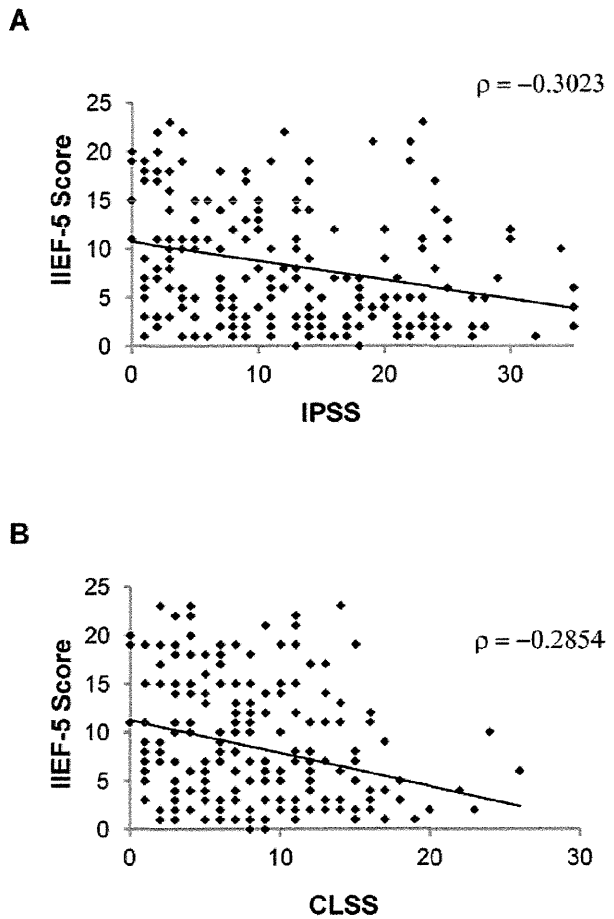

Figure 2. (A) Correlation of IPSS total score and IIEF-5 total score. (B) Correlation of CLSS total score and IIEF-5 total score. ρ: Spearman correlation coefficient.

correlated with the total IIEF-5 score (Figure 2A). The symptoms other than daytime frequency and incomplete emptying showed a significant relationship with the total IIEF-5 score (Table II). Likewise, the total CLSS scores significantly correlated with the total IIEF-5 score, with a Spearman rank correlation coefficient of −0.2854 ($p < 0.0001$, Figure 2B). The correlation between the individual symptoms of CLSS and the total IIEF-5 score is shown in Table II. The symptoms other than daytime frequency and incomplete emptying in the CLSS questionnaire showed significant relationship with the total IIEF-5 score. Interestingly, both bladder and urethral pain showed significant inverse correlation with the total IIEF-5 score ($p = 0.0168$ and 0.0051, respectively Table II). Furthermore, the multivariate analysis identified nocturia and urethral pain as independent factors for low IIEF-5 scores ($p = 0.00025$ and $p = 0.00547$,respectively, IIEF-5 total score = 11.68 − (1.884 × nocturia) − (1.676 × urethral pain), Table III)

Discussion

LUTS and sexual dysfunction are common in aging men. Recently, the association between LUTS and ED has been

Table II. Correlation between CLSS, IPSS, and IIEF score ($n = 220$).

	CLSS		IPSS	
	r	*p* value	*r*	*p* value
Daytime frequency	−0.0827	0.2215	−0.1217	0.0716
Nocturia	−0.2905	<0.0001	−0.3205	<0.0001
Urgency	−0.1628	0.0156	−0.1759	0.0089
Urgency incontinence	−0.201	0.0028	—	
Stress incontinence	−0.1752	0.0092	—	
Slow stream	−0.1655	0.014	−0.2115	0.0016
Straining	−0.2115	0.0016	−0.26	<0.0001
Interruption	—		−0.2055	0.0022
Incomplete emptying	−0.0962	0.1552	−0.1144	0.0965
Bladder pain	−0.1611	0.0168	—	
Urethral pain	−0.1882	0.0051	—	

r: Spearman's correlation coefficient. CLSS: Core lower urinary tract symptom score. IPSS: International prostate symptom score. IIEF: International index of erectile function. —: not addressed.

