TABLE 3 Hazard ratio (HR) to predict poor quality of life by univariate and multivariate analyses (N = 515)

	CLSS				IPSS			
	Univariate		Multivariate		Univariate		Multivariate	
	HR	*P* value	HR	*P* value	HR	*P* value	HR	*P* value
Daytime frequency	2.1	<0.001	1.4	0.011	1.9	<0.001	1.2	0.032
Nocturia	2.5	<0.001	1.8	<0.001	2.0	<0.001	1.6	<0.001
Urgency	2.8	<0.001	1.5	0.009	2.1	<0.001	1.5	0.001
Urgency incontinence	3.0	<0.001	2.0	<0.001	–		–	
Stress incontinence	1.7	<0.001	0.7	0.19	–		–	
Slow stream	1.9	<0.001	1.3	0.015	1.6	<0.001	1.1	0.34
Straining	2.1	<0.001	1.4	0.019	1.8	0.003	1.2	0.044
Interruption	–		–		1.9	0.002	1.1	0.24
Incomplete emptying	2.3	<0.001	1.6	0.001	2.0	<0.001	1.4	0.001
Bladder pain	3.2	<0.001	2.2	0.019	–		–	
Urethral pain	2.5	<0.001	1.9	0.025	–		–	

CLSS, core lower urinary tract symptom score; IPSS International prostate symptom score. –, not addressed.

assessment tool [1–5]. However, IPSS was originally designed for symptom assessment for BPH [1]. Given that almost all men develop histological hyperplastic lesions in the prostate [8], other pathological conditions such as neurological and inflammatory natures are certainly involved in symptom development in men [9,10]. The symptoms that are not included in IPSS but that are important, if any, should be appraised for LUTS assessment. The CLSS questionnaire is invented to meet the need for simple overall LUTS assessment without significant omission [7]. It can be used for the initial assessment or for post-therapeutic follow-up regardless of diseases and gender. In the present study, we compared the IPSS and CLSS questionnaires for men with LUTS.

Symptom scores of the two questionnaires were uniformly higher in men from the disease groups than in the controls. The scores correlated with poor QoL, indicating the clinical relevance of any LUTS. Multivariate regression analysis to predict the poor QoL identified nine symptoms as independent factors; daytime frequency, nocturia, urgency, urgency incontinence, slow stream, straining, incomplete emptying, bladder pain and urethral pain. All of these symptoms are addressed by the CLSS questionnaire. Meanwhile the IPSS questionnaire holds only six of them, and dismisses urgency incontinence, bladder pain and urethral pain. These dismissed symptoms had large hazard ratios in the regression model for the poor QoL, with bladder pain being the largest among the LUTS examined (2.2).

Intuitively, bladder/urethral pain may be present in invasive prostate cancer but should be uncommon in early prostate cancer and BPH. However, one-fifth of BPH men complained of pain in our sample. Treatment of BPH men with an α1-blocker improved the pain subscale in the Rand Medical Outcomes Study 36-item Health Survey [11]. Recent investigations have focused on the sensory afferent nerves, especially un-myelinated C fibres, in the development of LUTS [12]. C fibres are relatively inactive during normal voiding, but they play a critical role in conducting noxious stimuli such as pain [13]. Activation of C fibres has been shown in patients with BPH and OAB as well as in experimental bladder outlet obstruction models [14,15]. Transmitters interacting with C-fibres, such as nerve growth factor, prostaglandin and ATP [12], are implicated in urgency severity and therapeutic response [15]. Hence, it is conceivable that some men with BPH and/or OAB have pain symptoms. In addition, chronic prostatitis and interstitial cystitis, which are commonly associated with pain symptoms [16,17] may occur in or coexist in men with BPH. The IPSS or ICIQ-MLUTS, an extensive questionnaire to evaluate male LUTS, does not address pain symptoms [6]. In this regard, the CLSS questionnaire is more comprehensive than the other questionnaires. More detailed and more focused questions on pain symptoms may be needed in male LUTS assessment to characterize the nature of the pain and its relevance to specific conditions.

Limitation of the CLSS questionnaire and our study design should be mentioned. The advantage of CLSS, that it can capture core LUTS in a simple and non-disease-specific manner, is a reflection of its disadvantages. Once more detailed symptom assessment is required, CLSS may be less informative compared with a bladder diary or more focused questionnaires. Second, the CLSS questionnaire cannot replace questionnaires specific to a disease. For men with the definite diagnosis of BPH or OAB, for example, IPSS or OABSS (the overactive bladder symptom score) would be more appropriate assessment tools, respectively [2,18–20]. Third, this study is a cross-sectional investigation of Japanese men with clinical diagnoses rather than urodynamic diagnoses. Longitudinal studies and studies using cohorts of different cultural or clinical backgrounds are warranted to confirm the present study results.

In conclusion, the CLSS questionnaire is more comprehensive than the IPSS questionnaire for symptom assessment of men with various diseases/conditions, although both questionnaires can capture LUTS with possible negative impact on QoL.

CONFLICT OF INTEREST

None declared.

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Abbreviations: **OAB**, overactive bladder; **CLSS**, Core LUTS Score; **QoL**, quality of life; **ICIQ-MLUTS**, International Consultation on Incontinence Questionnaire for Male LUTS.

APPENDIX

Please circle the number that applies best to your urinary condition during the last week.

