CpG hypermethylation of *collagen type I α 2* contributes to proliferation and migration activity of human bladder cancer

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Abstract. In our microarray screening of methylated genes in bladder cancer (BC), the *collagen type 1 α 2* (*COL1A2*) gene was the most up-regulated among the 30,144 genes screened. We hypothesize that inactivation of the *COL1A2* gene through CpG methylation contributes to proliferation and migration activity of human BC. We subjected a bladder cancer cell line (BOY) and 67 BC specimens and 10 normal bladder epitheliums (NBEs) to conventional or real-time methylation quantitative polymerase chain reaction (PCR) and to real-time reverse transcriptase (RT)-PCR. We also established a stable COL1A2 transfectant for evaluating cell proliferation and migration activity. After 5-aza-dC treatment, the expression levels of *COL1A2* mRNA transcript markedly increased in BOY. Our cell proliferation assays consistently demonstrated growth inhibition in the COL1A2 transfectant compared with control and wild-type BOY cells (p<0.0001). Wound healing assays also showed significant wound healing inhibition in the COL1A2 transfectant compared to the counterparts (p=0.0016). We demonstrated by bisulfite DNA sequencing that the promoter hypermethylation of *COL1A2* was a frequent event in clinical BCs. The methylation index of *COL1A2* was significantly higher in the 67 BCs than in the 10 NBEs (p=0.0011). Conversely, *COL1A2* mRNA transcript was significantly lower in the BCs than in the NBEs (p=0.0052). The mechanism of COL1A2 down-regulation in BC is through CpG hypermethylation of the promoter region. *COL1A2* gene inactivation through CpG hypermethylation may contribute to proliferation and migration activity of BC.

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Introduction

Bladder cancer (BC), one of the five most common malignancies in the United States, is the 2nd most common tumor of the genitourinary tract and the 2nd most common cause of death in patients with urinary tract malignancies (1). As in other human cancers, loss of function of tumor suppressor genes and activation of oncogenes are two major alterations that promote BC initiation and progression (2). Recent intensive study of the epigenetic change of gene promoter hypermethylation has shown that it is an important molecular mechanism contributing to tumor suppressor gene inactivation in human cancer (3). There are numerous methods for large-scale screening for the detection of methylated genes in the human genome (4,5). However, most of them are technically complex (6). Our laboratory has developed a simple technique that combines microarray data from BC specimens with those from BC cells treated with pharmacological demethylating agent (5-aza-dC) (7). In this study, we used for the first time this combination analysis and identified 24 candidate hypermethylated genes. Among these genes, *collagen type I α 2* (*COL1A2*) gene was identified as the most up-regulated one.

Collagen type I, the most abundant molecule within the collagen family, consists of a heterotrimer with two α1 chains and one α2 chain. It is ubiquitous in skin, bone, tendon, dentin, etc (8). This type of collagen represents the major fibrillar component of the stroma in most solid malignancies, such as hepatoma, malignant melanoma and colorectal cancer (9-11). A particular extracellular matrix in stroma is necessary for the growth of tumors. The components of this matrix, in particular collagen type I, can be produced by both tumor cells and stromal fibroblast cells (11,12). In general, cancer cells producing type I collagen tend to have low tumorigenic potential (11-17). However, in some aggressive tumors, collagen type I is overexpressed and plays a role in tumor development (18,19). Thus, the functional role of collagen type I remains controversial and studies for collagen type I in BC are lacking in the literature. In this study, we evaluated the methylation status of *COL1A2* promoter and the expression of *COL1A2* mRNA in clinical BCs. Moreover, we established

COL1A2 transfectant and compared its proliferation and migration activity with those of the parental BC cells.

Materials and methods

Tissue samples. Tissue specimens were collected from 67 BC patients who had undergone radical cystectomy or transurethral resection of BC (TUR-Bt) at Kagoshima University Hospital between 2000 and 2005. Also used were 10 normal bladder epithelium (NBE) samples from patients with prostate cancer who had undergone radical prostatectomy. The background of patients is summarized in Table I. Of the 67 patients, seven were lost to follow-up. Written prior informed consent was obtained from all patients for use of their samples and clinical and pathological data. Each tumor was staged in accordance with the tumor-node-metastasis (TNM) staging system (20). A total of 77 fresh tissue samples [67 BCs and 10 normal bladder epitheliums (NBEs)] were frozen and stored at -80°C until being processed for DNA and RNA extraction.

Cell culture and 5-aza-dC treatment. The BC cell lines, KK47, T24, UMUC and BOY, which were established in our laboratory were maintained in minimum essential medium supplemented with 10% fetal calf serum and L-glutamine in a humidified atmosphere of 5% CO_2 and 95% air at 37°C (21). To screen for epigenetic alterations of gene methylation, the BOY cells were treated with the demethylating agent 5-aza-dC (1 μM). Cultured cells were harvested after four days of exposure to 5-aza-dC and the RNA was extracted.

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 was precipitated with ethanol. The total RNA was isolated from the frozen fresh tissues and BOY cells with Isogen (Nippon Gene, Tokyo, Japan) following the manufacturer's protocol. The concentrations of DNA and RNA were determined spectrophotometrically; their integrity was verified by gel electrophoresis. The RNA quality was checked in BioPhotometer™ (Eppendorf, Tokyo, Japan).

Antisense RNA (aRNA) amplification. We amplified aRNA from 5 μg of total RNA using the Amino Allyl MessageAmp™ aRNA amplification kit (Ambion, Austin, TX). We amplified single-stranded cDNA using an oligo-dT primer bearing a T7 promoter, converted the product into double-stranded cDNA, purified this cDNA and then performed amplification from double-stranded cDNA templates following the manufacturer's protocol.

