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Methylation of a CpG Island within the Uroplakin Ib Promoter: A Possible Mechanism for Loss of Uroplakin Ib Expression in Bladder Carcinoma[1]

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Abstract

Uroplakin Ib is a structural protein on the surface of urothelial cells. Expression of uroplakin Ib mRNA is reduced or absent in many transitional cell carcinomas (TCCs) but molecular mechanisms underlying loss of expression remain to be determined. Analysis of the uroplakin Ib promoter identified a weak CpG island spanning the proximal promoter, exon 1, and the beginning of intron 1. This study examined the hypothesis that methylation of this CpG island regulates uroplakin Ib expression. Uroplakin Ib mRNA levels were determined by reverse transcription polymerase chain reaction and CpG methylation was assessed by bisulfite modification of DNA, PCR, and sequencing. A correlation was demonstrated in 15 TCC lines between uroplakin Ib mRNA expression and lack of CpG methylation. In support of a regulatory role for methylation, incubating uroplakin Ib–negative lines with 5-aza-2′-deoxycytidine reactivated uroplakin Ib mRNA expression. A trend between uroplakin Ib mRNA expression and CpG methylation was also observed in normal urothelium and bladder carcinomas. In particular, loss of uroplakin Ib expression correlated with methylation of a putative Sp1/NFκB binding motif. The data are consistent with the hypothesis that methylation of specific sites within the uroplakin Ib promoter may be an important factor in the loss of uroplakin Ib expression in TCCs.

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Keywords: CpG methylation, uroplakin, bladder carcinoma, promoter, gene expression.

Introduction

Uroplakin Ib belongs to the tetraspanin family of membrane proteins [1–3] and forms plaque complexes with uroplakins Ia, II, and III along the asymmetric unit membrane (AUM) of umbrella cells of mammalian bladders [4–6]. Formation of these plaques by specific interactions between the four uroplakins provides the luminal surface of the bladder with strength, flexibility, and an impermeable barrier to prevent urine from invading underlying tissues. Uroplakin Ib is also required for export of uroplakin III from the Golgi apparatus to form mature plaques on the apical surface of urothelial cells [7]. Because complexes of uroplakin Ib and III are essential for the assembly of plaques on the mature fully differentiated urothelium, these data support the hypothesis that uroplakin Ib has a key functional role in driving the final stages of urothelial differentiation. Although initially considered to be urothelium-specific, recent reports have suggested that uroplakin Ib is also expressed in the cornea and conjunctival epithelium [8], trachea, placenta, pancreas, and kidney [9]. However, given that a definitive biological function for uroplakin Ib is yet to be determined, the importance of these observations is not clear.

Reduced expression by varying degrees or a disordered pattern of uroplakin expression is common in transitional cell carcinomas (TCCs). For example, there is loss of uroplakin II protein in 60% of TCCs [10]. Similarly, a disordered localization or loss of uroplakin III protein occurs in a high proportion (50%) of invasive, but not noninvasive, TCCs [11]. More recent studies have examined the feasibility of using changes in uroplakin expression as markers for primary and metastatic urothelial carcinomas. Thus far, preliminary results have been encouraging. For example, circulating uroplakin II mRNA–positive cells were detected in 3 of 29 (10.3%) patients with superficial cancers, 4 of 14 (28.6%) patients with muscularly invasive cancers, 2 of 5 (40.0%) locoregional node–positive patients, and 6 of 8 (75.0%) patients with distant metastases, although the percentage of the primary tumors positive for uroplakin II was not reported [12]. In another large immunohistochemical study using paraffin-embedded tissues, expression of uroplakin III was detected in 60% of primary tumors and 53% of metastases [13].

In a previous study using Northern analysis, we reported a high frequency (60%) of loss or downregulation of uroplakin

Abbreviation: TCC, transitional cell carcinoma

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Ib mRNA expression in samples of bladder TCC [14]. The small sample size precluded accurate correlations between levels of uroplakin Ib expression and cancer stage; however, the preliminary data suggested that those tumors without uroplakin Ib expression were frequently invasive in nature. Consistent with this idea, a loss of uroplakin Ib expression in 50% of invasive TCCs, but not in early-stage tumors, has been reported in a study using *in situ* hybridization [15]. The significance of this loss to bladder carcinoma progression and to invasive disease is not yet clear, but could reflect a loss of differentiation and may also be a useful prognostic indicator.