How many times do you typically urinate				
1 From waking in the morning until sleeping at night?	0	1	2	3
	≤7	8–9	10–14	≥15
2 From sleeping at night until waking in the morning?	0	1	2	3
	No	1	2–3	≥4
How often do you have the following symptoms?				
	Never	Rarely	Sometimes	Often
3 A sudden strong desire to urinate, which is difficult to postpone	0	1	2	3
4 Leaking of urine because you cannot hold it	0	1	2	3
5 Leaking of urine, when you cough, sneeze, or strain	0	1	2	3
6 Slow urinary stream	0	1	2	3
7 Need to strain when urinating	0	1	2	3
8 Feeling of incomplete emptying of the bladder after urination	0	1	2	3
9 Pain in the bladder	0	1	2	3
10 Pain in the urethra	0	1	2	3

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Aberrations of a cell adhesion molecule CADM4 in renal clear cell carcinoma

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Renal clear cell carcinoma (RCCC) is the most frequent subpopulation of renal cell carcinoma and is derived from the proximal uriniferous tubules. We have previously reported that an actin-binding protein, 4.1B/DAL-1, is expressed in renal proximal tubules, whereas it is inactivated in 45% of RCCC by promoter methylation. In the lung and several epithelial tissues, 4.1B is shown to associate with a tumor suppressor protein, CADM1, belonging to the immunoglobulin-superfamily cell adhesion molecules. Here, we demonstrate by immunohistochemistry that another member of the CADM-family protein, CADM4, as well as 4.1B is expressed specifically in human proximal tubules, while CADM1 and 4.1N, another member of the 4.1 proteins, are expressed in the distal tubules. Immunoprecipitation analysis coupled with Western blotting revealed that CADM4 associated with 4.1B, while CADM1 associated with 4.1N in the lysate from normal human kidney, implicating that a cascade of CADM4 and 4.1B plays an important role in normal cell adhesion of the proximal tubules. On the other hand, CADM4 expression was lost or markedly reduced in 7 of 10 (70%) RCC cell lines and 28 of 40 (70%) surgically resected RCCC, including 10 of 16 (63%) tumors with T1a. CADM4 expression was more preferentially lost in RCCC with vascular infiltration ($p = 0.04$), suggesting that loss of CADM4 is involved in tumor invasion. Finally, introduction of CADM4 into an RCC cell line, 786-O, dramatically suppressed tumor formation in nude mice. These findings suggest that CADM4 is a novel tumor suppressor candidate in RCCC acting with its binding partner 4.1B.

Key words: CADM4, renal clear cell carcinoma, cell adhesion, 4.1B/DAL-1

Abbreviations: CADM1: cell adhesion molecule 1; CADM4: cell adhesion molecule 4; DAL-1: deleted in the adenocarcinoma of the lung; RCC: renal cell cancer; RCCC: renal clear cell carcinoma; RT-PCR: reverse transcription-polymerase chain reaction; TSLC1: tumor suppressor in lung cancer 1; TSLL2: TSLC1-like molecule 2

Additional supporting information may be found in the online version of this article.

Brief description of the novelty and impact of the paper: This is the first demonstration that loss of CADM4, found in 70% of RCCC, is one of the most frequent molecular alterations so far reported in RCCC. Furthermore, restoration of CADM4 expression into an RCC cell line strongly suppresses tumor formation in nude mice. These findings suggest that CADM4 is a novel tumor suppressor candidate in human RCCC.

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Renal cell carcinoma (RCC) is a common malignancy from the urinary organs worldwide with an incidence of 13,000 and a mortality of 5,200 reported in Japan in 2007.[1] RCC can be classified into several histological subtypes, including renal clear cell carcinoma (RCCC), papillary renal cell carcinoma, chromophobe renal cell carcinoma, collecting duct carcinoma and unclassified renal cell carcinoma. RCCC and papillary carcinoma are known to be derived from the proximal uriniferous tubules, whereas chromophobe and collecting duct carcinomas are from distal tubules and collecting ducts, respectively.[2] Nephrons are composed of the proximal tubules, loops of Henle, distal tubules and collecting ducts and are implicated in highly specified roles with distinct membrane functions of each portion, including ion transport. Therefore, understanding the molecular features of the precursor cells would be prerequisite to understand the characteristics of each subtypes of RCC.

RCCC represents around 75% of all RCC and often shows aggressive phenotype, including frequent metastasis to distal organs and resistance to any therapeutic approaches, such as chemotherapy and radiotherapy. Like many other human cancers, RCCC develops and progresses toward malignancy through multiple genetic and epigenetic aberrations. From the viewpoint of genetic alterations, however, RCCC is a rather unique tumor, because the incidence of *RAS* mutation or *TP53* inactivation is exceptionally low in comparison with that in other solid tumors.[3] The most frequent genetic

alteration so far reported in RCCC is the inactivation of the *VHL* gene. Loss of the VHL protein leads to an inappropriate accumulation of hypoxia-inducible mRNA, such as VEGF, which appears to be responsible for the hypervascular nature of RCCC.[4] Another characteristic and clinically important feature of RCCC is the high incidence of metastasis even in the relatively early stages of tumors. Disruption of the cell adhesion machinery is an initial step of cancer invasion and metastasis. In fact, in previous studies, it has been demonstrated that alterations of E-cadherin or integrins are frequently observed in RCCC.[5,6]

We previously identified a tumor suppressor gene, *CADM1/TSLC1*, in human nonsmall cell lung cancer (NSCLC).[7] The *CADM1* is expressed in most epithelial tissues, while its expression is frequently lost in many tumors, including NSCLC or prostate cancer.[8] CADM1 belongs to the immunoglobulin superfamily cell adhesion molecules and carries three immunoglobulin loops in the extracellular domain, a single transmembrane domain and a short cytoplasmic domain. Subsequent analysis has shown that CADM1 forms a unique subfamily within IgCAMs together with its homologous proteins, CADM2, CADM3 and CADM4, in which CADM2 and CADM3 are only expressed in the nerve systems.[9,10] We have reported that CADM4 is expressed in the brain, lung, large and small intestines and urinary organs and that CADM4 could act as a tumor suppressor in prostate cancer.[11]