Dye coupling and microarray hybridization. Oligoarrays (AceGene® Human Oligo Chip 30K (http://hitachisoft.jp/dnasis/ex_acegene/index.html), Hitachi Software Engineering Co. Ltd., Yokohama, Japan) spotted with 30,144 genes were used for dye coupling, for BOY study before (Cy3) and after (Cy5) 5-aza-dC treatment and for microarray hybridization. Pellets were formed with ethanol-precipitated aRNA (5 μg) and 5 μl of CyDye™ (GE Healthcare Bio-Sciences, Buckinghamshire, UK); 5X fragmentation buffer was added after purification. After further refinement, we obtained concentrated

Table I. Patient characteristics.

Bladder cancer (BC)	
Total number	67
Median age (range)	72.6 (40-100) years
Gender	
Male	53
Female	14
Stage	
Tis	2
Superficial (pTa)	13
Invasive (≥pT1)	44
Unknown	8
Grade	
G1	5
G2	32
G3	30
Operation	
Cystectomy	8
TUR-Bt	59
Pre-treatment	
TUR-Bt	5
Chemotherapy	2
BCG	1
Recurrence	
Recurrence (+)	19
Recurrence (-)	41
Follow-up period (range)	476 (5-1440) days
Normal bladder epithelium (NBE)	
Total number	10
Median age (range)	66 (58-77) years
Gender	
Male	10
Female	0

coupled aRNA. A hybridization solution was added to the microarrays and this was followed by 18-h incubation at 50°C.

cDNA preparation and conventional RT-PCR. We synthesized first-strand cDNA with 1 μg of total RNA using oligo-dT primers of the reverse transcription (RT) system (Promega, Tokyo, Japan). For conventional RT-PCR, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was the internal control. The primer sequences and RT-PCR conditions are listed in Table II. The PCR products were electrophoresed on 2.0% agarose gels.

Real-time quantitative RT-PCR. Gene-specific PCR products were assayed continuously using a 7900 real-time PCR system (Applied Biosystems, Foster City, CA) following the manufacturer's protocol. The initial PCR step was a 10-min hold at 95°C; the cycles (n=40) consisted of a 15-sec denaturation step at 95°C followed by a 1-min annealing/extension step at 63°C. All reactions were performed in

Table II. Primer sequence and PCR conditions.

|	Primer sequence (5'-3')	Product size (bp)	Annealing temperature, time (cycle)
Conventional RT-PCR			
COL1A2-sense	GGAGCTCCAAGGACAAGAAA	118	58°C, 30 sec (35)
COL1A2-antisense	ATGAAGGCAAGTTGGGTAGC		
G3PDH-sense	GAAGGTGAAGGTCGGAGTCA	255	55°C, 30 sec (26)
G3PDH-antisense	CACTTGATTTTGGAGGGATCTC		
Conventional MSP (COL1A2)			
Pan-sense	GAGATTTGTAAATTTTGTTTATGT	161	48°C, 60 sec (45)
Pan-antisense	TAAACACTTTTAAAACTTTCAAAAAA		
MSP-sense	GGGTTGTAGAGTATTTCGACG	101	55°C, 30 sec (40)
MSP-antisense	ACTCGTTATCTACATACCTCCG		
USP-sense	GGGTTGTAGAGTATTTTGATGTGT	101	55°C, 30 sec (40)
USP-antisense	CTAACTCATTATCTACATACCTCCA		
Real-time QMSP (COL1A2)			
QMSP-sense	GGGTTGTAGAGTATTTCGACG	101	55°C, 60 sec (45)
QMSP-antisense	ACTCGTTATCTACATACCTCCG		
Probe	TTAAATTTGGAAAGGGCG		
Real-time QMSP (MYOD1)			
QMSP-sense	ATTTATGTGGTGGGTGGATTTTGGT	117	55°C, 60 sec (45)
QMSP-antisense	ATCCCCTCTCTATCCCCAACC		
Probe	TCCCTTCCTATTCCTAAATCCAACCTAAATACCTCC		
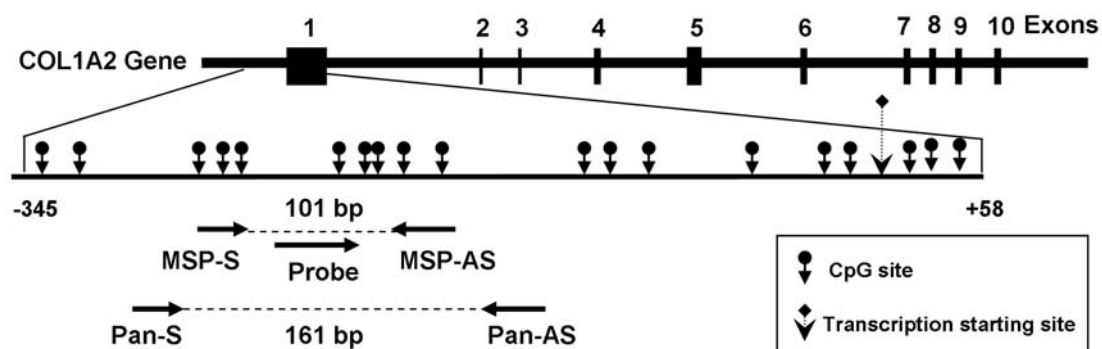
Figure 1. Schematic representation of the location of CpG sites and primers designed within the functional promoter of the *COL1A2* gene. First, universal PCR was performed with the universal primers Pan-sense (S) and Pan-antisense (AS), which do not carry any CpG sites. Conventional MSP and USP were with primer sets MSP-S and MSP-AS, and USP-S and USP-AS, respectively, and the universal PCR product was used as a template. For TaqMan quantitative methylation-specific PCR (QMSP), the fluorescent probe and primer sets were designed to hybridize to the amplified DNA region.