Table III. The result of multivariate regression model.

	p value
Daytime frequency	—
Nocturia	0.00025
Urgency	—
Urgency incontinence	—
Stress incontinence	0.07183
Slow stream	—
Straining	0.10179
Incomplete emptying	0.22512
Bladder pain	—
Urethral pain	0.00547

investigated in community-based studies [1–5,11–13]. Using the IPSS questionnaire as an assessment tool for LUTS, these studies showed a significant association between ED and LUTS including storage and voiding symptoms [1–5,11–13]. We have recently developed the CLSS questionnaire to assess core or important symptoms in various pathological conditions [8]. With questions on incontinence and pain, the CLSS questionnaire is more useful as an assessment tool of male LUTS than the IPSS questionnaire at the initial stage [9]. In the present study, using the IPSS and CLSS questionnaires, we tried to determine the relationship of LUTS scores with sexual function as measured by the IIEF-5 questionnaire.

The results confirmed the significant relationship between IPSS and ED. Among IPSS and CLSS symptoms other than daytime frequency and incomplete emptying showed significant correlation with the total IIEF-5 score. Importantly, both bladder and urethral pain showed significant inverse correlation with the total IIEF-5 score. In addition, nocturia and urethral pain were identified as independent factors for low IIEF-5 score by a multivariate regression analysis. These results indicate that evaluation of pain symptoms is indispensable for urological assessment in men with pelvic health problems.

Urogenital pain may be caused by various conditions. Chronic prostatitis is one of the most common conditions; about 40% of men with prostatitis experienced urethral or bladder pain in this study.

Concerning the relationship between pain and ED, testicular pain has been shown to impair sex drive and satisfaction, and perineal pain increased patients' sexual problems [14]. In another study, pelvic pain was associated with sexual anxiety, lack of interest in sex, and orgasm, and erectile difficulties [15]. These reports imply that urogenital pain could be a major cause of ED.

Despite the fact that accumulating evidence has identified LUTS as a risk factor for ED in aging men, the precise etiology between these two disorders remains to be clarified. Suggested mechanisms include a decrease of nitric oxide/nitric oxide synthase in the endothelium, increased Rho-kinase activity and calcium sensitivity, an adrenergic receptor imbalance, and autonomic hyperactivity [16,17]. Urogenital pain may induce the release of endorphins, which in turn act on mμ opiate receptors, strongly suppressing libido and sexual function. Opiates have been shown to decrease libido and sexual function by suppressing luteinizing hormone secretion and subsequently serum testosterone [18–20]. In these studies, blockade of mμ opiate receptors recovered sexual function [20]. Psychological effects of urogenital pain may interfere with sexual function [21]. Men with chronic prostatitis are known to experience depression that definitely impairs sexual function [22].

The limitations of our study include 1) an inadequate sample number, which might underestimate significance of certain symptoms, 2) the cross sectional nature of investigation, and 3) inclusion of Japanese men only. Longitudinal studies and/or studies using cohorts of different cultural or clinical background are warranted to confirm the results of this study.

In conclusion, urethral pain was identified as an independent factor for low IIEF-5 score. CLSS rather than IPSS would be a more appropriate tool for LUTS evaluation in men with pelvic health problem. Looking at LUTS symptoms would be mandatory in men with ED. In case with urethral pain, further examination might be considered.

Declaration of Interest: The authors declared no conflict of interest.

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Original Article: Clinical Investigation

Management trends, angioembolization performance and multiorgan injury indicators of renal trauma from Japanese administrative claims database

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Abbreviations & Acronyms
AE = angioembolization
AIS = Abbreviated Injury Scale
CI = confidence interval
DPC = Diagnosis Procedure Combination
ICD-10 = International Classification of Diseases and Related Health Problems, Tenth Revision
Nx = nephrectomy
RR = rate ratio

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Objectives: To show the characteristics and therapeutic trends of renal trauma in Japan using a nationwide database.