We have also demonstrated that CADM1 associates with an actin-binding protein, 4.1B/DAL-1, through the FERM-binding motif in the cytoplasmic domain.[12] Frequent loss of 4.1B in lung adenocarcinoma, breast cancer and meningioma suggested that 4.1B could be a tumor suppressor.[13,14] 4.1B is a member of the 4.1-family proteins with 4.1R, 4.1N and 4.1G and shows significant homology with ezrin, radixin and moesin as well as merlin, which is the responsible gene product in neurofibromatosis type 2. In lung and breast cancer and meningioma, frequent abrogation of the cell adhesion machinery composed of CADM1 and 4.1B has been demonstrated.[15–19] In the mouse nephron, 4.1B is expressed in the proximal uriniferous tubules, while 4.1N is expressed in the distal tubules. On the other hand, 4.1R expression is only restricted to a portion of ascending limb of the loop of Henle, while no 4.1G expression is observed in the nephron.[20] Therefore, among the 4.1 family proteins, we chose 4.1B and 4.1N as possible molecules involved in renal tumorigenesis. In addition, we have previously demonstrated that 4.1B is frequently inactivated by promoter methylation, providing a prognostic factor in RCCC.[21] However, the normal partners of the membrane protein associated with 4.1B in RCCC have not been reported yet.

Here, we examined the tissue-specific expression of CADM4, CADM1, 4.1B and 4.1N proteins in human nephrons and demonstrated that CADM4 was expressed and interacted with 4.1B in human proximal uriniferous tubules that are the precursor cells of RCCC. The high incidence of loss of CADM4 expression in cell lines and primary tumors from RCCC, together with the suppressor activity in the tumorigenicity of RCC cells by CADM4, strongly suggests that CADM4 is a novel tumor suppressor candidate involved in RCCC in cooperation with 4.1B.

Material and methods

Cell lines

Human RCC cell lines, ACHN, 786-O and 769-P were obtained from the American Type Culture Collection (Rockville, MD); VMRC-RCW and Caki-1 cells, from the Japanese Collection of Research Bio-resources (Tokyo, Japan); OS-RC-2, RCC10RGB, TUHR4TKB, TUHR10TKB and TUHR14TKB cells, from the Riken Cell Bank (Tsukuba, Japan). Cells were cultured according to the suppliers' recommendations.

Surgical specimens

Forty pairs of cancerous and adjacent noncancerous tissues of RCCC were surgically resected at the University of Tokyo Hospital after written informed consent from each patient was obtained. Analyses of human materials were carried out according to the Guidelines of the Ethics Committee of the University of Tokyo (authorization No. 2566). Pathological diagnosis was performed by urological pathologists (A. G. and T. M.).

Antibodies

A rabbit polyclonal antibody (pAb) against CADM4/TSLL2 (Bc-2) was raised against 13 synthetic polypeptides of the C terminus of CADM1 coupled with keyhole limpet hemocyanin and purified with an affinity column (MBL, Nagoya, Japan) as described previously.[11] The CADM1 antibodies used in this study were two rabbit polyclonal antibodies (pAbs) against the cytoplasmic domain, C-18,[22] and number 6 and a chicken monoclonal antibody (mAb) against the ectodomain, 3E1.[23] A rabbit pAb against 4.1B/DAL-1 was described previously.[21] A mouse mAb against 4.1N and a goat pAb against GAPDH (V-18) were purchased from BD Biosciences (Franklin Lakes, NJ) and from Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

Immunohistochemistry

Sequential sections of 4-μm thick from human RCCC and noncancerous kidney tissues of the same patients were cut from the paraffin blocks. The sections were deparaffinized, autoclaved in Histofine pH 9 (Nichirei Biosciences, Japan) at 121°C for 20 min, cooled down to room temperature and incubated with 0.3% H_2O_2/methanol for 30 min and with 5% normal donkey serum in 0.02% NaN_3/PBS for 30 min. These sections were incubated with the indicated primary antibodies and visualized by Envision kit/HRP (DAB) (Dako, Glostrup, Denmark) according to the manufacturer's recommendations. All sections were counterstained with hematoxylin. Elastica van Gieson (EVG) staining was also used to assess the vascular permeation of tumors.

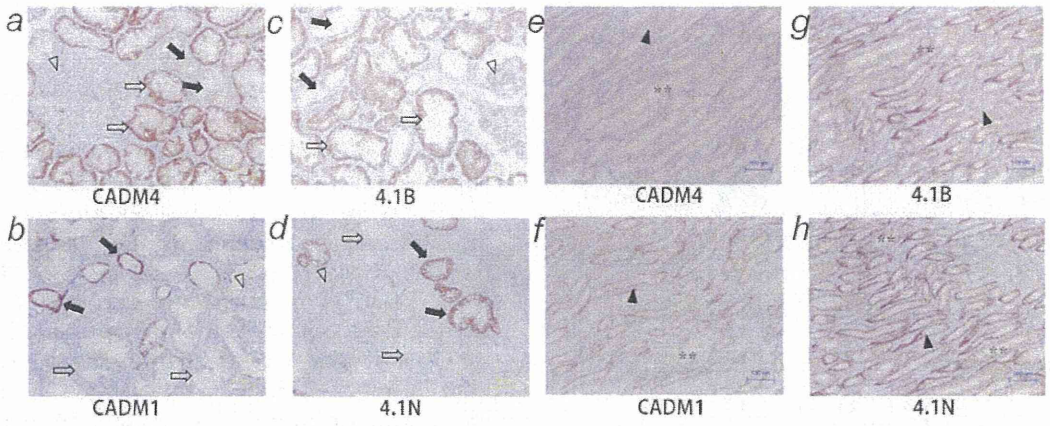

Figure 1. Immunohistochemical analyses of CADM4 (*a* and *e*), CADM1 (*b* and *f*), 4.1B (*c* and *g*) and 4.1N (*d* and *h*) proteins in normal human renal cortex (*a–d*) and medulla (*e–h*). Expression of CADM4 and 4.1B is detected in the proximal convoluted tubules (*a* and *c*), whereas that of CADM1 and 4.1N is detected in the distal convoluted tubules (*f* and *h*). Open and closed arrows indicate the proximal and the distal convoluted tubules, respectively, whereas open and closed arrowheads indicate the glomerulus and the loops of Henle, respectively. Asterisks and double asterisks indicate the collecting duct in the cortex and that in the medulla, respectively. The bar indicates 50 μm (*a–d*) or 100 μm (*e–h*).