Currently, the molecular mechanisms that normally regulate expression of uroplakin Ib and that might be altered in urothelial tumor progression are unknown. One common mechanism involved in silencing gene expression in tumors and tumor cell lines is hypermethylation of CpG islands within the 5′ promoter regions of genes, an epigenetic mechanism in which DNA methyl transferases act in concert with other proteins, such as methylation-dependent binding proteins and histone deacetylases (reviewed in Ref. [16]). Promoter hypermethylation has also been shown to be an important mechanism for specific gene silencing in bladder cancer [17–21].

In the current study, we show that the proximal promoter region of the uroplakin Ib gene contains a weak CpG island and address the hypothesis that CpG methylation of the promoter might be responsible for the downregulation of uroplakin Ib expression in bladder carcinomas. Our data demonstrate that methylation of specific CpG sites, including putative binding sites for Sp1 and NFκB located within the CpG island, is associated with absent or greatly reduced uroplakin Ib mRNA expression in normal nonurothelial tissues *versus* bladder tumor cell lines and TCCs.

Materials and Methods

Bladder Carcinoma Cell Lines and Tissues

The TCC-Sup, Sca-BER, J82, and T24 cell lines derived from TCCs and the SV40-transformed uroepithelial cell line SV-HUC-1 were all obtained from ATCC (Rockville, MD). Dr. D. Leavesley (Royal Adelaide Hospital, Adelaide, South Australia, Australia) kindly provided the 5637 cell line; characterization of TCC lines BL13, BL17/0/×1, BL17/2, BL17/5, and BL28 has been described previously [22–24]. HT1376, SD, SW1710, VM-Cub1, and VM-Cub3 cell lines were provided by Dr. Marc-Oliver Grimm (Department of Urology, Heinrich-Heine University, Dusseldorf, Germany). RT112 cells were obtained from Prof. Pamela Russell (Oncology Research Centre, Prince of Wales Hospital, Sydney, Australia). SV-HUC-1 cells were grown in F12 medium supplemented with 10% fetal bovine serum. Other lines were maintained in Dulbecco's modified Eagle's medium, pH 7.4, supplemented with 15% fetal calf serum (FCS) for VMCub-1, VM-Cub3, HT1376 SD, SW1710, and RT112 or RPMI 1640 with 10% FCS for all remaining cell lines, at 37°C in an atmosphere of 5% CO_2.

Samples of normal tissue (renal pelvis, ureter, and colon) and bladder tumor (GI/GII, GII, GIII, and carcinoma *in situ*) were obtained with informed consent at operation, snap-frozen in liquid nitrogen, and stored at −80°C. Frozen sections were stained with hematoxylin and eosin to identify urothelial tissues, colonic epithelium, or carcinoma, and microdissected prior to analysis. The patients who supplied the normal samples of urothelium did not suffer from any malignant urological diseases. Peripheral blood mononuclear cells were obtained from a normal volunteer.

Isolation of DNA and RNA from Cell Lines and Tissue Samples

DNA was isolated from tissues and cell lines using a salting-out method [25]. Total cellular RNA was isolated from tissues or cell cultures when 80% to 90% confluent using TriZol (Invitrogen, Carlsbad, CA) or TriReagent (Sigma Aldrich, Castle Hill, New South Wales, Australia), respectively, according to the manufacturers' instructions. After phenol/chloroform extraction to remove residual DNA, 2 μg was used to prepare cDNA.

Expression of Uroplakin Ib mRNA by Semiquantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA (2 μg) was used to prepare cDNA in 19 μl of RNAse-free water with 1 μl of 100 mM random hexamer, 6 μl of MMLV RT 5 × reaction buffer, and 1.5 μl of 10 mM dNTP mix. After incubation at 72°C for 3 minutes, 0.6 μl of RNAsin (1 U/μl) and 2 μl of MMLV reverse transcriptase (13.3 U/μl; MBI Fermentas, Vilnius, Lithuania) were added, and incubation was continued at 37°C for 2 hours, then 50°C for 10 minutes, before termination at 95°C for 5 minutes. In each subsequent PCR reaction, 2 μl of cDNA was used. Uroplakin Ib levels were determined by amplification of a 741-bp product using forward and reverse primers (F1 and R1; Table 1) in a PCR reaction containing 5 μl 10 × buffer, 0.5 μl of 25 mM dNTP, 4.0 μl of each primer at 20 pmol/μl, 1.0 μl (5 U) of Taq polymerase (ABgene; Epsom, Surrey, UK), and 3.0 μl of 25 mM $MgCl_2$. Amplification conditions were 94°C for 4 minutes, followed by 27 cycles (except for 5637 in 5-azaC studies, which used 35 cycles) of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 75 seconds. A final step of 72°C for 10 minutes was used to complete the reaction. To verify RNA quality and to ensure equal loading of cDNA into the reactions, levels of GAPdH were

Table 1. Sequences of Primers Used in RT-PCR and Methylation Analyses.