Methods: All renal trauma cases from the Diagnosis Procedure Combination database during 6 months of each year from 2006 to 2008 were included in the analysis. The following variables were considered: demographics, ambulance use, comorbid trauma, interventions, mechanism of injury and the Abbreviated Injury Scale. Patients were divided into two groups by trauma range: limited to rib, abdomen and pelvis (group A) or more extended (including supradiaphragmatic regions or lower extremities; group B). Rib fracture impact was assessed as a predictor of comorbid organ trauma. The incidences of angioembolization failure and nephrectomy were also evaluated.

Results: A total of 1505 renal trauma cases (1014 and 491 in groups A and B, respectively) were identified. Comorbid trauma in the liver, spleen and lumbar/pelvic fractures were 7.4%, 5.6% and 5.1% in group A and 24.0%, 11.2% and 17.5% in group B, respectively. The rates of angioembolization (and its failure proportion), nephrectomy, transfusion and mortality were 7.9% (12.5%), 3.3%, 15.6% and 1.1% in group A, and 17.1% (11.9%), 2.6%, 28.3% and 8.1% in group B, respectively. Risks of coincident traumas in the liver, spleen and pelvic fracture were 2.23, 2.35 and 2.72 times higher if a rib fracture was observed. The incidences of renal trauma and nephrectomy (per 100 000 person-years) were estimated as 2.06 and 0.063, respectively.

Conclusions: Angioembolization failure is not rare, and nephrectomy is an important last resort. Patients with comorbid rib fracture should be explored for coincident traumas.

Key words: embolization, kidney, mortality, nephrectomy, trauma.

Introduction

During the past two decades, non-operative management has become widely accepted as the preferable approach to renal trauma with advancing technology of transcatheter AE.[1–3] Although traumatic nephrectomy is becoming rare, it is still an effective last resort, and physicians should not hesitate to carry out nephrectomy if the need arises.

To reveal the characteristics of renal trauma involving multiple comorbid organ traumas and a therapeutic choice strategy is very useful for physicians' decision-making algorithms. To our knowledge, only one report from the USA has been published that describes the therapeutic trend and outcome of AE based on a nationwide cohort.[1] A risk assessment of traumatic nephrectomy is also informative for people who have only a single kidney or who face undergoing nephrectomy for urological malignancy.

The present study showed today's characteristics and therapeutic trends of renal trauma, and evaluated the incidence of traumatic nephrectomy in both the male and female general

population in Japan using the DPC database, a nationwide administrative database.

Methods

DPC database

The nature of the DPC database has been previously described.[4–6] In brief, this database collects inpatient administrative claims data in Japan that contain: (i) main diagnoses, comorbidities at admission and complications after admission accompanied by ICD-10 codes; (ii) surgical procedures accompanied by original Japanese K-codes; and (iii) discharge status. The numbers of patients in the database were 1.08-, 2.99- and 2.86 million in 2006, 2007 and 2008, respectively, and the number in 2008 represented approximately 40% of all acute care inpatient hospitalizations in Japan.

Sampling strategy

The database held the data between 1 July and 31 December 2006 to 2008 (6 months during each year), and we used it. The patients included in the present study were those who were diagnosed with "injury of kidney" (ICD-10 code; S37.0). Given the anonymous nature of the data collection process, informed consent was not required. Study approval was obtained from the Institutional Review Board of University of Occupational and Environmental Health, Fukuoka, Japan. The following information was extracted for each patient: sex, age, use of ambulance, comorbidities at admission, use of transfusion, mortality and therapeutic procedures related to renal trauma. AE failure was defined as requirement for subsequent therapy, which suggests a failure to control bleeding, urine leakage or other undefined problems. We also obtained the mechanism of injury (blunt or penetrating) and the Abbreviated Injury Scale (AIS, 1998) at the abdomen, both of which were voluntary items. AIS is a component of the Injury Severity Score, which is an anatomical scoring system developed for quick evaluation of multiple injuries and critical care. AIS is an ordinal scale ranging from 1 (minor injury) to 6 (non-survival injury).[7–9]