Immunoprecipitation and Western blotting

Human RCCC and noncancerous renal tissues were treated with a lysis buffer [50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4] with protease inhibitors [200 μM AEBSF, 10 μM leupeptin, 1 μM pepstatin A] and centrifuged at 3,000 rpm at 4°C for 10 min to obtain the tissue lysates as the supernatants. For direct Western blotting, an aliquot of the tissue lysates (1 μg) was applied in each lane in a 4–12% gradient SDS–PAGE (Invitrogen, Carlsbad, CA). For immunoprecipitation, an aliquot of tissue lysates (1–2 mg) was incubated with an appropriate primary antibody for 30 min at 4°C, and then protein A-Sepharose 6MB (GE Healthcare, Buckinghamshire, UK) was added and further incubated for overnight at 4°C. Immunoprecipitates were rinsed with the lysis buffer three times, suspended in a sample buffer containing 2% SDS and incubated for 5 min at 100°C. The samples were fractionated in 4–12% gradient SDS–PAGE, transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) and incubated with an appropriate primary antibody. The binding of the primary antibody was detected with ECL™ Western Blotting Detection Reagent (GE Healthcare) using a peroxidase-conjugated secondary antibody (GE Healthcare).

Reverse-transcription PCR

Total cellular RNA was extracted from 786-O cells using an RNeasy Mini kit (QIAGEN, Valencia, CA). One microgram of total cellular RNA was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen) with oligo(dT) primers. A CADM4 fragment of 128 bp was amplified using 0.5 μmol/l of primers 5′-TAGTGGGCATGGTCTGGTG-3′ and 5′-TTTCC TCTTGTGTCCGTCG-3′. A 4.1B fragment of 153 bp was amplified using 0.5 μmol/l of primers 5′-GTAGTGGTCCATA AAGAGACAGAGA-3′ and 5′-GATACAAGTCAGTTGGGT TAGAAGA-3′, whereas a β-actin fragment of 646 bp was amplified using 0.1 μmol/l primers 5′-AAATCTGGCACCA CACCTT-3′ and 5′-AGCACTGTGTTGGCGTACAG-3′.

Restoration of CADM4 expression by 5-aza-2′-deoxycytidine

About 1×10^5 of 786-O cells were seeded at day 0, treated with 5-aza-2′-deoxycytidine (5-aza-CdR; 10 μM; Sigma-Aldrich, St., MO) or PBS as a control for 24 hr on days 2 and 5 and collected on day 8 as reported previously.[7,24]

Expression of CADM4 in an RCC cell line

A vector expressing the whole-coding sequence of human CADM4 (pcTSLL2/CADM4) was described previously.[11] 786-O cells were transfected with a pcTSLL2/CADM4 or an empty vector, pcDNA3.1 (Invitrogen) using Lipofectamine LTX (Invitrogen) according to the manufacturer's instructions, selected against 500 μg/ml G418 sulfate (Invitrogen) and three independent cell clones were then obtained.

Tumorigenicity analysis

A suspension of 1×10^5 cells in 0.2 ml of PBS was injected subcutaneously into one to two sites on the backs of 6-week female BALB/c nu/nu mice. Tumor growth was assessed by measuring the xenografts in two dimensions twice a week. Tumor volumes were calculated according to the formula (volume) = 1/2 × (long axis) × (short axis).[2] All animal

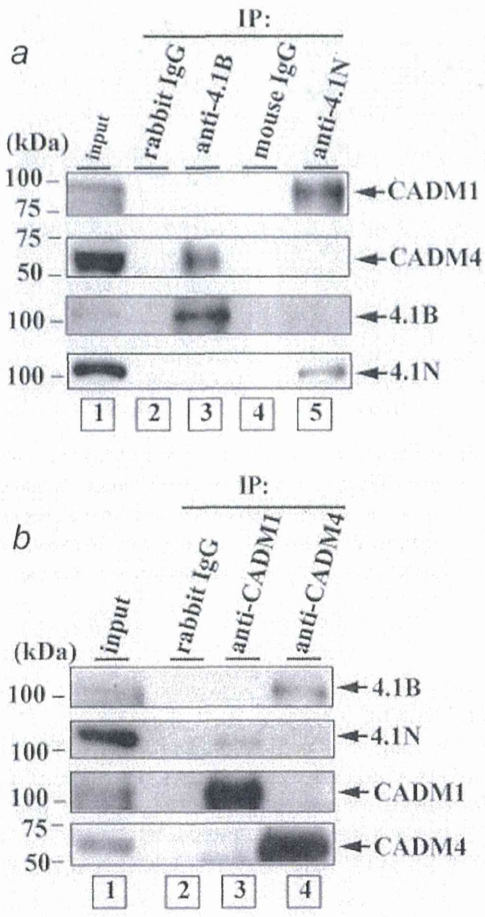

Figure 2. Interaction of CADM4 with 4.1B and CADM1 with 4.1N. (*a*) Total lysates of normal human kidney were immunoprecipitated with control rabbit IgG (lane 2), anti-4.1B pAb (lane 3), control mouse IgG (lane 4) and anti-4.1N pAb (lane 5), and binding proteins were detected by immunoblotting using anti-CADM1 pAb, anti-CADM4 pAb, anti-4.1B pAb and anti-4.1N pAb (top to bottom). An aliquot of the tissue lysates (5 μg) was loaded as a control (lane 1). (*b*) Total lysates of normal human kidney were immunoprecipitated with control rabbit IgG (lane 2), anti-CADM1 pAb (lane 3) and anti-CADM4 pAb (lane 4), and binding proteins were detected by immunoblotting using anti-4.1B pAb, anti-4.1N pAb, anti-CADM1 pAb and amti-CADM4 pAb (top to bottom). An aliquot of the tissue lysates (5 μg) was loaded as a control (lane 1).

experiments were performed in accordance with the institutional guidelines.