triplicate and a negative control lacking cDNA was included. For quantitative analysis, *Glucuronidase ß* (*GUSB*) was used as an internal control because it had shown the smallest Cy5/Cy3 fluctuation in our microarray analysis. The relative mRNA expression examined was normalized to the amount of GUSB in the same cDNA using the standard-curve method provided by the manufacturer. For TaqMan probes and primers for *COL1A1*/*COL1A2* and *GUSB*, we used assay-on-demand gene expression products (Applied Biosystems). For normal bladder controls we prepared cDNA samples from 2 different lots of human bladder total RNA (Chemicon International, Inc., Temecula, CA, USA). All reactions were performed in duplicate.

Conventional methylation-specific PCR. Genomic DNA (100 ng) from BCs and NBEs was subjected to sodium bisulfite modification using the CpGenome™ DNA modification kit (Millipore, Billerica, MA). According to the promoter information of *COL1A2* (22), methylation-specific (MSP) and unmethylation-specific (USP) primers were designed based on the functional promoter sequence using MethPrimer software (http://www.urogene.org/methprimer). An initial PCR product was created with universal primers (Pan-S and Pan-AS), of which the forward and reverse primers carry no CpG sites; this was followed by a second nested PCR with primers specific for MSP or USP (Fig. 1). The primer sequences and PCR conditions are listed in Table II. In each

assay, the absence of a DNA template served as the negative control. The obtained MSP and USP products were analyzed by electrophoresis in 2% agarose gels.

Real-time quantitative methylation-specific PCR. For TaqMan quantitative methylation-specific PCR (QMSP), the fluorescent probe and primer sets were designed to also hybridize to the amplified region of DNA (Fig. 1). Myogenic differentiation-1 (MYOD1) primer sequences were used as an internal control. The first PCR was amplified with universal primers (Pan-S and Pan-AS) using 3 μl of bisulfite-treated DNA in a total volume of 20 μl. The first PCR step consisted of initial denaturation (10 min, 95°C) followed by 15 cycles of a 1-min denaturation step at 95°C, an annealing step at 48°C and an extension step at 72°C. A second round of nested QMSP was then carried out using 1 μl of the first PCR product. The PCR cycle was programmed up to 45 cycles and all reactions were performed in duplicate. The primers and probe sequences are listed in Table II. Serial dilutions of CpGenome universal methylated DNA (Millipore, Tokyo, Japan) were used to construct a calibration curve. The methylation index was defined as the quantity of fluorescence intensity derived from *COL1A2* promoter amplification divided by the fluorescence intensity from MYOD1 amplification, multiplied by 1000.

Bisulfite DNA sequencing analysis. Bisulfite-modified DNA was amplified using a pair of universal primers (Pan-S and Pan-AS) 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-S primer was carried out following the manufacturer's instructions (Applied Biosystems).

Construction of COL1A2 expression vectors and transfection to BOY. The *COL1A2* vector was constructed by inserting full-length *COL1A2* cDNA into the *Xba*I and *Bam*HI restriction site of the pBApo-CMV Neo™ vector (Takara Bio, Otsu, Japan). The *COL1A2*- and the non-targeting (control) vector were transfected into BOY cells by calcium phosphate co-precipitation. The BOY cells were split and grown in selective medium with 1000 mg/l of G418. After 2 weeks, G418-resistant colonies were chosen and expanded in medium containing 100 mg/l of G418.

Immunoblotting. Total protein lysate was prepared with detergent lysis buffer in the presence of a protease inhibitor. The 20 μg of protein lysate was separated by NuPAGE on 4-12% bis-tris gel (Invitrogen, Tokyo) and transferred into a polyvinylidene fluoride (PVDF) membrane. Immunoblotting was done with diluted (1:1000) polyclonal COL1A2 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 echochemiluminescence detection system (GE Healthcare Bio-Sciences).

Immunocytochemistry. For COL1A2 immunostaining we used cell monolayers grown (48 h, 37°C, 5% CO$_2$) on poly-L-lysine-coated chamber glass (Asahi Techno Glass Corp., Tokyo,

Japan), rinsed once with phosphate-buffered saline (PBS), and treated sequentially for 3 min each with 70-, 90- and 100% acetone at -20°C. After 30-min immersion in blocking buffer [0.01 M PBS (1.0 l) + goat serum (20 ml) + Triton-X (2 ml) + bovine serum albumin (1.0 g)], we added polyclonal antibody for COL1A2 (sc-8786; 1:500 dilution) overnight. Immuno-staining was visualized with fluorescein isothio-cyanate (FITC)-labeled second antibody (#111-096-003, Jackson Immuno Research) and photographed with an all-in-one digital microscope (Keyence Japan, Osaka, Japan). For the control, all experiments were performed without the primary antibody.

3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. COL1A2 transfectant, control and wild-type BOY cells were seeded at a density of 10,000 cells/well in 96-well microplates and incubated for 24, 48, 72, 96 or 120 h. Then, 50 μl of 1-mg/ml MTT in PBS were added to each well and the plates were incubated for an additional 4 h. The resulting formazan was dissolved with 100 μl of dimethyl-sulfoxide (DMSO) after aspiration of the culture medium. The plates were placed on a plate shaker for 5 min and read immediately at 570 nm using a microplate reader (MPR-A4i; Tosho, Tokyo, Japan).