Descriptive and statistical analysis

The patients were divided into two groups according to the range of "severe trauma": in group A, "severe trauma" was restricted to the ribs, abdomen, and/or pelvis; in group B, "severe trauma" ranged from the supradiaphragmatic region to the lower extremities. "Severe trauma" was defined as corresponding to the ICD-10 code of bone fractures, organ injury, open wound, crushing injury and traumatic amputation of a body part (Appendix I). We then calculated the incidence of coincidence of abdominal and pelvic trauma with and without a comorbid rib fracture. Furthermore, among the cases with abdominal AIS scores of 3 (severe injury) or more, we compared the AE failure rate between level I trauma centers and lower-level centers. Finally, the incidences of renal trauma and traumatically imperative nephrectomy were calculated by a person-year method.

Univariate comparisons of each variable were carried our using the χ^2-test. The threshold for significance was $P < 0.05$. All P-values and 95% CI were calculated using PASW version 18.0 (SPSS, Chicago, IL, USA).

Results

Among the 8.42 million inpatients in the study population, 1505 with renal trauma were identified. Table 1 shows the comorbidities, therapeutic procedures and clinical characteristics of renal trauma patients. Males accounted for 72%, and the median age was 41 years (range 0–96 years). Patients in group B required longer hospital stays, more frequently injured other organs, and had higher transfusion rates and mortality than did patients in group A. Overall, renal trauma patients had another trauma in an abdominal organ at the rate of 15.8% in group A and 37.5% in group B ($P < 0.001$). AE was more frequently required in group B (7.9% in group A and 17.8% in group B, $P < 0.001$), and no significant differences were observed in their failure rates (12.5% and 11.9%, respectively; $P = 0.907$) or nephrectomy rates (3.3% and 2.6%, respectively; $P = 0.521$). Considering only ambulance users ($n = 792$), the overall rates of AE, nephrectomy and mortality increased to 17.6%, 4.4% and 5.5%, respectively. Information about mechanisms of renal trauma was available from 320 patients. Blunt and penetrating injuries were assigned to 304 (95%) and 16 (5%) patients, respectively.

In Table 2, we considered a rib fracture to be a predictor of coincident organ injury. If a rib fracture was present, risks of coincident traumas of the liver and spleen, and fractures of lumbar and pelvic bones were observed 2.23 (95% CI, 1.72–2.89), 2.34 (1.64–3.33), 2.45 (1.75–3.42) and 2.74 (1.85–4.05) times higher, respectively.

Figure 1 shows therapeutic trends for renal trauma. A total of 46 nephrectomies were carried out, and their previous procedures were one AE ($n = 10$), two AE ($n = 1$), open repair ($n = 1$) and no treatment ($n = 34$). Interval dates between previous intervention and nephrectomy were 0 ($n = 9$), 1 ($n = 2$) and 7 ($n = 1$) days. Among 230 patients who underwent AE, 177 (76.9%) underwent the first intervention on the hospitalization day, and 37 (16.0%) underwent the first intervention the next day. Two patients underwent arteriovenous fistula constructions for hemapheresis.

AIS was obtained from 52 patients who underwent AE, and 47 were assigned an AIS of 3 or more. Among these 47 patients, AE failure occurred in two of 40 (5.0%) in level I trauma centers, and in two of seven (28.5%) in lower-level hospitals ($P = 0.046$).

To estimate the incidence of renal trauma, we first calculated the presumable cohort population in the present study.