Results

Cell-type-specific expression of the CADM- and 4.1-family proteins in human kidney

To understand the physiological and pathological roles of these proteins in the kidney, precise patterns of expression were examined in human normal kidneys by immmunohistochemical staining using specific antibodies against CADM1, CADM4, 4.1B and 4.1N. As shown in Figure 1*a*, CADM4 is specifically expressed at the cell–cell attachment sites in the proximal convoluted tubules. 4.1B is also expressed along the cell membrane in the proximal tubules as reported previously (Fig. 1*c*).[20,21] However, neither CADM1 nor 4.1N gives any signals in the proximal tubules (Figs. 1*b* and 1*d*). In the distal convoluted tubules, on the other hand, CADM1, but not CADM4, is expressed along the cell membrane (Figs. 1*a* and 1*b*). Expression of 4.1N, but not 4.1B, is also detected in the distal tubules (Figs. 1*c* and 1*d*). In addition, signals of 4.1B and 4.1N are detected in the loops of Henle or the collecting ducts, whereas the CADM1 signal is detected in the ascending limbs of the loops of Henle. 4.1B expression is also observed in the glomerulus as reported previously.[21] In contrast, CADM4 is expressed exclusively in human proximal tubules as summarized in Supporting Information Table 1. Taken together, the findings clearly indicate that CADM4 and 4.1B are expressed in the proximal tubules, while CADM1 and 4.1N are expressed in the distal tubules in human kidney.

Interaction of CADM4 and 4.1B protein

CADM1 associates with 4.1B through its FERM-binding motif in normal epithelial tissues.[12] Coincident expression of CADM4 and 4.1B in the proximal tubules and that of CADM1 and 4.1N in the distal tubules prompted us to examine the possible association of each pair of proteins by immunoprecipitation coupled with Western blotting. As shown in Figure 2*a*, when the lysate of normal human kidney was immunoprecipitated with an antibody against 4.1B and immunoblotted with an anti-CADM4 antibody, specific signals of about 55 kDa corresponding to CADM4 were detected (Fig. 2*a*, lane 3). However, no CADM1 protein was coprecipitated when the same immunoprecipitate was blotted with an anti-CADM1 antibody (Fig. 2*a*, lane 3). Inversely, when normal kidney lysate was immunoprecipitated with an anti-CADM4 antibody and immunoblotted with an anti-4.1B antibody, distinct signals corresponding to 4.1B were detected (Fig. 2*b*, lane 4). However, no 4.1N protein was coprecipitated with CADM4 (Fig. 2*b*, lane 4). On the other hand, when normal kidney lysate was immunoprecipitated with an anti-4.1N antibody and then immune-blotted with an anti-CADM1 antibody, CADM1 signals were detected (Fig. 2*a*, lane 5). However, no CADM4 protein was co-immunoprecipitated with 4.1N. Moreover, 4.1N, but not 4.1B, was inversely co-immunoprecipitated with an anti-CADM1 antibody (Fig. 2*b*, lane 3). These results indicate that CADM4 associates with 4.1B, while CADM1 associates with 4.1N in normal human kidney cells, corresponding to the pattern of their tissue-specific expression.

Frequent loss of CADM4 and 4.1B expression in human RCC cells and RCCC tumors

Because RCCC is derived from the proximal uriniferous tubules, possible alteration in the expression of CADM4 as well as 4.1B was examined by Western blotting. As shown in

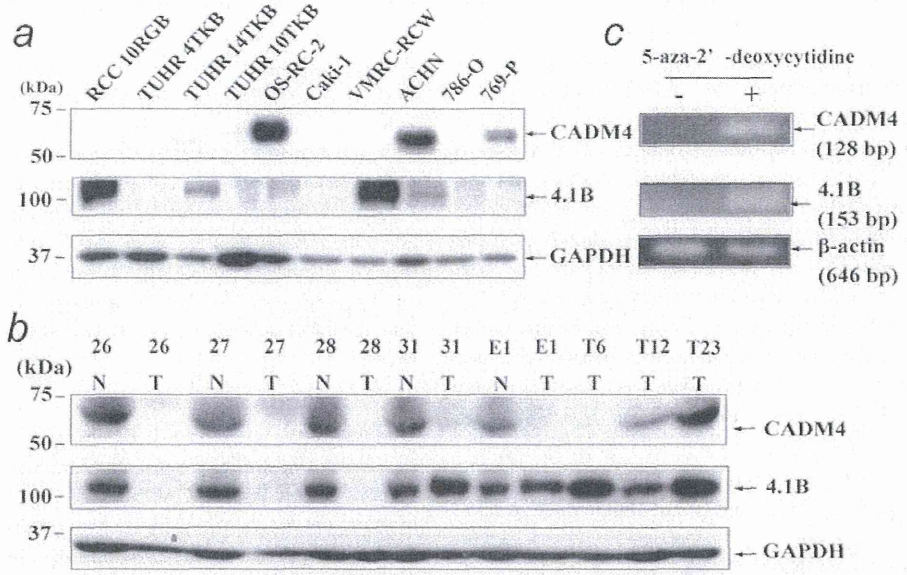

Figure 3. Loss of expression of CADM4 and 4.1B proteins in RCCC and restoration of CADM4 expression by 5-aza-2′-deoxycytidine. (*a* and *b*) Immunoblotting of CADM4 and 4.1B proteins in 10 RCC cell lines (*a*) and the primary tumors of RCCC (*b*). An aliquot of the tissue lysates (1 μg) was subjected to 4–12% SDS–PAGE and detected by anti-CADM4 pAb (upper), anti-4.1B pAb (middle) and anti-GAPDH mAb as a control (lower). N and T in (*b*) indicate noncancerous renal tissues and tumor tissues, respectively, whereas the number indicates individual patients. (*c*) Reverse transcriptase-PCR analysis of the CADM4 (upper), 4.1B (middle) and beta-actin as a control (lower) in 786-O cells treated with or without 5-aza-2′ deoxycytidine.

