unmethylated DNA Sets (Millipore, Billerica, MA, USA) were used as the positive and negative controls for the methylated alleles, respectively.

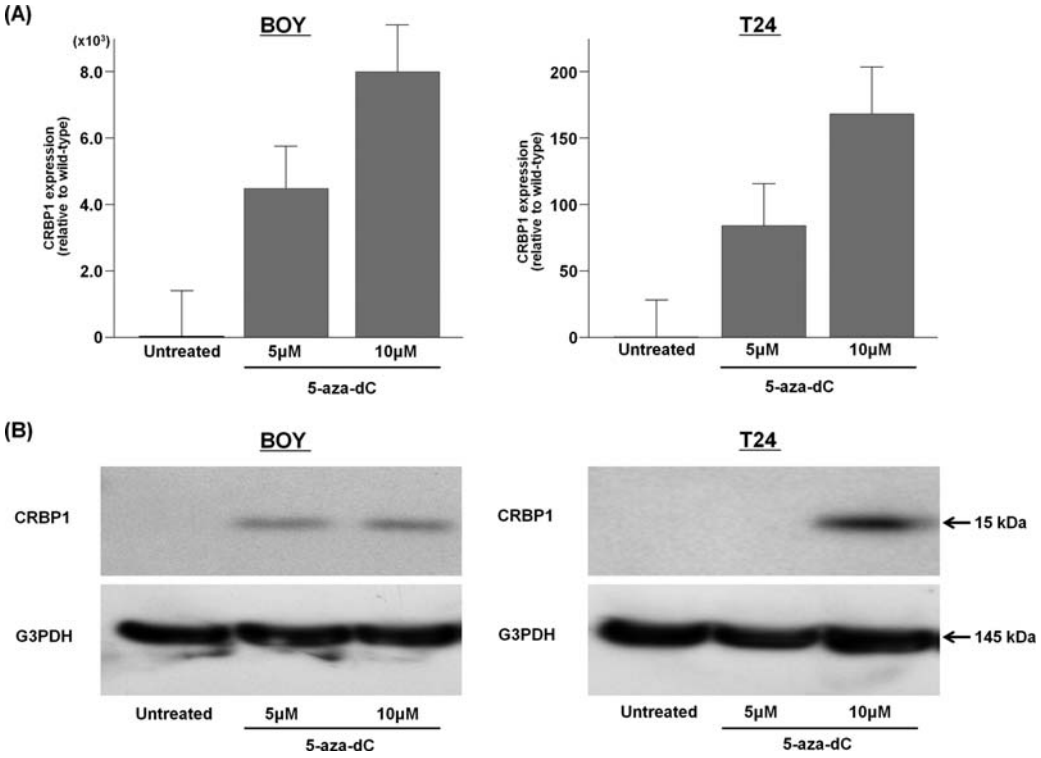

Figure 2. CRBP1 expression in BC cells before and after treatment with 5-aza-dC. (A) CRBP1 mRNA expressions were markedly increased in both cell lines after 5-aza-dC treatment in a dose-dependent manner. (B) CRBP1 protein bands (15 kDa) were restored in both cell lines after 5-aza-dC treatment.

Bisulfite DNA sequencing analysis. Bisulfite-modified DNA was amplified using a pair of universal primers, PAN-S1 and PAN-AS1, followed by a second nested PCR with the primers, PAN-S2 and PAN-AS2, in a total volume of 20 μl. The PCR conditions are shown in Table II. Direct bisulfite DNA sequencing of the PCR products using a forward PAN-S2 primer was carried out following the manufacturer's instructions (Applied Biosystems).

Immunoblotting. Total protein lysate was prepared with a detergent lysis buffer in the presence of a protease inhibitor. The 20 μg of protein lysate were separated by using NuPAGE on a 4-12% Bis-Tris gel (Invitrogen, Tokyo) and transferred into a PVDF membrane. Immunoblotting was done with a diluted (1:200) polyclonal *CRBP1* antibody (sc-8786, Santa Cruz Biotechnology, Santa Cruz, CA). After being washed, the membrane was incubated with donkey anti-goat IgG horseradish peroxidase conjugate (Bio-Rad, Hercules, CA). Specific complexes were visualized with an echochemi-luminescence detection system (GE Healthcare, Fairfield, CT, USA).