Table 1 Characteristics of patients with renal trauma

Trauma range	Rib, Abdomen and pelvis (group A)	More extended (group B)
Total	1014	491
Median age, years (range)	41 (2–96)	42 (0–96)
Median length of stay, days (IQR)	11 (7–18)	19 (11–35)
Male (%)	719 (70.9)	366 (74.5)
Ambulance use (%)	397 (39.2)	395 (80.4)
Trauma (%)		
Rib fracture†	109 (10.7)	265 (54.0)
Overall abdominal organs	160 (15.8)	184 (37.5)
Liver	75 (7.4)	118 (24.0)
Spleen	57 (5.6)	55 (11.2)
Pancreas	14 (1.4)	6 (1.2)
Stomach or bowel	11 (1.1)	10 (2.0)
Abdominal vessel	4 (0.4)	15 (3.1)
Pelvic organs	16 (1.6)	14 (2.9)
Lumbar or pelvic fracture	52 (5.1)	86 (17.5)
Transfusion (%)	158 (15.6)	139 (28.3)
Mortality (%)	11 (1.1)	40 (8.1)
Intervention (%)		
AE	80 (7.9)	84 (17.1)
AE failure‡	10 (12.5)	10 (11.9)
Open repair	16 (1.6)	7 (1.4)
Nephrectomy	33 (3.3)	13 (2.6)

†Rib fractures included intrathoracic severe traumas. (S223–5, S25.x–28.x.) ‡AE failure was defined as a requirement of subsequent therapy. The proportion was expressed as a percentage of total AE.

Table 2 Risk rate ratios of other organ trauma with versus without rib fracture

	Rib fracture		RR (95%CI)
	No	Yes	
Total	1131	374	
Liver (%)	111 (9.8)	82 (21.9)	2.23 (1.72–2.89)
Spleen (%)	63 (5.6)	49 (13.1)	2.34 (1.64–3.33)
Lumber fracture (%)	68 (6.0)	55 (14.7)	2.45 (1.75–3.42)
Pelvic fracture (%)	49 (4.3)	44 (11.8)	2.74 (1.85–4.05)
Other abdominal or pelvic trauma (%)	86 (7.6)	41 (11.0)	1.45 (1.01–2.06)

We utilized the DPC database to collect inpatient data for 6 months of each year, and coverage rates (pi) were 27.4%, 44.2%, and 42.7% of all acute hospitalizations in 2006, 2007, and 2008, respectively. According to National Census data[10] the general population in Japan (Ni) is was 127.7, 127.7, and 127.6 million in 2006, 2007, and 2008, respectively. Therefore, a presumable general population cohort ($\Sigma[Ni \times pi/2]$) was 72.9 million person-years. The incidences of renal trauma and traumatic nephrectomy (per 100 000 person-years) were then estimated as 2.06 (95% CI, 1.95–2.16) and 0.063 (0.044–0.081), respectively.

Discussion

We showed today's characteristics and therapeutic trends of renal trauma using a large population based database. First, we found that rib fracture was associated with higher occurrences of coincident liver and spleen trauma (rate ratios of 2.23 and 2.35, respectively). Higher risk for spleen than liver trauma is a rationale in the consideration of a longer distance between the right kidney and the ribs, than between the left kidney and the ribs. This tendency mirrors published reports by Shweiki *et al.* who examined 476 cases and showed that

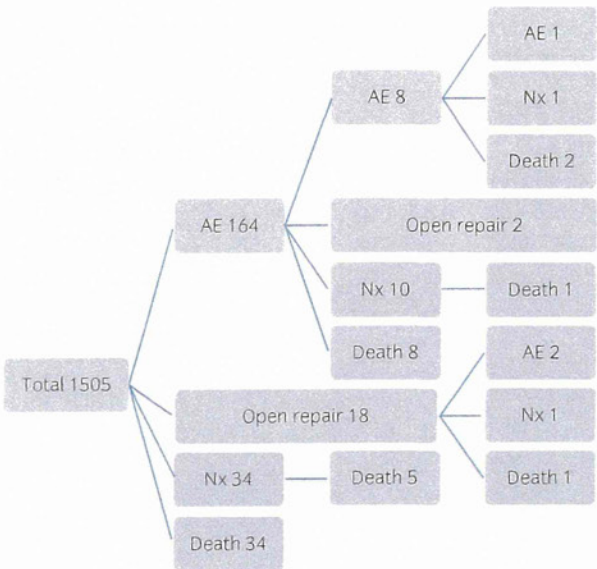

Fig. 1 Repeated procedures undergone by patients with renal trauma.