Figure 3*a*, 7 of 10 (70%) RCC cell lines showed loss or marked reduction in CADM4 expression. In addition, 6 of 10 (60%) from the same panel of RCC cells lost or markedly reduced 4.1B expression. In total, 9 of 10 (90%) RCC cells lost either CADM4 or 4.1B expression, suggesting that the disruption of the CADM4-4.1B cascade is an extremely frequent event in RCC. We have previously shown that 4.1B is inactivated by methylation of the gene promoter in RCCC. Therefore, to examine the possible involvement of promoter methylation in silencing of the *CADM4* gene, 786-O cells completely lacking CADM4 expression were treated with 5-aza-2′ deoxycytidine for 24 hr twice as described in Material and methods section. As shown in Figure 3*c*, CADM4 mRNA was restored in 786-O cells, suggesting that promoter methylation could be involved in at least a subset of RCC cell lines.

Next, we examined the expression of CADM4 in primary RCCC surgically resected and pathologically diagnosed at the University of Tokyo Hospital. Western blotting revealed that 28 of 40 (70%) primary RCCC lost CADM4 expression, while noncancerous renal tissues from the same patients expressed a significant amount of CADM4 protein (Fig. 3*b*). Loss of CADM4 expression was observed at high frequency even in RCCC at relatively early stages, including 10 of 16 (68%) tumors with T1a or 5 of 6 (83%) tumors with Fuhrman's grade 1. Interestingly, CADM4 expression was preferentially lost in RCCC with vascular infiltration (15/17, 88%) relative to those without vascular infiltration (13/23, 57%; $p = 0.04$; Table 1). These findings suggest that loss of CADM4 is a relatively early event in renal tumorigenesis and could be involved in vascular infiltration. Histological features are shown in Supporting Information Figure 1 for tumors with and without CADM4 expression. In tumors lacking CADM4 expression, inconspicuous vascular infiltrations (Supporting Information Figs. 1*a* and 1*c*) were manifested by EVG stain in large- and small-sized veins (Supporting Information Figs. 1*b* and 1*d*). In contrast, vascular infiltration was not identified even by EVG stain in tumors expressing CADM4 (Supporting Information Figs. 1*e* and 1*f*). In addition to CADM4, loss of 4.1B expression was detected in 19 of 40 (48%) primary RCCC by Western blotting (Fig. 3*b*). In total, the loss of expression of CADM4 or 4.1B occurred in 32 of 40 (80%) of RCCC. Interestingly, average size of the tumors lacking expression of either CADM4 or 4.1B or both was significantly larger than that of the tumors expressing both CADM4 and 4.1B ($p = 0.028$) (Table 2). No pathological changes, however, were observed between the tumors lacking both CADM4 and 4.1B and those lacking either of them, supporting an idea that CADM4 and 4.1B proteins act in the same cascade of cell adhesion.

Suppression of tumorigenicity of an RCC cell line, 786-O, by CADM4

To understand the biological function of CADM4 in RCCC, we transfected a CADM4 expression vector into an RCC cell

line, 786-O, completely lacking endogenous CADM4 expression, and obtained three independent transfectants (786/CADM4-1~3). As shown in Figure 4*a*, these cells stably expressed a significant amount of CADM4 protein. On the other hand, the amounts of 4.1B protein in these transfectants were quite low and almost the same as those in parental 786-O and 786/V cells (data not shown). 786/CADM4-1~3 cells showed essentially similar morphology to 786/V or parental 786-O cells, although cell populations showing a flatter morphology appeared to be more prominent in 786/CADM4-1~3 cells (Figs. 4*b* and 4*c*). On the other hand, 786/CADM4-1~3 cells did not show a dramatic difference in cell proliferation *in vitro* relative to 786/V or parental 786-O cells when analyzed by an MTS assay (data not shown). Finally, the tumor-forming activity of these cells *in vivo* was examined by injecting them into the back of BALB/c nu/nu mice. As shown in Figure 4*d*, 786/V cells developed palpable tumors around 3 weeks after injection (average latency: 17.2 days), and the tumors grew into large tumors with an average volume of 268 mm^3. In contrast, most of the 786/CADM4 cells did not form palpable tumors until 4 weeks after injection (average latency: 33.1 days). Moreover, the growth of the developed tumors was slow, forming much smaller masses with an average volume of 21 mm^3, indicating that restoration of CADM4 significantly suppresses tumor formation by an RCC cell line, 786-O. This finding provides more evidence that CADM4 acts as a novel tumor suppressor candidate in RCCC.

Table 1. Pathological parameters and loss of CADM4 expression in RCCC

Parameters	No. of Tumors Lost CADM4/No. of Tumors Examined (%)	
T-Classification		
1a	10/16 (63)	NS
1b	9/13 (69)	
2	2/3 (67)	
3a	3/4 (75)	
3b	4/4 (100)	
Fuhrman's Grade		
1	5/6 (83)	NS
2	16/24 (67)	
3	7/9 (78)	
4	0/1 (0)	
Vascular Infiltration		
(–)	13/23 (57)	*
(+)	15/17(88)	

*$p = 0.04$.
NS: not significant.