Primer	Sequence 5′–3′
F1	TGTTCGTTGCTTCCAGGGCCTGC
R1	AGTAGAACATGGTACCCAGGAGAACC
5′-Methyl	GAAAGCGATGAGTGTGGTTGTTAAGGTGT
OuterA68	CCAACCCTTAAACCCGAAAAATTCCCTAC
GAPdH-5′	CCACCCATGGCAAATTCCATGGCA
GAPdH-3′	TCTAGACGCAGGTCAGGTCCACC

analyzed using forward and reverse primers (GaPdH-5′ and GAPdH-3′; Table 1), which amplified a 600-bp product. GAPdH was not amplified from the RNA template in the absence of reverse transcriptase (data not shown), demonstrating that neither the genomic sequence nor the GAPdH pseudogene was amplified under these PCR conditions. The reaction mix was similar to that used for uroplakin Ib and amplification conditions were: 94°C for 4 minutes, followed by 24 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 90 seconds; and a final step of 72°C for 10 minutes. Reaction products (5 μl of GAPdH; 15 μl of uroplakin Ib) were separated on a 1.5% agarose gel at 60 V, alongside *Hin*dIII/*Eco*RI markers and viewed using ethidium bromide. Images were analyzed with the Kodak 1D Image Analysis System (Eastman Kodak Company, Rochester, NY).

5-Aza-2′-deoxycytidine (5-azaC) Treatment of Cell Lines

All cell lines were initially seeded in 10-cm culture dishes (3×10^5 cells/dish) in 10 ml of medium (RPMI + 10% FCS, T24, 5637; DMEM + 15% FCS, VMCub 3, SW1710) and incubated at 37°C and 5% CO_2 in a humidified atmosphere. After incubation for 24 hours, increasing concentrations (0.025, 0.125, 2.5, and 5.0 μM) of 5-azaC (Sigma Aldrich) diluted in PBS or PBS alone were added to each dish and incubation continued. After 24 hours, the medium was removed and replaced with fresh medium containing 5-azaC, and the medium changes continued every 3 days if required, until control dishes without 5-azaC were confluent. Under these conditions, VMCub3 and SW1710 were harvested 3 days after drug addition; 5637 cells were harvested 4 days after drug addition and T24 cells were harvested 6 days after drug addition. Total RNA was isolated and converted to cDNA, as described above.

Bisulfite Modification of DNA, Amplification, and Cloning of the 327-bp Product

DNA was bisulfite-modified according to a previously published protocol [26] with minor modifications. Briefly, 1 μg of DNA was treated with sodium bisulfite (4.4 M) plus urea (5.75 M) and hydroquinone (0.33 mM) for 20 cycles of 55°C for 15 minutes followed by 95°C for 30 seconds, before desalting using the Qiaquick PCR Purification kit (Qiagen GmbH, Hilden, Germany). Modified DNA was desulphonated by incubation in 0.33 M NaOH for 15 minutes at 37°C and purified by ethanol precipitation. Primers 5′-methyl and outerA68 (Table 1) were designed to amplify specifically bisulfite-modified DNA but not unmodified sequence (data not shown). PCR was performed on bisulfite-modified DNA using HotStar Taq polymerase (Qiagen). PCR conditions consisted of an initial denaturation at 95°C for 15 minutes, followed by five cycles where the annealing temperature was progressively reduced by 1°C for each cycle from 68°C to 64°C. Each cycle commenced with 96°C for 1 minute, then the annealing step was carried out for 45 seconds followed by 72°C elongation for 45 seconds. The PCR continued with 30 cycles at 96°C for 1 minute, 63°C for 1 minute, and 72°C for 1 minute, followed by a final 72°C extension for 5 minutes. PCR products were purified using a WIZARD kit (Promega, Madison, WI) and those generated from TCC cell lines were sequenced in both directions using primers 5′-methyl and outerA68 (Table 1). PCR products generated from tissue samples were cloned into the pGEM-Teasy plasmid vector (Promega) and 10 individual clones sequenced as above. Sequencing was carried out using Dyenamic ET terminators (Amersham Biosciences, Little Chalfont, Bucks, UK), and ABI Prism, Version 3.4.1 technology (Applied Biosystems, Foster City, CA).

DNA Sequence Analysis

The CpG island in the uroplakin Ib promoter was identified by eye using a previously described approach [26]. Uroplakin Ib promoter sequences were analyzed using the Internet-based transcription factor binding site search programs, Matinspector (http://www.generegulation.com) and TESS (www.cbil.upenn.edu/cgi-bin/tess).