Wound healing assay. COL1A2 transfectant, control, and wild-type BOY cells were used in the 'wound healing' assay to test the alteration of cell motility and migration. Cells were initially seeded uniformly onto 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. Micro-photographs 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.

Collagen analysis. Collagen was extracted from conditioned medium by pepsin digestion and separated by repeated salt fractionation, as described previously (10). The concentration of NaCl is 0.7 M and purified collagen was analyzed by sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) using 2-15% gradient gel. Human Collagen Type I® (Applied Cell Biotechnologies, Yokohama, Japan) was used as a positive control for distinguishing α1- from α2 chain of type I collagen.

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 was Expert StatView (version 4, SAS Institute Inc., Cary, NC); for the comparison test among the three groups, the non-adjusted statistical level of significance of p<0.05 corresponds to a Bonferroni-adjusted level of p<0.0167. The molecular functions of the up-regulated genes after 5-aza-dC treatment were referenced using Gene Ontology (http://www.gene ontology.org/) and GeneCards (http://www.genecards.org/index.shtml).
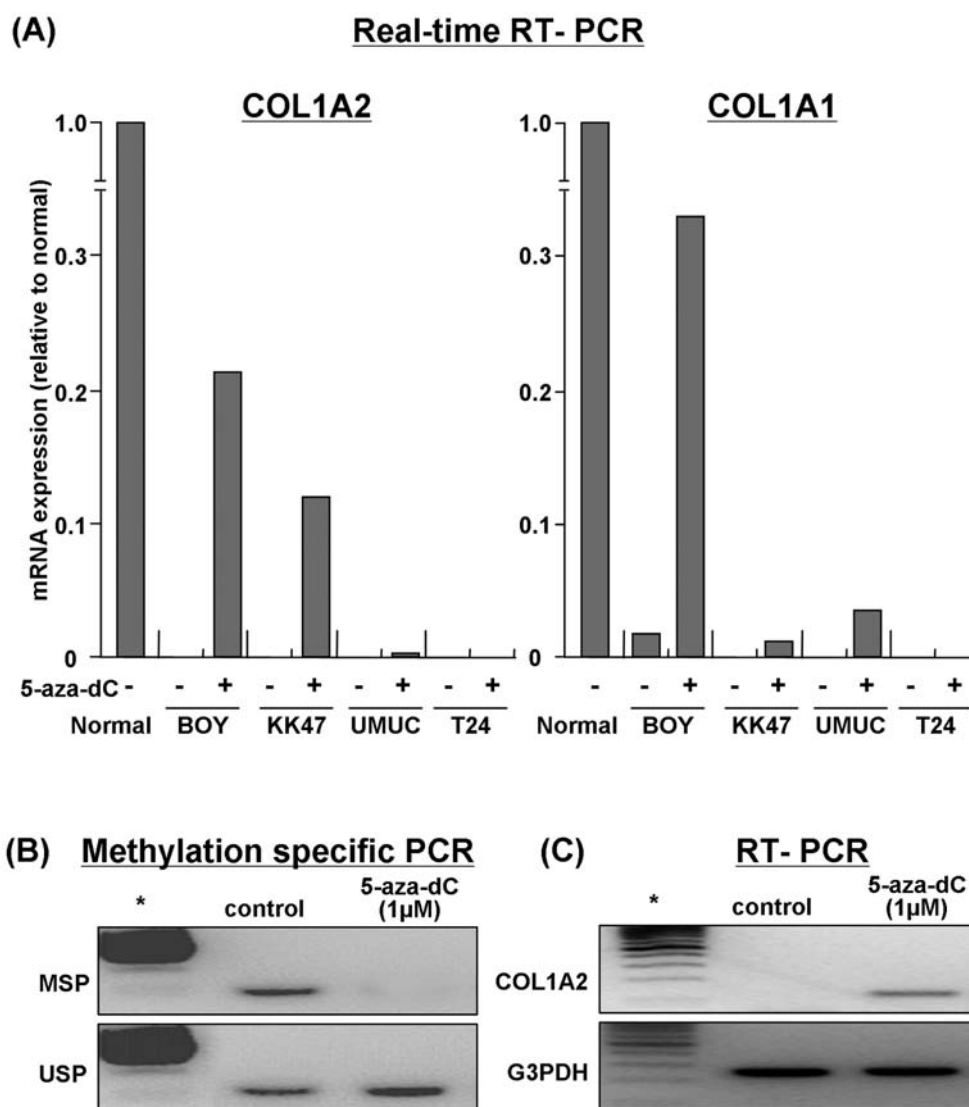
Figure 2. *COL1A1/COL1A2* mRNA expression in BC cells before and after treatment with 5-aza-dC (A). Methylation-specific PCR (MSP) (B) and conventional RT-PCR (C) demonstrated that promoter hypermethylation of *COL1A2* gene was markedly decreased and that the mRNA transcript of the *COL1A2* gene was markedly increased after 5-aza-dC treatment in BOY cells. PCR conditions and primer sequences are described in Table II. *100 bp DNA ladder.

Results

Identification of genes up-regulated in 5-aza-dC-treated BOY cells. Among the 30,144 genes, we identified 24 that were up-regulated >1.5-fold in 5-aza-dC-treated compared to untreated BOY cells by filtering the down-regulated genes in the BCs used in our previous microarray (7) (Table III). Among these, 15 harbored CpG islands in their putative promoter regions. Among the 24 candidate genes, we focused on *COL1A2* and *COL1A1* because these genes are the top 2 up-regulated one (6.01- and 4.03-fold) of all the 24 genes tested.