Discussion

In the present study, we initially demonstrated the cell-type-specific expression of CADM- and 4.1- family proteins in human nephrons by immunohistohemistry. CADM4 and 4.1B are expressed in the proximal uriniferous tubules, while CADM1 and 4.1N are expressed in the distal tubules. Such distinct patterns of expression have not been reported in other organs, including the lung, where CADM1, CADM4, 4.1B and 4.1N are all expressed in the pulmonary epithelial cells. Cell-type-specific expression of these proteins in the nephron, therefore, suggests that the cell adhesion machinery of CADM- and 4.1- proteins might play specific roles in each uriniferous tubule, for example, those related to the ion transport or re-absorption of specific molecules, although some of these proteins are also expressed in the loops of Henle or the collecting ducts in human kidney (Fig. 1). Next, by immunoprecipitation analysis coupled with Western blotting, we demonstrated that the CADM4 protein associated with 4.1B, while CADM1 associated with 4.1N in normal human kidney. Previous studies have reported that CADM1 associates with 4.1B through the FERM-binding motif in epithelial cells.[12] In addition, CADM3 is shown to associate with 4.1N in neuronal cells.[25] These results suggest that both CADM1 and CADM4 molecules have the potential to associate with both 4.1B and 4.1N. However, the clear demonstration in the present study of the specific interaction between

Table 2. Expression status of CADM4 and 4.1B and pathological characters of RCCC

Expression Status of CADM4/4.1B	No. of Tumors	Average Size (mm^3)		No. of Tumors (%) with T1		No. of Tumors (%) with Vascular Infiltration		No. of Tumors (%) with Metastasis
+/+	8	40 ± 1	*	7 (88)	NS	1(13)	NS	0 (0)
+/– and –/+	17	62 ± 33		9 (53)		11 (65)		3 (18)
–/–	15	49 ± 24		9 (60)		5 (33)		3 (20)
Total	40	53 ± 28		25 (63)		17 (43)		6 (15)

*$p = 0.028$.
NS: not significant.

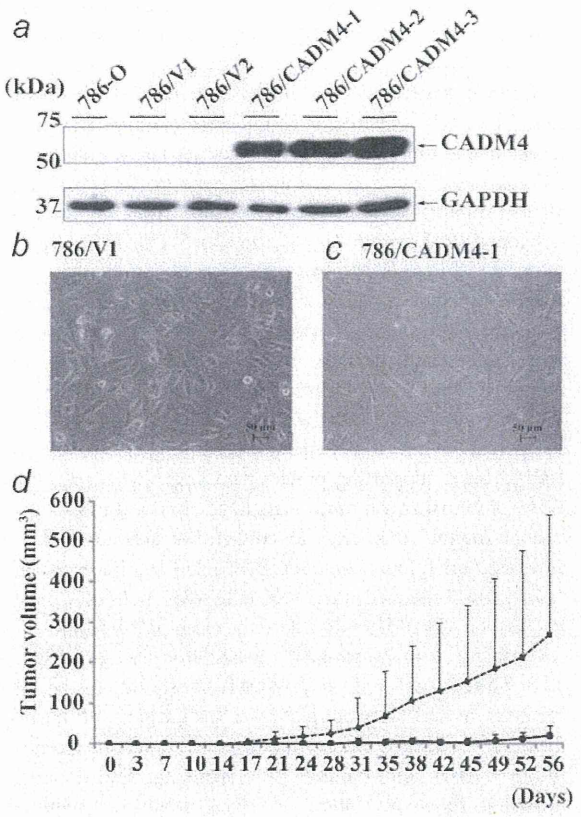

Figure 4. Suppression of tumorigenicity of 786-O cells in nude mice by CADM4. (*a*) Western blotting of CADM4 protein (upper) and control GAPDH protein (lower) in parental 786-O cells, 786-O cell clones transfected with a vector alone (786/V1, 786/V2) and 786-O cell clones transfected with CADM4 (786/CADM4-1, 786/CADM4-2 and 786/CADM4-3). (*b* and *c*) Morphology of 786/V1 (*b*) and 786/CADM4-1 (*c*) cells under phase-contrast microscopy. The bar indicates 50 μm. Original magnification, ×100. (*d*) Tumor formation in nude mice. The average volume of tumors that formed at 18 sites was determined at the indicated times after injection of 10^5 cells from the 786/V (dashed line) and 786/CADM4 (solid line) cells. $^*p = 0.00012$.

CADM4 and 4.1B and between CADM1 and 4.1N in human kidney lysates strongly supports the finding that CADM4 and 4.1B are co-expressed in the proximal tubules while CADM1 and 4.1N are coexpressed in the distal tubules.

In the previously study, we have shown the frequent inactivation of 4.1B in RCCC.[21] Therefore, in this study, we examined whether CADM4 could also act as a tumor suppressor in RCCC. This hypothesis was supported by two lines of evidence (1) the frequent loss of CADM4 expression in primary tumors and cell lines from RCCC and (2) the suppression of the suppression of subcutaneous tumor formation of a human RCC cell line, 786-O, in nude mice by the introduction of CADM4. It is quite noteworthy that over 70% of primary RCCC tumors lost CADM4 expression in Western blotting or immunohistochemistry. The loss of CADM4 is, therefore, one of the most frequent molecular alterations so far reported in RCCC. It is well known that the *VHL* gene is inactivated in about 80% of RCCC.[26] However, mutation of the *VHL* and inactivation of the CADM4-4.1B cascade appears to be independent at least in 7 RCC cell lines with characterized VHL status as summarized in Supporting Information Table 2. This finding could be consistent with possible distinct functions of a cell adhesion molecule, CADM4 and a transcriptional silencer, VHL, although mutation of the *VHL* in 40 primary RCCCs remains to be examined. The clinicopathological features of the tumors revealed that loss of CADM4 expression already occurred in the early stage of RCCC tumors with T1a or Fuhrman's grade 1, suggesting that loss of CADM4 is a relatively early event in renal carcinogenesis. A more important finding is the significant association of the loss of CADM4 with the vascular infiltration of RCCC. Considerable portions of RCCC, especially a subset of those successfully resected by surgical operation, often contain the lesions of vascular infiltration, which is known to provide one of the prognostic markers of an RCCC patient.[27] Tumors lacking CADM4 expression might have the potential to metastasize to the distant organs through vascular infiltration, even though the relevant tumors are in the early clinicopathological stages on the basis of the TNM classification. Further studies as to the recurrence of RCCC in these patients would be required to answer this hypothesis.