Results

The Proximal Promoter Region of the Uroplakin Ib Gene Contains a CpG Island

In a recent study [27], a 235-bp fragment of the proximal uroplakin Ib promoter and encompassing exon 1 was shown to be sufficient to drive expression of a luciferase reporter gene construct in urothelial cell lines. Although binding motifs for several protein factors were identified within this promoter fragment, such as AP-1, Sp1, and AP-4 [27], the importance of these proteins to promoter activity was not investigated. As part of ongoing studies to identify possible mechanisms that might be involved in downregulation of the uroplakin Ib gene in TCCs, we examined uroplakin Ib promoter sequences for the presence of a possible CpG island using the method described by Dobrovic et al. [26] and for putative transcription factor binding motifs using Internet-based programs, TESS (www.cbil.upenn.edu/cgi-bin/tess) and MatInspector (http://www.generegulation.com).

The uroplakin Ib promoter region contained a weak CpG island of approximately 400 bp spanning the proximal promoter, the first exon of 86 bp in length, and the beginning of intron 1 (Figure 1). This island contains 13 CpG sites, has a CpG:GpC ratio of 0.4, and is 54% GC-rich, compared with a CpG:GpC ratio of > 0.6 and a GC content of 60% to 70%, which classically defines a CpG island [28]. Figure 1 also illustrates the location of putative transcription factor binding sites within this region of the uroplakin Ib proximal promoter. In addition to those motifs previously reported [27], possible binding motifs for Sp1, NFκB, c-ets-1, and TFIID were identified.

Uroplakin Ib mRNA Levels in Bladder Cancer Cell Lines Can Be Induced by 5-azaC

To determine if methylation of the uroplakin Ib promoter might be involved in regulating uroplakin Ib expression, uroplakin Ib mRNA levels were first examined by RT-PCR in normal bladder and a large series of bladder cancer cell