Construction of CRBP1 expression vectors and transfection to BC cell lines. The *CRBP1* vector was constructed by inserting full-length *CRBP1* cDNA into the *Bam*HI and *Xba*I restriction sites of the pBApo-CMV NeoTM vector (Takara Bio, Otsu, Japan). The *CRBP1*- and the non-targeting (control) vectors were transfected into the BOY and T24 cells by calcium phosphate co-precipitation. Both cells were split and grown in a selective medium with 1000 mg/l of G418. After two weeks, G418-resistant colonies were chosen and expanded in a medium containing 100 mg/l of G418.

XTT assays. For quantification of the cell proliferation of the CRBP1 transfectants, the controls and the wild-type BOY and T24 cells, the absorbance was measured at 492 nm after the cells had been exposed to XTT, which was metabolized by mitochondrial dehydrogenase to yield a formazan dye. In brief, the cells were transferred to 96-well plates at $3x10^3$ cells/well and incubated for 72 h. Then, a 50 μl solution of XTT (0.3 mg/ml) that contained 1.25 μM of PMS was added to each well and the absorbance was measured after a further 4 h of incubation at 37˚C.

Wound-healing assay. The CRBP1 transfectants, controls and the wild-type BOY and T24 cells, were used in the 'wound-healing' assay to test the alteration of cell motility and migration. Cells were initially seeded uniformly into 60-mm culture plates with an artificial 'wound' carefully created at 0 h. A P-20 pipette tip was used to scratch the subconfluent cell monolayer. Microphotographs were taken at 0, 6 and 12 h. Quantitative analysis of the percentage of wound healing was calculated using the distance across the wound (n=20) at 0, 6 and 12 h, divided by the distance measured at 0 h for each cell line.

Statistical analysis and annotation of gene function. The relationship between two groups and the numerical value obtained by real-time RT-PCR was analyzed using the Mann-Whitney U test. The relationships between three groups and the numerical value were analyzed using the Bonferroni-adjusted Mann-Whitney U test. The analysis software used was Expert StatView (version 4, SAS Institute Inc., Cary, NC, USA). The non-adjusted statistical level of significance of $p<0.05$, corresponded to the Bonferroni-adjusted level of