Tumor-forming activity in nude mice has been considered to be the classic and most established criteria to assess the malignant phenotype of cultured cancer cells. On the basis of this criterion, 786-O is a malignant RCC cell for its tumorigenicity in nude mice as reported previously.[28] In this study, CADM4 appears to suppress tumor growth, as shown in Figure 4*d*, where the average volume of developed tumors at 56 days from 786/CADM4 cells (21 mm^3) is much smaller than that from 786/V cells (268 mm^3). Moreover, tumorigenicity in nude mice was recently re-evaluated as a method to assess the stemness of cancer cells. From this point of view, 786-O cells appear to contain a considerable number of cancer stem cells, because tumors developed in all 18 injection sites of 10^5 of 786/V cells with a relatively short latency averaging 17.2 days. In contrast, the restoration of CADM4 expression appears to decrease the subpopulation of cancer stem cells, because 10^5 of 786/CADM4 cells failed to develop tumors in 4 of 18 injection sites even at 56 days after injection. Moreover, the average latency of tumor formation was 33.1 days, much longer than that in 786/V cells. These results suggest that CADM4 suppresses not only the tumor growth but also the size of the cancer stem cell population in 786-O cells. However, these tumors only grew locally at the injected sites, and none of the tumors showed invasion or metastasis to adjacent or distant organs until 56 days after injection. These results suggest that even 786/V cells did not recapitulate the vascular infiltration of human RCCC in nude mouse-model, although we did not confirm absence of the vascular infiltration in these tumors.

It has been reported that 4.1B, Timp-3, RASSF1 and several other tumor suppressor genes are inactivated by promoter

methylation at high frequency in RCCC.[21,29–31] In this study, treatment of RCC cells with 5-aza-2′ deoxycytidine restored CADM4 expression in 786-O cells lacking endogenous CADM4 expression, suggesting that promoter methylation is involved in at least a portion of RCCC. We failed, however, to examine the detailed state of the *CADM4* gene promoter in RCC cell lines or primary RCCC by bisulfate sequencing or the COBRA method probably due to the extraordinary CpG-rich structure of the *CADM4* gene promoter. Therefore, the molecular cause of the aberration of CADM4 molecule in primary RCCC remains to be elucidated. In addition to the promoter methylation, loss of heterozygosity (LOH) on 19q13.2, where the *CADM4* gene is mapped, could be involved as observed in many other tumor suppressor genes, including the *CADM1*.[32] Inactivating mutations, including point mutations, frameshift and insertions/deletions might be additional molecular mechanisms to inactivate the *CADM4* gene, although inactivation through such mechanisms is relatively rare in the case of the *CADM1*.[33] It is interesting that chromosomal region 19q13, on which the *CADM4* gene is localized, also show LOH frequently in gliomas, suggesting that a similar mechanism connected to cell adhesion could play a role in neurogenic tumorigenesis.[34,35] On the other hand, 4.1B expression was lost in about one half of RCCC as we reported previously.[21] In this study, we confirmed using a distinct series of samples that 48% of primary RCCC tumors showed loss or marked reduction of 4.1B expression. In total, 32 of 40 (80%) primary RCCC showed loss or marked reduction of either CADM4 or 4.1B, indicating that disruption of the CADM4-4.1B cascade is one of the most frequent events in RCCC. We have previously reported that CADM4 could be a tumor suppressor candidate in prostate cancer on the basis of the frequent loss of CADM4 expression (6 of 9) in primary prostate cancer as well as the suppression of tumor-forming activity by CADM4 in a prostate cancer cell line, PPC-1.[11] As shown previously, CADM4 is expressed in a quite unique spectrum of tissues, such as the brain, lung, large and small intestines and urinary organs, including the kidney, ureter, bladder and prostate. The involvement of CADM4 in both RCCC and prostate cancer suggests that the malignant tumors of uroepithelial origin might have a common target cascade, at least in part, in their carcinogenic processes.

Genetic evidence of the involvement of CADM4 in RCCC would be finally obtained if RCCC were developed in mice deficient in the *Cadm4* gene. In this connection, a report of conditional knock-out mice of the *Nf2* gene in the proximal convoluted tubules is noteworthy, because 100% of these mice developed RCC within 6 - 10 months.[36] The *NF2* gene is responsible for neurofibromatosis type 2, a familial cancer affected by bilateral eighth-cranial-nerve tumors, as well as meningiomas, shwannomas and gliomas. The *NF2* gene encodes an actin-binding protein, merlin, which shows significant homology with ezrin, radixin and moesin, in addition to the 4.1 family proteins.[37] Further analyses by Morris *et al.*[33] indicated that EGFR was hyperactivated in RCC developed in $Nf2^{-/-}$ mice, supporting previous findings that merlin inhibits EGFR internalization and signaling physiologically, whereas loss of merlin could lead to constitutive activation of EGFR and resultant tumor formation in the kidney. Recently, several studies demonstrated that cell adhesion molecules, such as NCAM or CADM1, interact with receptor thyrosine kinases and modify their signaling.[38,39] In addition, loss of merlin and 4.1B protein, as well as CADM1, is shown to be deeply involved in the development and progression of meningiomas.[14,40] CADM4 could also associate with several receptor tyrosine kinases and modify their signaling. Further analyses, including those of $Cadm4^{-/-}$ mice, would be required to understand the role of CADM4 in human renal carcinogenesis.

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