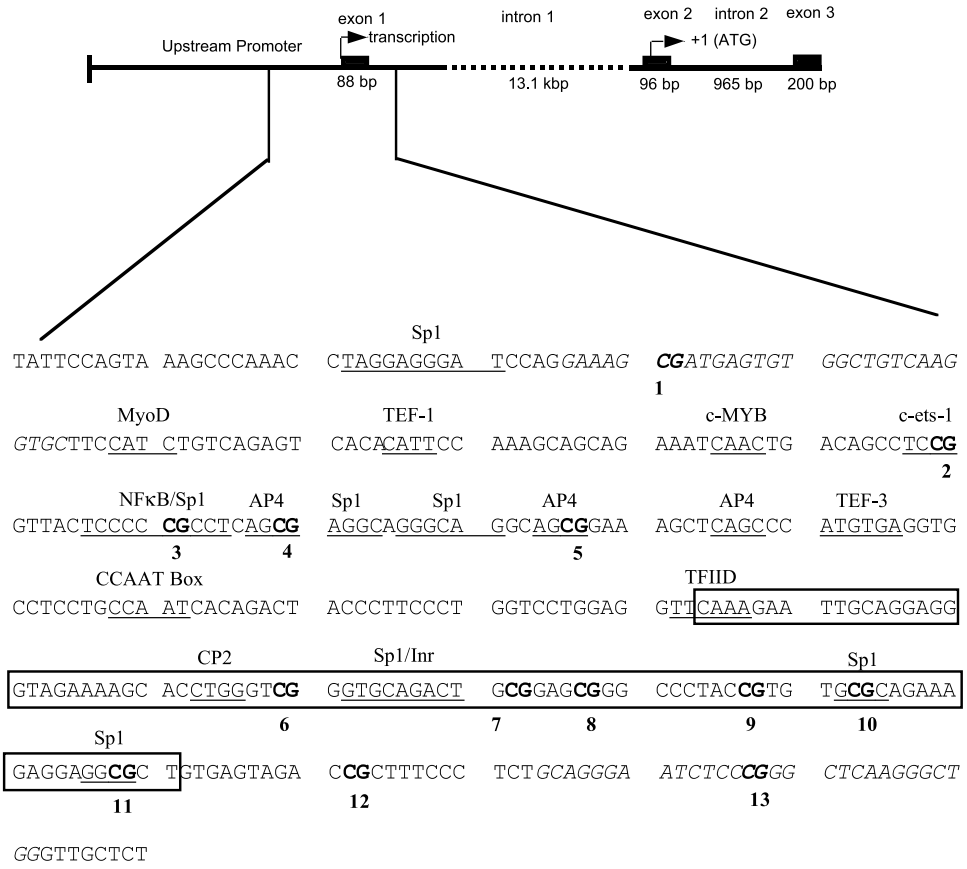


Figure 1. *Identification of a CpG island within the proximal promoter region of the uroplakin Ib promoter. Schematic illustration of the uroplakin Ib gene and promoter region around the transcription start site as described previously [27]. The sequence encompassing the CpG island is shown in the bottom panel. Within this 370-bp fragment, the CpG pairs are marked in bold type and numbered 1 to 13, with the numbering shown below the sequence. The sequence in italics defines the location of the primers used to amplify bisulfite-modified DNA for analysis of CpG methylation. Exon 1 is boxed. The underlined sequences represent core sequences for putative transcription factor binding sites as determined by transcription factor prediction programs, TESS and MatInspector. A potential CCAAT box is also marked.*

lines (Figure 2*A*). Normal bladder expressed moderate amounts of uroplakin Ib mRNA, as did TCC-derived cell lines BL13, BL17/0/X1, BL17/2, BL17/5, and BL28. Both RT112 and HT1376 expressed abundant amounts of uroplakin Ib mRNA. However, SD, VMCub1, 5637, and the transformed urothelial cell line SVHUC-1 expressed uroplakin Ib weakly and there was no detectable uroplakin Ib mRNA in any of the four remaining cell lines (J82, VMCub3, SW1710, and T24). When reverse transcriptase was omitted, uroplakin Ib was not amplified (data not shown).

Based on these observations, representative uroplakin Ib–negative cell lines T24, VM-Cub3, and SW1710 were incubated with increasing doses of 5-azaC (an inhibitor of the methylase enzyme) and expression of uroplakin Ib mRNA was assessed by RT-PCR. Results illustrated in Figure 2*B* clearly show that 5-azaC induced a dose-dependent reactivation of uroplakin Ib mRNA expression in all cell lines. Low levels of uroplakin 1b mRNA in the TCC cell line 5637 can only clearly be detected using a high number of PCR cycles (35). Under these conditions. there was a clear increase in levels of uroplakin Ib mRNA following treatment with 5-azaC. Taken together, these data suggest that promoter methylation might be a potential regulatory mechanism controlling uroplakin Ib mRNA levels in bladder cancer cell lines.

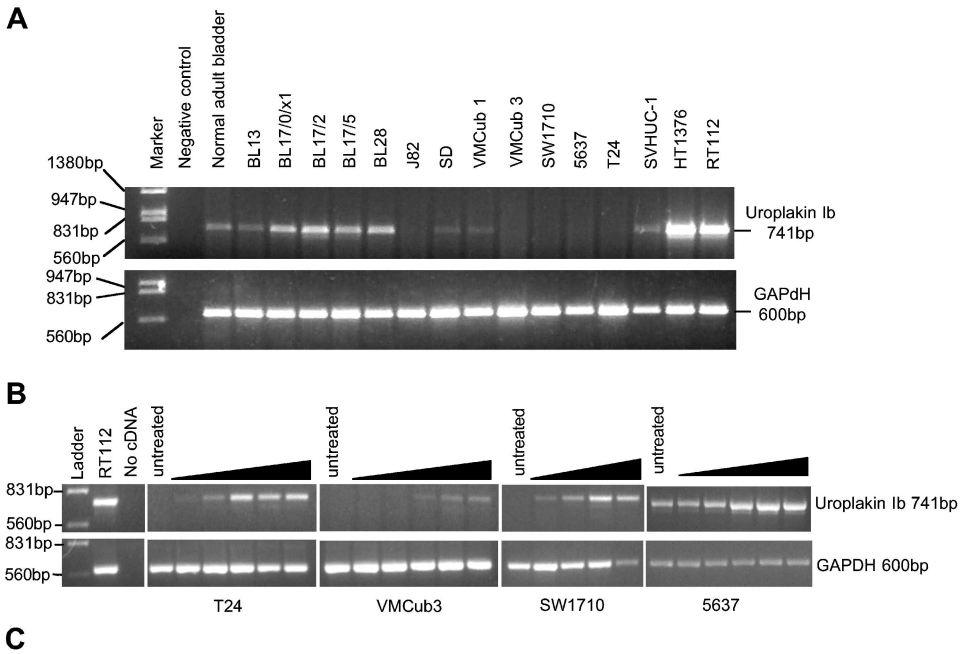
A Possible Trend between Methylation of the CpG Island in the Uroplakin Ib Proximal Promoter and Loss of Uroplakin Ib Expression in TCC Cell Lines