the odds ratios of liver and spleen trauma in the presence of any low rib fracture were 2.11 and 2.42, respectively.[11]

We also clarified today's therapeutic trend. Overall, 89% of all cases underwent no intervention. In general, conservative management for renal trauma is widely used today, and it is reported that most grade I and II, and stable parenchymal grade III and IV injuries were managed without any interventions.[2,12]

In contrast, nephrectomy was carried out in 3.1% of all patients with renal trauma and 6.7% of patients initially undergoing AE. This shows that timely nephrectomy remains an important last resort despite the fact that AE has advanced. Nephrectomy rates vary widely among countries. For example, Dobrowolski *et al.* reported that the rate of 73% in Poland was much higher than that of 11.3% in the USA.[13] He stated that this difference might be affected by hospital access, availability of computed tomography with contrast medium, and other social factors, such as traffic accident frequency and gun regulation. Kuan *et al.*[7] reviewed 8465 cases of renal trauma in the National Trauma Data Bank of the USA between 1994 and 2003, and mentioned that the nephrectomy rate and mortality were 7.3% and 11.3%, respectively, which were higher than the present study, and that blunt mechanisms represented 81.4% of cases, which was lower than the present. Besides racial variants and differences in database backgrounds, we infer that social factors play an important role in this discrepancy. In developed countries, traffic accidents could account for many renal traumas besides falls.[3] Traffic accident fatalities per 100 000 population are 11.01 and 3.85 in the USA[14] and Japan,[15] respectively. Guns are strictly regulated in Japan, and in the present study, only one case had trauma related to gunshot.

The AE performance difference is also interesting. From the USA, Hotaling *et al.* showed that among grade IV and V renal trauma cases, level I trauma centers achieved a significantly higher diagnostic angiography success rate than those of other hospitals.[1] In the present study, we attempted a similar analysis by using the AIS score as a severity indicator; however, few cases involved the AIS, especially those in the lower-level emergency hospitals. Although level I trauma centers barely achieved a favorable result ($P = 0.046$), further data accumulation is required.

Incidences of renal trauma and traumatic nephrectomy in Japan were 2.06 and 0.063 per 100 000 person-years, respectively. This is useful information, especially for people who have only a single kidney or who face selecting either nephrectomy or renal-sparing surgery for urological malignancy. Although the associated life expectancy is >20 years, physicians could explain the traumatic nephrectomy risk as <0.002%. Of course, however, renal failure derived from chronic kidney disease is a separate issue. After the nephrectomies, arteriovenous fistula for hemapheresis were required in two patients (0.13% overall and 0.25% in ambulance users). Another report from the USA[7] stated that dialysis was required in 0.46% of cases (perhaps including transit dialysis), which is unlikely to make much of a difference to the present study.

There were several limitations in the present study. Because the definition of trauma is completely subjective to the doctor's judgment, and comorbidity/complication lists were limited to up to four diagnoses, an underestimation of comorbidities or complications could have occurred. Especially in life-threatening cases, diagnoses with less critical priority might be unlikely to be reported. Furthermore, the present study focused on inpatient cases only, and consequently there might be a sample bias toward patients who are more seriously ill. Furthermore, because critically traumatized patients are likely to be transported to large hospitals, and because there is a bias toward large hospitals in the DPC database,[16,17] the calculation could be an overestimation. Finally, several important clinical parameters, such as causes of trauma, renal trauma grading and laboratory or imaging test results, were not recorded in the database. Despite these limitations, using the nationwide database enabled us to show today's therapeutic trends of renal trauma based on real-world data.