Verification of altered COL1A2 and COL1A1 mRNA expression in BC cells before and after treatment of 5-aza-dC and methylation status of the COL1A2 gene promoter. Four BC cell lines (BOY, KK47, UMUC and T24) were subjected to real-time RT-PCR to evaluate the mRNA expression levels of *COL1A2* and *COL1A1* (Fig. 2A). Before 5-aza-dC treatment, no mRNA expression of *COL1A2* was observed,

but we found certain expression levels of *COL1A1* in BOY cells. In contrast, marked expression was observed in normal human bladder RNA. After 5-aza-dC treatment, *COL1A2* and *COL1A1* mRNA expression was overwhelmingly increased especially in BOY cells, which had been subjected to cDNA microarray analysis mentioned above. We found no retrieval of the mRNA expression in T24 cells. Therefore, we selected BOY cells for further experiments. The MSP and USP assays for *COL1A2* for the BOY cells before and after treatment with 5-aza-dC are shown in Fig. 2B. Treatment with 5-aza-dC eliminated the MSP-positive band in BOY cells (Fig. 2B). Conventional RT-PCR also demonstrated that the expression level of *COL1A2* mRNA transcripts was markedly increased in BOY cells treated with 5-aza-dC (1 μM) compared to that of the untreated control (Fig. 2C).

Identification of COL1A2 expression in transfected cells and effect of COL1A2 overexpression on cell growth. Because we found no mRNA expression of *COL1A2* in wild-type BOY cells and marked retrieval of the gene expression after the

Table III. Top 20 methylated genes in BC.

| No.	Symbol	Gene name	Accession	UniGene	Location	Function	Fold changes	CpG island
1	*COL1A2*	α 2 type 1 collagen	NM_000089	Hs.489142	7q22.1	Skeletal development	6.01	Yes
2	*COL1A1*	α 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 via 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*	α-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	Unk
14	*NICAL*	Hypothetical protein flj11937	NM_022765	Hs.33476	6q21	Signal transduction	1.95	Yes
15	-	Immunoglobulin m heavy chain	AF267819_1	-	-	-	1.95	Unk
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*	α 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

Unk, unknown.
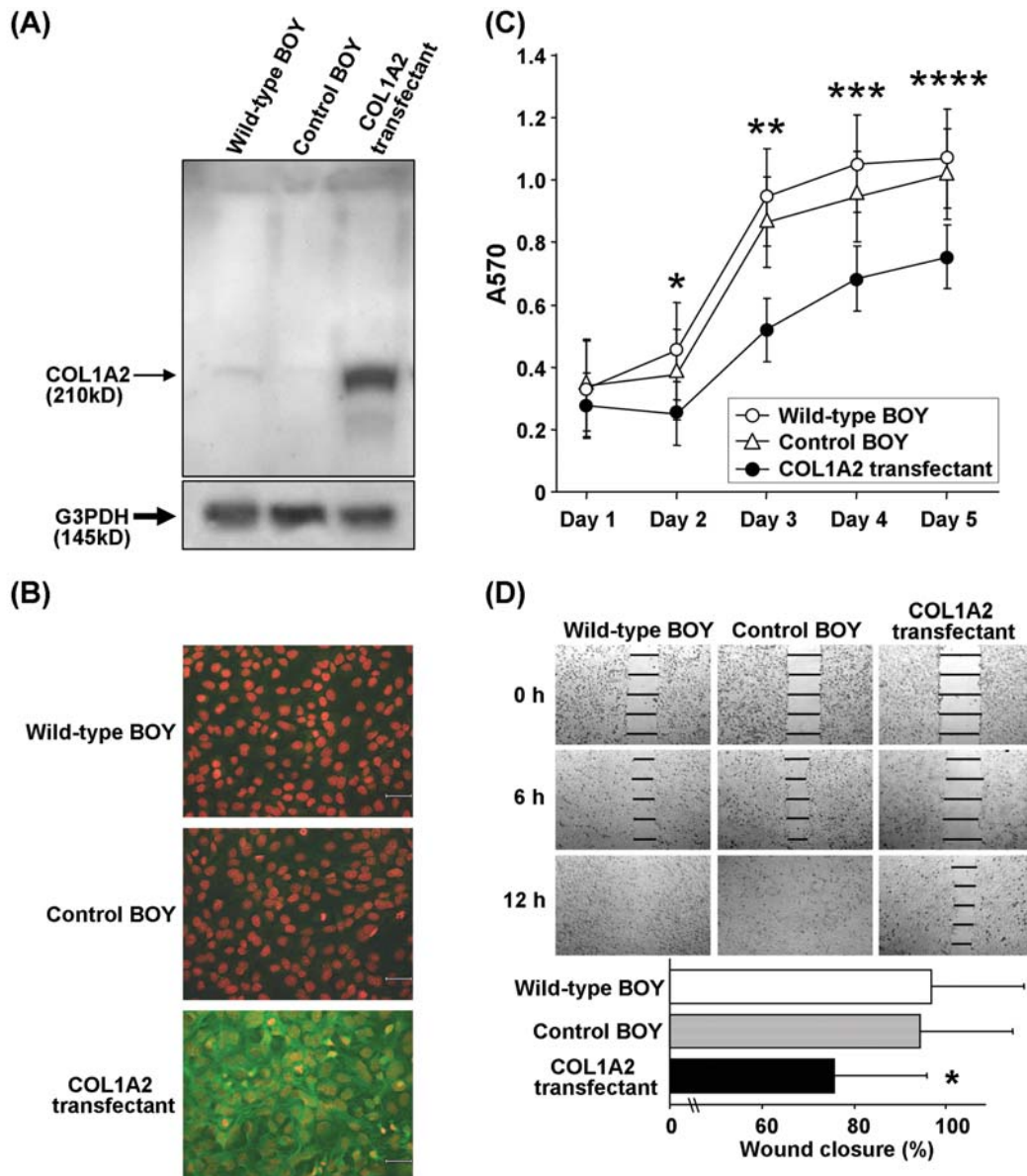
Figure 3. Effect of COL1A2 transfection on the growth of BOY cells. (A) COL1A2 expression determined by immunoblotting. Lane 1, wild-type BOY cells; lane 2, cells treated with the control vector; lane 3, COL1A2-transfected BOY cells. (B) COL1A2 protein expression visualized by immunocytochemistry. In the COL1A2 transfectant, COL1A2 protein was overwhelmingly expressed compared to wild-type and control BOY cells. (C) Cell growth determined by MTT assay. Compared to wild-type and control BOY cells, cell growth was significantly reduced in the COL1A2 transfectant (p<0.0001 day 2, 3, 4 and 5). (D) Percentage of wound closure corresponds to the distance between wound edges in at least three randomly chosen regions (mean ± SD) relative to the distance at time 0 h for each cell. A significant wound healing inhibition was observed in the COL1A2 transfectant after 12 h (p<0.0016) compared with wild-type and control BOY cells.