To examine more closely the relationship between methylation and uroplakin Ib expression, DNA from each of the bladder cell lines was bisulfite-treated and a 327-bp fragment was amplified, encompassing the CpG island within the proximal uroplakin Ib promoter (Figure 1). The bisulfite modification reaction was essentially complete in all samples, as all cytosine residues not associated with CpG sites were converted to thymidine residues (determined by sequencing; data not shown). The methylation status of 10 CpG pairs located in the proximal promoter, exon 1, and the beginning of intron 1 (Figure 1) is illustrated in Figure 2*C*, where closed circles represent methylated cytosines and open circles represent unmethylated cytosines. CpG sites 1 and 13 are located within the PCR primers, so their methylation was not analyzed. A marked trend was evident in the majority of cell lines (12/15; 80%) between an absence of methylation at most CpG pairs and readily

detectable levels of uroplakin Ib mRNA. This correlation was particularly strong at six CpG pairs in the promoter region (nos. 3–8), three of which are putative binding sites for Sp1. CpG site 3 is of particular interest as it is a putative recognition site for both Sp1 and NFκB. However, methylation of CpG pair 2, at the 5′ end of the CpG island, and CpG pairs 10 and 11, located at the 3′ end of exon 1 and in intron 1, showed no clear correlation with a lack of uroplakin Ib mRNA expression, suggesting that these CpG residues were not important in mediating expression of uroplakin Ib mRNA.

Relationship between Uroplakin Ib mRNA Levels and CpG Island Methylation in Normal Tissues and Bladder Carcinomas

Studies of the relationship between uroplakin Ib mRNA expression and promoter methylation were then extended to a series of tissue samples. Figure 3*A* illustrates patterns of uroplakin Ib mRNA in normal urothelial tissues and in four specimens of TCCs of varying stage and grade. Urothelium from a normal renal pelvis expressed uroplakin Ib mRNA, but expression was not detected in normal ureter. Uroplakin Ib mRNA was clearly detected in early-stage tumor samples



C

CpG number	2	3	4	5	6	7	8	9	10	11	Uroplakin Ib mRNA
Putative Motif	c-Ets1	NFκB Sp1	Sp1	AP4	Sp1				Sp1	Sp1	
HT1376	●	❍	❍	❍	❍	❍	❍	●	●	●	Yes
RT112	❍	❍	❍	❍	❍	❍	❍	❍	❍	❍	Yes
BL17/0/X1	❍	❍	❍	❍	❍	❍	❑	❍	❍	❍	Yes
BL17/2	●	●	●	●	●	●	●	●	●	●	Yes
BL17/5	❍	❍	❍	❍	❍	❍	❍	❍	❍	❍	Yes
BL28	●	●	❍	●	❍	❍	●	❍	●	●	Yes
SVHUC-1	●	●	❍	❍	❍	❍	●	❍	●	●	Yes
BL13	●	●	❍	❍	❍	❍	❍	❍	❍	❍	Yes
SD	●	●	●	●	●	●	●	●	●	●	Yes
VM-Cub1	●	●	●	●	●	●	●	●	●	●	Yes
J82	❑	●	●	●	●	●	●	●	●	●	No
VM-Cub3	❍	●	●	●	●	●	●	●	●	●	No
SW1710	●	●	●	●	❍	●	●	●	●	●	No
5637	●	●	●	●	❑	●	●	●	●	●	No
T24	❍	●	●	●	●	●	●	●	●	●	No

Figure 2. *Relationship between uroplakin Ib mRNA levels in bladder cancer cell lines and methylation of the uroplakin Ib promoter. (A) Total RNA was isolated from normal bladder and cultures of TCC cell lines, converted to cDNA, and used in RT-PCR analysis of uroplakin Ib and GAPdH mRNA levels, as described in Materials and Methods section. Negative control lanes contained no RNA or cDNA. (B) T24, VMCub3, 5637, and SW1710 cells were treated with increasing doses of 5-azaC (0.025–5 μM) for up to 6 days. Total RNA was isolated, converted to cDNA, and used to examine uroplakin Ib and GAPdH mRNA levels by RT-PCR, as described in Materials and Methods section. (C) Methylation analysis of 10 CpG pairs within the proximal promoter region and exon 1 of the uroplakin Ib gene in transitional cell carcinoma cell lines. DNA was isolated and bisulfite-modified, and the CpG island was amplified by PCR followed by direct sequencing of the products, as described in Materials and Methods section. (●) Methylated cytosine; (○) unmethylated cytosine; (□) equivocal data. Equal peak heights were observed on replicate sequencing traces, suggesting heterogeneity of methylation status at these sites.*