We showed that angioembolization failure is not rare and that nephrectomy is still an effective last resort. Patients with a comorbid rib fracture should be carefully examined for coincident organ trauma.

Conflict of interest

None declared.

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Appendix I

ICD-10 definition of "severe trauma"

Region	
Supradiaphragm (including upper extremities)	S02.x, S04–9.x, S11.x, S12.x, S14.x, S15.x, S17–9.x, S21.x, S22.0–5, S22.5, S22.8, S22.9, S24–9.x, S41.x, S42.x, S47–9.x, S51.x, S52.x, S57-9.x, S61.x, S62.x, S67–9.x
Overall abdominal organs	S36.x
Liver	S36.1
Spleen	S36.3
Pancreas	S36.2
Stomach or bowel	S36.4–7
Abdominal vessel	S35.0–3, S35.5, S35.7–9
Pelvic organs	S37.1–9, S38.x, S39.x
Lumbar fracture	S32.0, S32.7, S32.8
Pelvic fracture	S32.1, S32.3–5, S32.7. S32.8
Low extremities	S71.x, S72.x, S77–9.x, S81.x, S82.x, S87–9.x, S91.x, S92.x, S97–9.x
Multiple or unspecified regions	T01.x, T02.x, T04–6.x, T09.1, T09.2–6, T09.8, T09.9, T11.0–6.x, T11.8, T11.9, T13.0–6, T13.8–4.9

BJUI
BJU INTERNATIONAL

Assessment of lower urinary tract symptoms in men by international prostate symptom score and core lower urinary tract symptom score

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Study Type – Therapy (symptom prevalence)
Level of Evidence 2a

What's known on the subject? and What does the study add?
The International Prostate Symptom Score (IPSS) has been most commonly used for the symptom assessment of men with lower urinary tract symptoms (LUTS). However, LUTS in men are so variable that they may not be fully captured by the IPSS questionnaire alone. This study has demonstrated that the Core Lower Urinary Tract Symptom Score (CLSS) questionnaire, which addresses 10 important symptoms, is an appropriate initial assessment tool for LUTS in men with various diseases/conditions.

OBJECTIVE

- International Prostate Symptom Score (IPSS) has been commonly used to assess lower urinary tract symptoms (LUTS). We have recently developed Core Lower Urinary Tract Symptom Score (CLSS). *The aim of this study is to compare IPSS and CLSS for assessing LUTS in men.*

PATIENTS AND METHODS

- Consecutive *515* men fulfilled IPSS and CLSS questionnaires.
- IPSS QOL Index was used as the QOL surrogate.
- The clinical diagnoses were BPH ($n = 116$), BPH with OAB wet ($n = 80$), prostate cancer ($n = 128$), prostatitis ($n = 68$), underactive bladder ($n = 8$), others ($n = 72$), and controls (e.g., occult blood) ($n = 42$).
- Simple statistics and predictability of poor QOL (QOL Index 4 or greater) were examined.

RESULTS

- All symptom scores were significantly increased in symptomatic men compared with controls. Scores of corresponding symptoms of two questionnaires were significantly correlated ($r = 0.58–0.85$, all $P < 0.0001$).
- A multivariate regression model to predict poor QOL indicated *nine* symptoms (daytime frequency, nocturia, urgency, urgency incontinence, *slow stream*, straining, incomplete emptying, bladder pain and urethral pain) as independent factors.
- The hazard ratios for bladder pain (*2.2*) and urgency incontinence (*2.0*) were among the highest.
- All the nine symptoms are addressed in CLSS, while three symptoms (urgency incontinence, bladder, and urethral pain) are dismissed in IPSS.