demethylating agent, we established a COL1A2 transfectant from the cells. Immunoblotting showed a 210 kDa COL1A2 protein in the COL1A2 transfectant at levels >400-fold higher than controls (Fig. 3A). Immunocytochemical examination showed that COL1A2 protein was highly expressed in COL1A2-transfected cells, whereas it was absent in control and wild-type BOY cells (Fig. 3B). To evaluate the relationship between *COL1A2* expression and cell growth, we performed MTT assays using COL1A2 transfectant, control and wild-type BOY cells. Three independent MTT assays consistently demonstrated significant growth inhibition in COL1A2 transfectant compared with control and wild-type BOY cells on day 2, 3, 4 and 5 (p<0.0001) (Fig. 3C). To examine the cellular migration ability, we performed wound

healing assays using COL1A2 transfectant, control and wild-type BOY cells. Three independent wound healing assays showed significant wound healing inhibition in COL1A2 transfectant after 12 h of treatment (p<0.0016) (Fig. 3D).

Verification of collagen products in cell culture medium of COL1A2 transfectant and detection of intracellular COL1A1/ COL1A2 mRNA expression in the transfectant. To examine whether induced COL1A2 was secreted to extra-cellular matrix (ECM), collagen products of the transfectant was assayed by pepsin treating medium and separated on SDS gel electrophoresis. We found both collagen chain (α1 and α2) were secreted to the culture medium of COL1A2 transfectant, whereas no collagen chain was detected in that of the control
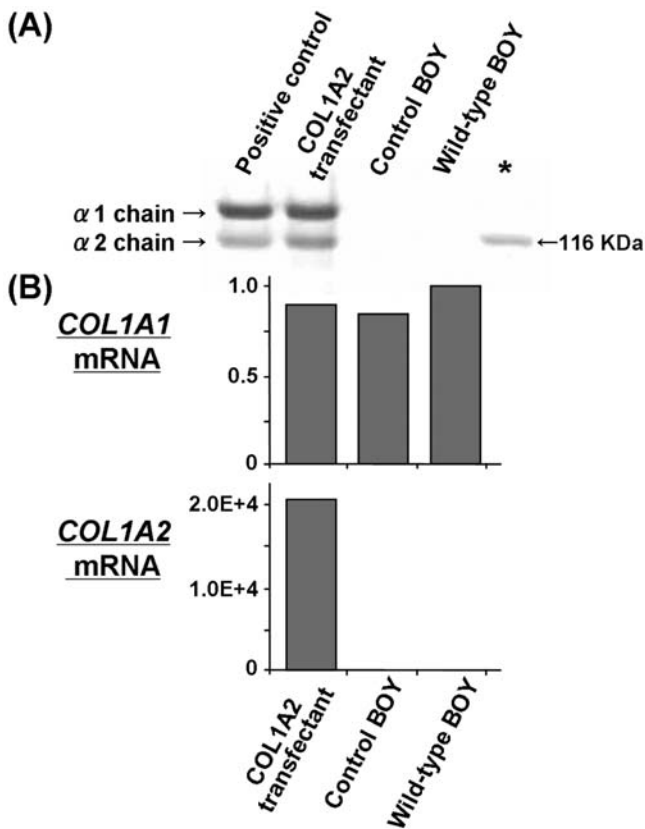
Figure 4. Collagen secretion in cell culture medium of COL1A2 transfectant, control and wild-type BOY cells. (A) α1- and α2 chain of type I collagen secretion pattern in cell culture medium. Lane 1, positive control; lane 2, COL1A2-transfected BOY cells; lane 3, cells treated with the control vector; lane 4, wild-type BOY cells. Positive control indicates heterotrimer formation of human type I collagen. *Protein marker. (B) Detection of intracellular *COL1A1/COL1A2* mRNA expression of COL1A2 transfectant, control and wild-type BOY cells. Y-axis indicates mRNA expression levels relative to wild-type BOY.

and wild-type BOY cells (Fig. 4A). The ratio of α1 to α2 in secreted collagen of COL1A2 transfectant was close to 2 to 1 ratio, as was the same ratio of the heterotrimer-positive control. We found that mRNA expression of *COL1A1* was still conserved in COL1A2 transfectant, as was similarly in control or wild-type BOY cells (Fig. 4B, upper). The expression levels of *COL1A2* mRNA in COL1A2 transfectant was ~20,000-fold higher than that in control or wild-type BOY cells (Fig. 4B, lower).