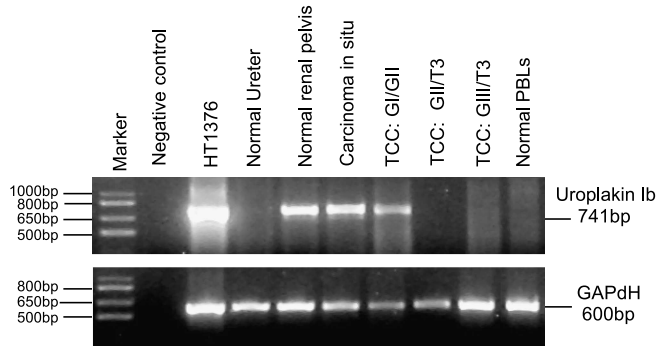
(carcinoma *in situ* and GI/GII) but not in the higher-grade samples (GII/T3 and GIII/T3). Furthermore, in two nonurothelial tissues, uroplakin Ib mRNA was not detected either in peripheral blood mononuclear cells from a normal volunteer or from normal colonic epithelium (data not shown).

The methylation status of the uroplakin Ib promoter was then examined using DNA isolated from these tissue samples. PCR products, amplified from bisulfite-modified DNA, were subcloned into the plasmid vector pGemT-Easy, and 10 individual clones were propagated and sequenced for each tissue sample. Data summarized in Figure 3*B* illustrate the percentage of clones showing methylated cytosine residues at each CpG pair. There was considerable heterogeneity in methylation status at certain CpG sites (e.g., at CpG pair 5); however, overall patterns of methylation were similar to those observed in cell lines, with a correlation between reduced levels of methylation at CpG pairs 3 to 8 and expression of the gene. For example, in all tissue samples that expressed uroplakin Ib mRNA, CpG pair 3 was poorly methylated (0–20% of clones), whereas in two thirds of samples in which uroplakin Ib mRNA was not expressed, this site was methylated in 80% to 90% of clones. A similar result was observed at CpG site 4, where 0% to 10% of clones were methylated in all tissues expressing the gene, but 0% to 70% (average 42%) were methylated in tissues lacking expression. In agreement with the results observed in TCC cell lines, the most 5′ CpG pair and the four CpG pairs at the most 3′ end of the sequence were also almost always highly methylated irrespective of uroplakin Ib expression status, indicating that these residues might not play a role in controlling expression of the gene. Methylation of the uroplakin Ib promoter in the two samples of nonurothelial tissue in which uroplakin Ib mRNA could not be detected showed that all (in normal peripheral blood mononuclear cells) and 9 of 10 CpG pairs (in colonic epithelium) were always heavily methylated, raising the possibility that promoter methylation might play a role in silencing this gene in nonurothelial tissues.

Discussion

In this study, a weak CpG island was identified within the uroplakin Ib gene, which spans the proximal promoter region, exon 1, and the beginning of intron 1. We subsequently investigated the possibility that methylation of this region might be important in regulating uroplakin Ib expression and a contributing factor to loss of uroplakin Ib expression in TCCs. Data generated in the current study have provided

A



B

CpG position	2	3	4	5	6	7	8	9	10	11	Age of subject	Uroplakin Ib mRNA
Putative TF Binding	c-Ets1	NFκB Sp1	Sp1		Sp1				Sp1	Sp1		
Normal ureter	80	90	20	60	30	20	80	20	80	80	67	No
Normal renal pelvis	100	20	10	20	20	80	70	90	100	90	79	Yes
TCC: Ca in situ	50	10	10	20	10	60	60	80	100	40	82	Yes
TCC: T2/GI/GII	100	0	0	100	0	0	0	100	100	100	82	Yes
TCC: T3/GII	100	80	70	80	50	80	60	100	100	100	79	No
TCC: T3/GIII	100	0	0	0	0	90	0	100	100	100	79	No
Normal Colonic Epithelium	100	90	90	90	0	100	90	100	100	100	82	No
Normal PBMCs	30	100	40	80	40	100	100	100	100	100	35	No

Figure 3. *Analysis of uroplakin mRNA expression and uroplakin Ib promoter methylation in samples of normal tissues and transitional cell carcinomas. (A) Total RNA was isolated from the indicated tissues, converted to cDNA, and used in RT-PCR analysis of uroplakin Ib and GAPdH mRNA levels, as described in Materials and Methods section. Negative control lane contained no cDNA. (B) DNA was isolated from the indicated tissues, and following bisulfite modification and PCR amplification of the uroplakin Ib CpG island, products were cloned into pGEM-Teasy and 10 individual clones were isolated and sequenced as described in Materials and Methods section. Results presented are the percentages of clones that were methylated at each position in the CpG island.*

evidence to support this hypothesis. Firstly, in cell lines derived from TCC and which express no detectable uroplakin Ib mRNA, expression can be reactivated by treatment with 5-azaC. Examination of TCC cell lines demonstrated a strong correlation between methylation of specific residues within the CpG island and a loss of uroplakin Ib mRNA expression. Finally, in DNA isolated from normal urothelium and TCC samples, as well as in nonurothelial normal tissues (normal colonic epithelium and peripheral blood mononuclear cells), there was a similar correlation between methylation of the CpG island and an absence of uroplakin Ib mRNA. Taken together, these data support the notion that methylation of this CpG island within the uroplakin Ib proximal promoter region may be an important determinant of uroplakin Ib expression.