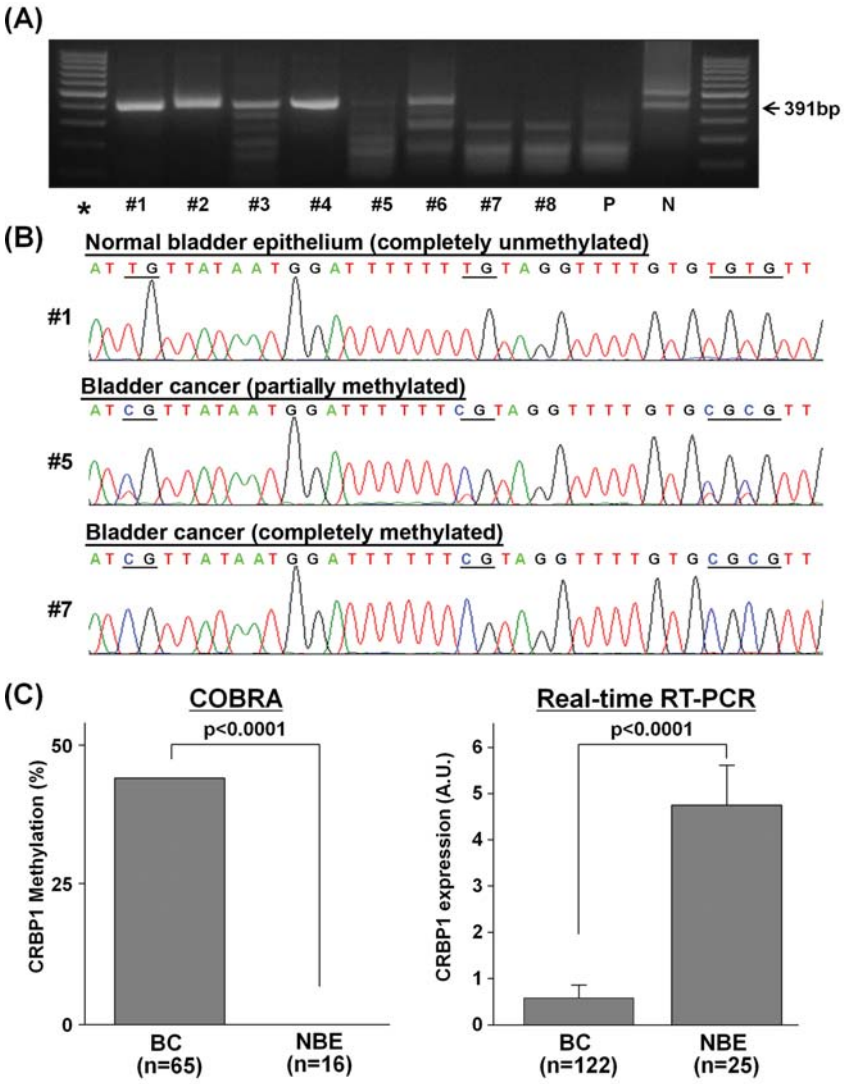

Figure 3. Methylation status of *CRBP1* promoter region in clinical specimens. (A) COBRA classified the specimens into 'unmethylated' (lanes 1, 2 and 4), 'partially methylated' (lanes 3, 5 and 6), and 'completely methylated' (lanes 7 and 8). Universally methylated DNA was respectively used as the positive control (lane P) and unmethylated DNA was used as the negative control (lane N). *100-bp DNA ladder. (B) Bisulfite DNA sequencing confirmed 'unmethylated' (#1), 'partially methylated' (#5), and 'completely methylated' (#7). Bars under the sequence indicate CpG sites. The results of the bisulfate DNA sequencing were consistent with those obtained by COBRA. (C) Methylation status and mRNA expression of CRBP1 in clinical specimens. (C, left) The promoter hypermethylation of the *CRBP1* gene was detected in 43% of the BCs, whereas it was not detected in any of the NBEs. (C, right) CRBP1 mRNA expression in the BCs was significantly lower than that in the NBEs. All experiments were carried out in triplicate.

$p<0.0167$ in the comparisons test comparing the three groups. The molecular function of the up- and down-regulated genes was classified into 17 categories as referenced in the Gene Ontology Consortium (http://www.geneontology.org/index.shtml), i.e., apoptosis, anatomical structure development, cell cycle, cell differentiation and proliferation, gene expression, metabolic process, response to stimulus, regulation of gene expression, transport, regulation of apoptosis, cell proliferation, signal transduction and transcription, signal transduction, transport, transcription and others.

Results

Methylation status and altered CRBP1 expression in 5-aza-dC-treated BC cell lines. Fig. 1 shows the methylation status of BOY and T24 analyzed with the COBRA assay. As the PCR product contains three 'CGCG' motifs, which are targeted by the restriction enzyme (*Bst*UI), there could be four fragments (138, 105, 79 and 69 bp) if a sample has complete methylation in all CpG sites, whereas there could be a single band of 391 bp if a sample has complete unmethylation in these sites. If the CpG sites are partially methylated, the fragments could be observed in various combinations of these sizes. Before 5-aza-dC treatment, we found the four fragments (each of 138, 105, 79 and 69 bp) and a fragment of 253 bp (sum of 105, 79 and 69), but no 391-bp band was observed in either cell line. After 5 μM of 5-aza-dC treatment, a 391-bp band appeared in BOY with various fragments of 148 bp (sum of 79 and 69) and 253 bp (sum of 105, 79 and 69), while the band of 243 bp (sum of 138 and 105) in T24, indicated that complete demethylation was achieved by the demethylating agent even though methylated CpG sites partially remained (Fig. 1B). These cells were subjected to real-time RT-PCR to evaluate the mRNA expression levels of *CRBP1*. The expression levels of *CRBP1* mRNA were markedly increased in a dose-dependent manner by 5-aza-dC treatment (Fig. 2A).