COL1A2 mRNA expression and methylation status of the COL1A2 gene promoter in clinical BC samples. Representative results of MSP and USP assays for *COL1A2* in clinical BCs are shown in Fig. 5A. In our series, positive MSP bands were found in 42 of the 67 BCs (62.7%) and no visible MSP band was detected in all 10 NBEs (data not shown). Fig. 5B shows typical bisulfite DNA sequencing results for BC samples corresponding to samples 'U' and 'P' in Fig. 5A. In sample 'U', with a modest USP and no MSP band, there was a modest 'T' peak and no 'C' peak at each CpG site (Fig. 5B, upper). In sample 'P', with a modest USP and a strong MSP band, there was a modest 'T' peak along with a stronger 'C' peak at each CpG site (Fig. 5B, lower). We also performed QMSP and real-time quantitative RT-PCR to evaluate the methylation

status of the *COL1A2* promoter region and the expression levels of *COL1A2* mRNA. The methylation index of the BCs was significantly higher than that of the NBEs (BC, 42.3±28.9; NBE, 0.07±0.01, p=0.0011) (Fig. 5C, left). In contrast, the *COL1A2* mRNA expression level of the BCs was significantly lower than that of the NBEs (BC, 10.1±2.6; NBE, 15.8±3.8, p=0.0052) (Fig. 5C, right). We found no relationship between the histopathological cancer type and the methylation index or COL1A2 mRNA expression.

Discussion

A previous study demonstrated a gene expression profile of hypermethylated genes in BC cells treated with 5-aza-dC with an oligonucleotide microarray (23). However, these gene profiles from specific cancer cells are not always same as those from clinical cancer specimens. The CpG island array is another tool for high-throughput methylation analysis for detecting the aberrant methylation status of gene promoter in cancer specimens (4,5). However, the protocol for target preparation is complicated. Most of the reported methods are aimed at selective enrichment for the methylated fraction of the genome and use either methylation-sensitive restriction enzymes or antibodies that recognize 5-methyl cytidine (4,5). There are major problems with each reported method. Methods that rely on frequent sites of restriction enzymes (HpaII/ MspI) result in a high genome fraction to be amplified (high complexity), which limits PCR efficiency and ends up favoring non-CpG island DNA. The sensitivity of antibody-based methods is undetermined and possibly low. Finally, there are limited validation data on microarray results for a gold-standard technique (bisulfite-PCR). Thus, false data may lead researchers in the wrong directions. To address these issues, we identified the genes methylated in human BC by filtering the subset of genes restored after 5-aza-dC treatment in human BC cells from the down-regulated genes in the clinical BCs in our previous microarray (7). Our profile for methylated genes in BC cells is preferable because we used microarray data from two simple hybridizations. In addition, we confirmed that the promoter hypermethylation of *COL1A2*, which was at the top of our profile, was significantly frequent in BCs compared to that in NBEs. A significantly higher mRNA expression of *COL1A2* was also confirmed in our series of BCs and NBEs. There was no significant relationship in our cohort between the *COL1A2* expression and clinicopathological parameters including tumor stage, tumor grade and patient prognosis. Altered expression of the *COL1A2* is a frequent event, but it may function early in BC carcinogenesis.