A strong correlation was observed between expression of uroplakin Ib and a lack of methylation at specific CpG pairs in the proximal promoter region. In particular, methylation of CpG sites 3 and 4 correlated very strongly with a lack of uroplakin Ib mRNA expression. CpG site 3 forms part of potential binding motifs for both Sp1 and NFκB, and CpG 4 is in the core motif for binding of Sp1, making these transcription factors important candidates for regulatory roles in expression of the uroplakin Ib gene. Interestingly, in a recent study using plasmid reporter assays, a 235-bp promoter sequence, which includes the CpG island investigated in the current study, was shown to be a key determinant of uroplakin Ib reporter activity in urothelial cells [27]. The most completely methylated sites in all cell lines, bladder tumors, and nonurothelial tissues were clustered at the extreme ends of the CpG island—5′ CpG pair 2 and 3′ CpG pairs 9 to 11—which are located in exon 1 and the beginning of intron 1, suggesting that methylation of these regions is not important in regulation of expression.

The data presented in this study raise the possibility that promoter methylation may be a potential mechanism responsible for the loss of uroplakin Ib expression frequently detected in bladder carcinomas. Of the tumor samples examined, a carcinoma *in situ* (Tis) and a GI/GII tumor both expressed uroplakin Ib mRNA, and were almost entirely unmethylated at CpG sites 3 and 4. However, in GII and GIII tumor samples that showed no detectable uroplakin Ib expression, these sites were heavily methylated. Silencing of gene expression by methylation may, at least in part, account for aberrant uroplakin Ib mRNA levels observed in TCCs [9,14,15]. However, other mechanisms might also be involved because in one of the tumor samples in the current study, the CpG island remained unmethylated in the absence of uroplakin Ib expression. Increased methylation has also been recognized in several tissues during normal ageing [29], including the E-cadherin gene in bladder carcinoma [30]. The normal urothelial and carcinoma samples investigated in the current study were obtained from elderly subjects, raising the possibility that methylation of the uroplakin Ib promoter may also reflect ageing. However, one of the samples of normal urothelium from an elderly subject remained unmethylated.

Methylation-induced silencing of genes has been reported in many different tumors (including bladder carcinoma) including p16 [17] and the candidate bladder tumor-suppressor gene, *DBCCR1* [18]. In contrast, the widespread loss of *CD82 (KAI1)* expression in bladder carcinomas could not be attributed to promoter methylation [31]. A study by Salem et al. [32] in bladder carcinomas has demonstrated progressive increases in the degree of methylation in four CpG islands, including those located within the *PAX6*, *p16*, and *DBCCR1* genes. They correlated the degree of methylation with tumor stage, and showed that tumors showing aggressive behavior contained the most methylated sequences. Based on these data, they have suggested that a ''hypermethylator'' phenotype occurs in TCC, where multiple independent genes become progressively methylated, and that this deregulation may contribute to bladder carcinoma progression.

The methylation and expression status of the uroplakin Ib gene was also examined in colonic epithelium and normal peripheral blood mononuclear cells. These nonurothelial tissues both showed a high degree of methylation in the proximal promoter and expression of uroplakin Ib mRNA that could not be detected, raising the possibility that methylation might be an important mechanism by which the uroplakin Ib gene is silenced in nonurothelial tissues. One of our samples of normal ureter also did not show detectable uroplakin Ib expression and the proximal promoter was correspondingly highly methylated. Although the reasons for this result are not clear, a likely explanation is that the sample only contained small amounts of urothelial tissue and the patterns of uroplakin Ib expression and methylation reflected those of the underlying muscularis and lamina propria. There are precedents for methylation being involved in tissue-specific control of gene expression, including lymphoid and developmental-specific expression of the mouse terminal deoxynucleotidyl transferase gene [33] and placental-specific expression of the lactogen I gene [34]. A more extensive study of other tissues will be needed to provide stronger support for the hypothesis that methylation regulates tissue-specific patterns of expression of uroplakin Ib.

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