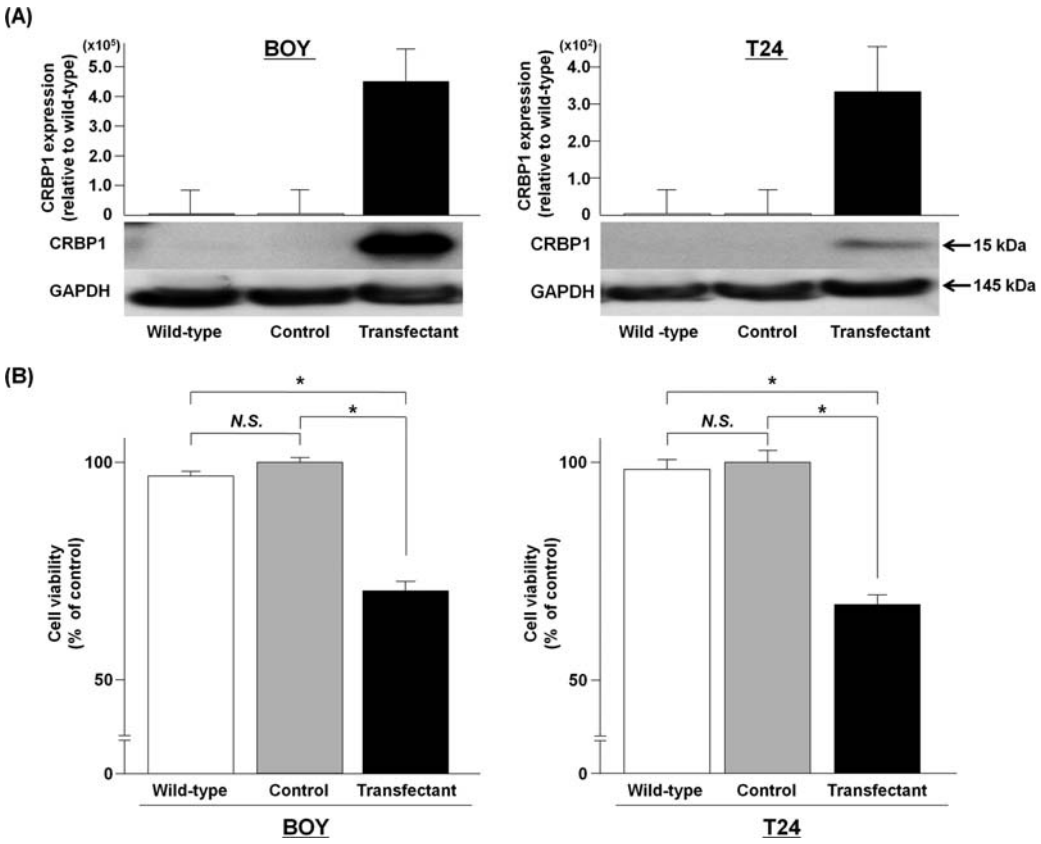

Figure 4. Effect of cell viabilities in CRBP1 transfectants by XTT assay. (A) CRBP1 expression determined by real-time RT-PCR and immunoblotting. (B) Cell viabilities determined by XTT assay. Cell viability inhibitions were observed in CRBP1-transfected BOY and T24 cell lines. *p<0.0001

Immunoblotting also demonstrated that the 15 kDa CRBP1 protein was restored after 5-aza-dC treatment in both cell lines (Fig. 2B).

DNA methylation status and mRNA expression of CRBP1 in clinical samples. Representative results of the COBRA assay in NBE (no. 1) and clinical BC (from nos. 2 to 8) samples are shown in Fig. 3A. A single band of 391 bp was observed in the sample nos. 1, 2 and 4, indicating that these CpG sites were completely unmethylated. The 391-bp band with various fragments was found in the sample nos. 3, 5 and 6 samples, indicating that these CpG sites were partially methylated. Sample nos. 7 and 8 had fragments without the 391-bp band, indicating these CpG sites were completely methylated. In Fig. 3B, we show the results of typical bisulfite DNA sequencing in the BC samples corresponding to COBRA. In sample no. 1, there was a modest 'T' and no 'C' peak at any CpG site, substantiating that this was a completely unmethylated sample, as indicated by COBRA. Sample no. 5, which was diagnosed as partially methylated by COBRA, disclosed a strong 'C' and modest 'T' peak at each CpG site, corroborating that it was partially methylated. In sample no. 7, a strong 'C' and no 'T' peak at any CpG site substantiated that it was a completely methylated sample.