Previous studies demonstrated that DNA methylation inhibits transcription of COL1A2 promoters (12,13). Consistent with their study, we also found COL1A2 is frequently down-regulated through promoter CpG hyper-methylation in human BC. Previous oligonucleotide microarray analyses showed down-regulation of *COL1A2* transcript in some human malignancies, such as BC, hepatoma and malignant melanoma (7,9,10). The functional role of the *COL1A2* gene remains controversial. It has been reported as an onco-related molecule as well as a potential tumor-suppressor gene (12,19). What is the significance of decreased collagen synthesis in cancer? Type I collagen consists of a
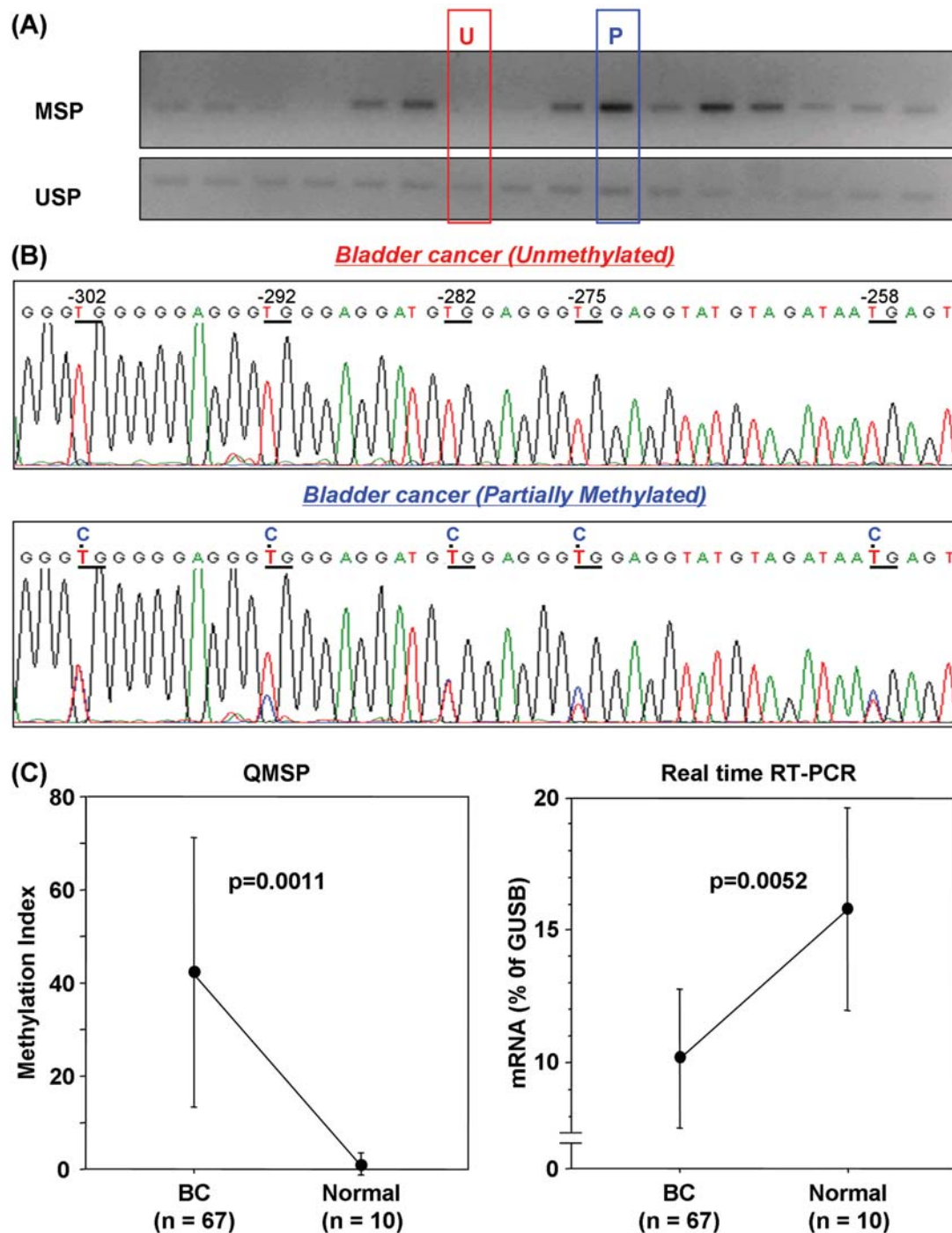
Figure 5. Methylation status of the *COL1A2* promoter and *COL1A2* mRNA expression in clinical samples. (A) Representative results of MSP (top) and USP (bottom) of BC samples. (B) Bisulfite DNA sequencing of samples with no (sample 'U') and strong (sample 'P') MSP bands. Sample 'U', which manifested no MSP and a modest USP band, exhibited a very low 'C' peak along with a modest 'T' peak at each CpG site (top). Sample 'P', which manifested a strong MSP and a modest USP band, exhibited a stronger 'C' peak along with a modest 'T' peak at each CpG site (bottom). PCR conditions and primer sequences are listed in Table II. (C) The *COL1A2* methylation index was significantly higher in the BCs than NBEs (p=0.0011) (left). Conversely, *COL1A2* mRNA expression was significantly lower in the BCs than NBEs (p=0.0052) (right).

heterotrimer with two similar procollagens: α1 chains (COL1A1) and α2 chain (COL1A2). In our profile, *COL1A1* and *COL1A2* are the top 2 up-regulated mRNA transcripts after treatment of the demethylating agent. Simultaneous inactivation of both genes by promoter methylation results in low expression levels of type I collagen protein, the major fibrillar component of the stroma. However, previous studies suggest that these procollagens may function independently regardless of a heterotrimer structure (12,15). Some investi-

gators have reported that repression of the COL1A2 protein due to hypermethylation of the gene promoter was observed in tumorigenic cell lines (11,13). Moreover, stable transfectant of the COL1A2 in a tumorigenic cell line showed a flatter morphology with increased adherence to the substratum, a reduced ability to proliferate in soft agar, slower growth kinetics and suppression of tumorigenicity (12,15). Other investigators have demonstrated that decreased collagen production by tumorigenic cells correlates with faster cell

growth, less adhesion to substratum and increased tumorigenicity of the transformed cells (16,17). However, in some malignancies, COL1A2 is overexpressed and plays a role in tumor development through angiogenesis and interactions with integrin or TGF-ß signaling pathways (18,19). To analyze the functional consequences of COL1A2 silencing in BC cells, we established a stable COL1A2 transfectant. Our cell proliferation assays consistently demonstrated growth inhibition in the COL1A2 transfectant compared with control and wild-type BC cells. Wound healing assays showed significant wound healing inhibition in the COL1A2 transfectant. Our collagen analysis revealed that heterotrimer collagen was produced in cell culture medium of the COL1A2 transfectant. Because we detected *COL1A1* mRNA expression inherent in COL1A2 transfectants as well as wild-type, induced COL1A2 seems to be secreted as heterotrimer formation (two α1- and one α2-chain) by coupling with COL1A1. Consistent with a previous study (12), these results suggest that cell growth- and migration inhibition may caused by increased cell adhesion to the heterotrimer collagen secreted in ECM of the COL1A2 transfectant. Hence, decreases in collagen production contribute to the tumorigenic phenotype by changing the cell matrix interactions. However, functional roles of the procollagen intracellular still remains to be elucidated. Further experiments will determine whether COL1A2 suppresses intracellular signal transduction pathways for tumorigenicity. In our clinical samples, we found no relationship between the histopathological cancer type and the methylation index or *COL1A2* mRNA expression. The cohort includes many samples from TUR-Bt. Pathological diagnosis regarding tumor stage or grade tends to be incorrect because of the tissue degradation by electric coagulation. It may influence statistical accuracy. To our knowledge, we demonstrated for the first time the silencing of COL1A2 in BC cells. We conclude that the mechanism of COL1A2 down-regulation in BC cells is through CpG hypermethylation of the promoter region. The fact that restored COL1A2 expression in the transfectant decreased cell growth and migration activity indicates that COL1A2 is a promising candidate for gene therapy of human BC.

Acknowledgements

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