COBRA and bisulfite DNA sequencing revealed that there was promoter CpG hypermethylation in 28 out of the 65 BCs (43%) and in none of the 16 NBEs (0%) (Fig. 3C). Conversely, *CRBP1* mRNA expression in the BCs was significantly lower than that in the NBEs (0.63±0.11 vs. 4.92±0.80, p<0.0001) (Fig. 3C). We analyzed the relationship between the clinicopathological factors, but there was no correlation between the methylation status or mRNA expression of *CRBP1* and the patient characteristics (data not shown).

Identification of CRBP1 expression in transfected cells and effect of CRBP1 overexpression on cell growth. As we found very low expression levels of *CRBP1* mRNA in wild-type BOY and T24 cells, we established CRBP1 transfectants from the BOY and T24 cells. The expression levels of *CRBP1* mRNA in the BOY/CRBP1 transfectants were ~450,000-fold higher than those in the control or wild-type BOY cells, and the levels in the T24/CRBP1 transfectants were 400-fold higher than those in the control or wild-type T24 cells (Fig. 4A). Immunoblotting vigorously detected a 15-kDa CRBP1 protein in the BOY/CRBP1 transfectants, and it was modestly detected in the T24/CRBP1 transfectants (Fig. 4A). In order to evaluate the relationship between *CRBP1* expression and cell growth, we performed XTT assays using the CRBP1 transfectants, control, and wild-type cells. Three independent XTT assays consistently demonstrated significant growth inhibitions in the CRBP1 transfectants compared to the control, and wild-type cells (% of cell viability in BOY on day 3: 68.8±0.7, 100±1.8 and 95.7±0.8, respectively, p<0.0001, and that in T24: 66.6±0.3, 100±1.8 and 99.5±0.8, respectively, p<0.0001) (Fig. 4B).

In order to examine cellular migration ability, we carried out wound-healing assays using the CRBP1 transfectants in comparison to their counterparts. Three independent wound-

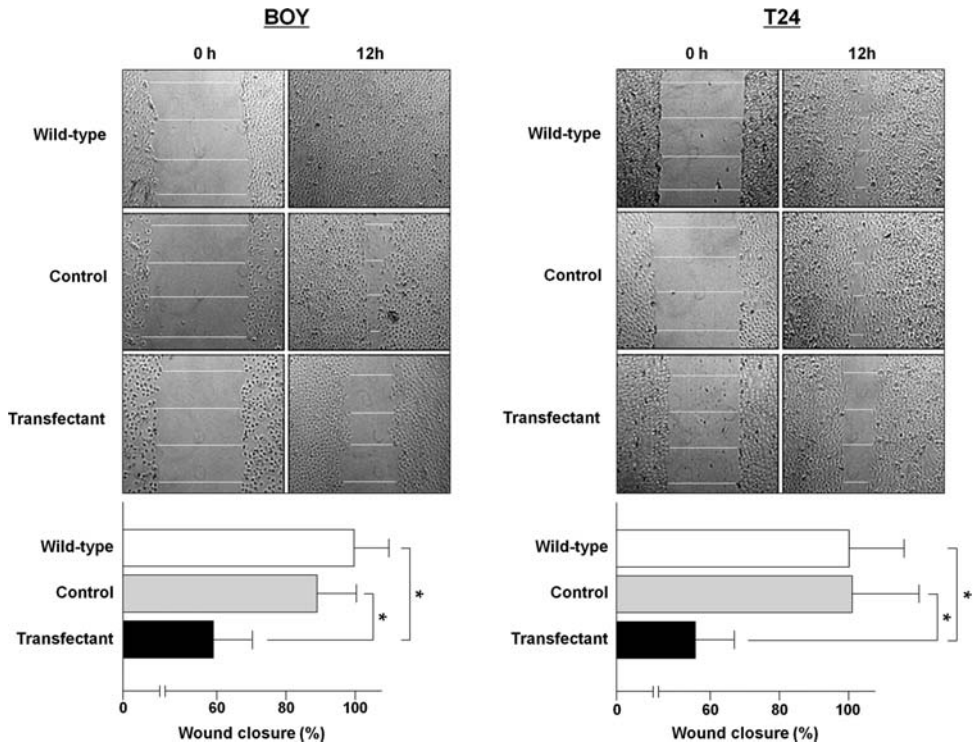

Figure 5. Percentage of wound closure corresponding to the distance between wound edges relative to the distance at time 0 h for each cell. Significant wound healing inhibition was observed in the CRBP1 transfectant after 12 h in comparison to the wild-type and the control. *p<0.0001.

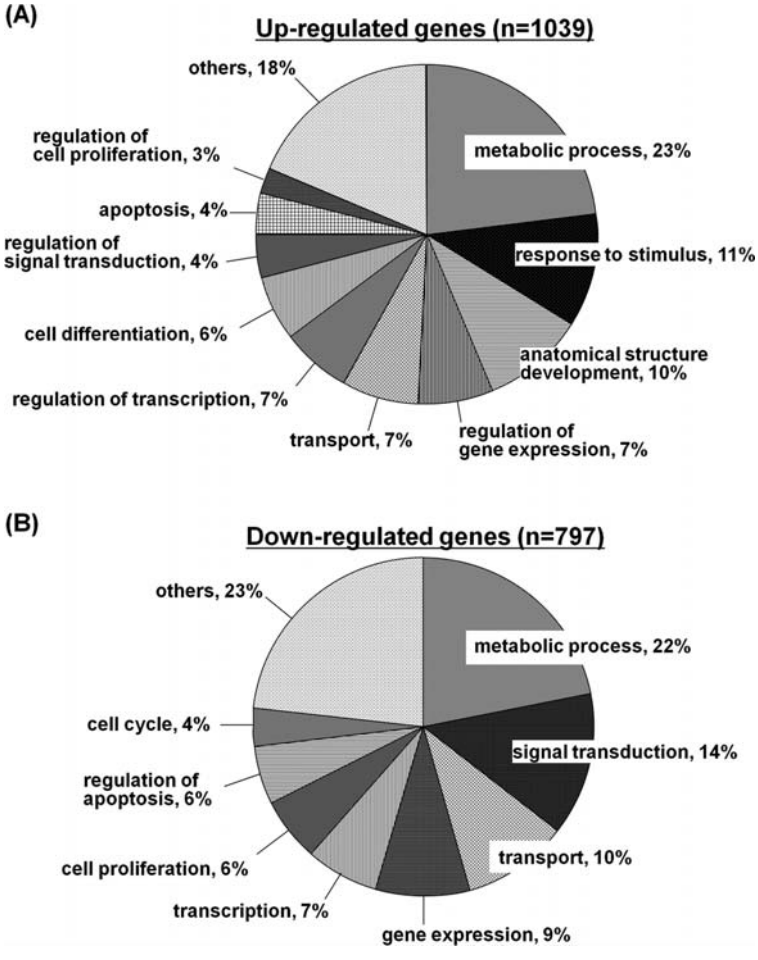

Figure 6. Distribution of altered expressions of functionally categorized genes in CRBP1 transfectants in comparison to the control transfectant. The functional features of the 1039 up- (A) and 797 down-regulated (B) genes were classified into 17 categories.

healing assays demonstrated significant cell migration inhibitions in the CRBP1 transfectants after 48 h of treatment (% of wound closure in BOY: 59.3±3.8, 89.1±1.9 and 100±10.8, respectively, $p<0.0001$, and that in T24: 57±7.5, 104.1±0.9 and 100±0.7, respectively, $p<0.0001$) (Fig. 5).

Gene expression analysis for CRBP1 overexpression. In order to gain further insight into which genes were affected by the activity of the *CRBP1* gene expression, we analyzed the gene expression profile of the *CRBP1* transfectants, in which the *CRBP1* protein was overwhelmingly expressed (Fig. 4A). We identified 1039 genes that were up-regulated >2-fold compared to the control transfectant (Fig. 6A). In contrast, 797 genes were down-regulated <2-fold (Fig. 6B). The functional annotations of the up- and down-regulated genes were classified into 17 categories (Fig. 6). Functionally, the up-regulated genes were involved in the metabolic process (23%), response to stimulus (11%), anatomical structure development (10%), regulation of gene expression (7%), transport (7%), regulation of transcription (7%), cell differentiation (6%), regulation of signal transduction (4%), apoptosis (4%), regulation of cell proliferation (3%) and others (18%) (Fig. 6A). The down-regulated genes were involved in the metabolic process (22%), signal transduction (14%), transport (10%), gene expression (9%), transcription (7%), cell proliferation (6%), regulation of apoptosis (6%), cell cycle (4%) and others (23%) (Fig. 6B).

Discussion

We have previously demonstrated that our profile for methylated genes in BC is preferable as we used microarray data from two simple hybridizations. In brief, we identified the genes methylated in human BC (Table III) by filtering the subset of genes restored after 5-aza-dC treatment in human BC cell lines from the down-regulated genes in the clinical BCs in our previous microarray (4). Based on this profile, we focused on the *CRBP1* gene in this study, and we confirmed that the promoter hypermethylation of *CRBP1* was significantly more frequent in BCs than in NBEs. Other sudies have also demonstrated that the promoter hypermethylation of *CRBP1* is a frequent event in various cancers, and that it inhibits the transcription of CRBP1 (10-18). However, there have not been many studies on the functional role of CRBP1 in BC. Consistent with previous studies on other malignancies, we also found *CRBP1* promoter hypermethylation and the frequent down-regulation of *CRBP1* mRNA in human BC. In addition, the demethylating agent restored *CRBP1* mRNA expression through the demethylation of the *CRBP1* promoter in BC cell lines, suggesting that the inactivation of the *CRBP1* gene by the promoter plays an important role in human BC development. The COBRA assays demonstrated that there were various methylation stages (complete and partial methylation as well as unmethylation) in the clinical BCs, suggesting that these degrees of methylation could contribute to a BC phenotype, which defines the malignant potential. These results led us to believe that it would be better to use a combination of RA and demethylating agents, such as aza-dC, for therapeutic strategies in patients with BC. There was no significant relationship in our cohort between the *CRBP1* expression and clinicopathological parameters including tumor stage and grade, and patient prognosis. However, a previous study demonstrated that CRBP1 gene expression was correlated with longer survival in laryngeal squamous cell carcinoma (23). The follow-up period was too short to evaluate prognosis in the present study.

It is known that *CRBP1* regulates intracellular RA transport, metabolism, and transcriptional activity (7). The loss of *CRBP1* could diminish RA transport and result in blockage of the transcription of RA-responsive genes that regulate cell proliferation. However, previous studies have supported the hypothesis that CRBP1 could be involved in the promotion of apoptosis through an increase in the RA metabolism in breast cancer cell lines (17,24,25). As the suppression of apoptosis played a key role in BC carcinogenesis (26), the observed low expression of CRBP1 in our study could disrupt the RA-mediated apoptosis pathway in BC. In clinical terms, the functional role of the *CRBP1* gene in human cancers still remains controversial. Many studies have shown that it is down-regulated and functions as a tumor suppressor gene (11-18), whereas it is up-regulated in cervical cancer (19). In breast cancer, CRBP1 down-regulation promoted the progression of tumors through the inhibition of RA receptor activity (24). In terms of BC, RA treatment effectively reduced the recurrence rate and increased the survival time of superficial BC patients (26,27). However, there have been no gain-of-function studies on the *CRBP1* gene. Therefore, we established CRBP1 transfectants using BC cell lines, and these significantly showed growth and migration inhibition in comparison to the controls. The data suggest that CRBP1 gene expression can cause a tumor suppressive function in BC through the intracellular RA metabolism. Further investigations are necessary to clarify whether these phenomena are critical in the development of BC.

In order to gain further insight into which genes are affected by *CRBP1* gene expression, we analyzed the gene expression profile of the *CRBP1* transfectants. The functional annotations of the up-regulated genes after CRBP1 transfection were distributed among 17 categories including apoptosis, anatomical structure development, cell cycle, and cell differentiation and proliferation. The up-regulated gene categories in the CRBP1 transfectants include tumor suppressive categories, such as anatomical structure development, cell differentiation and apoptosis. However, the down-regulated gene categories include oncogenic categories, such as signal transduction, transcription, cell proliferation and cell cycle. These results suggest that *CRBP1* activates other tumor suppressor genes and inactivates oncogenic genes in BC. Further investigations are required to test this hypothesis.

We concluded that the mechanism for CRBP1 down-regulation in BC cells occurs through the CpG hypermethylation of the promoter region. To the best of our knowledge, we demonstrate for the first time that the *CRBP1* gene can have a tumor suppressive function in human BC. The fact that the restored CRBP1 expression in the transfectants decreased cell growth and migration activity, indicates that this gene is a promising candidate for gene therapy of human BC.

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