CONCLUSION

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

KEYWORDS

LUTS, assessment, CLSS, IPSS, BPH

INTRODUCTION

Assessment of LUTS is highly important in the diagnosis and treatment of lower urinary tract dysfunction. The IPSS would be most commonly used as the assessment tool for men with BPH, overactive bladder (OAB), and after radical prostatectomy and prostatic radiotherapy [1–5]. However, LUTS associated with these conditions are so variable that they may not be fully captured by the IPSS questionnaire alone. For example, men with BPH or prostate cancer undergoing radiotherapy often have LUTS such as urgency, urgency incontinence and lower abdominal pain [5] although none of these symptoms are addressed by the IPSS questionnaire [1]. For this reason, a more comprehensive assessment tool is needed for the precise appraisal of LUTS in men. The International Consultation on Incontinence Questionnaire for Male LUTS (ICIQ-MLUTS) is designed to assess a variety of LUTS in men in a non-disease-specific

Age (year)	67.7 ± 11.1*
Serum PSA (ng/mL) (n = 397)	5.9 ± 16.9*
Prostate volume (mL) (n = 319)	31.5 ± 21.8*
Uroflowmetry (n = 178)	
Voiding volume (mL)	222 ± 148.4*
Peak flow rate (mL/s)	15.1 ± 24.3*
Residual volume (mL)	66.7 ± 136.1*
Diagnostic group	
BPH	116
BPH with OAB	80
Prostate cancer	128
T2N0M0	85
T3N0M0	27
M1	16
After radical prostatectomy	43
Androgen deprivation therapy	76
After radiotherapy	4
Others	9
Prostatitis	68
Type I	12
Type II	10
Type III	8
Type IV	38
Underactive bladder	8
Others	72
Control	42

TABLE 1
Characteristics of the study subjects (N = 515)

**Means ± SD. PSA, prostate-specific antigen; BPH, benign prostatic hyperplasia; OAB, overactive bladder.*

manner [6]. Unfortunately, this questionnaire is practically too extensive and does not address pain symptoms; a simpler and more appropriate questionnaire is desirable for the clinical practice.

Recently we have developed the Core LUTS Score (CLSS) questionnaire for core or important symptoms in various pathological conditions [7]. The CLSS questionnaire addresses 10 important symptoms selected from 25 symptoms defined by the ICS standardization committee (Table 2). These 10 symptoms were chosen as the most influential three symptoms by more than one-quarter of patients of nine common conditions/diseases [7]. This characterizes the CLSS questionnaire as an overall or non-disease-specific symptom assessment tool with possible multiple dimensions. The CLSS questionnaire has been confirmed for validity and reliability in male and female subjects [7]. In addition, the CLSS questionnaire can be user-friendly for patients because of the simplicity of its question wording and response scale. Here, we compared the IPSS questionnaire and the CLSS questionnaire for the assessment of LUTS in men.

PATIENTS AND METHODS

The study was approved by our institutional ethical committee. In all, 515 consecutive treatment-naive men who visited our hospital, a tertiary referral institution, for urological conditions between April 2009 and April 2010, were enrolled (Table 1). The subjects, aged from 14 to 91 years (mean 67.7 ± 11.1 years), were divided into disease groups by routine urological examinations: BPH (n = 116), BPH with OAB (n = 80), prostate cancer (n = 128), prostatitis (type I: n = 12, II: n = 10, III: n = 8, IV: n = 38), underactive bladder (n = 8), and others (n = 72). Men from the control group (n = 42) were subjectively free of LUTS and comprised occult blood (n = 35), retroperitoneal fibrosis (n = 4) and non-functioning adrenal tumour (n = 3). Patients with BPH with urgency incontinence were regarded as BPH with OAB. Men with high serum levels of PSA underwent a prostate biopsy to exclude cancer and questionnaire data from before the biopsy were used for analysis.

The subjects were asked to response to two self-administered questionnaires for IPSS and CLSS on the same occasion.

The IPSS questionnaire comprises seven questions on LUTS (incomplete emptying, frequency, intermittency, urgency, weak stream, straining and nocturia) and an additional question to yield quality of life (QoL) index, which was scored from 0 (delighted) to 6 (terrible) and used as the QoL surrogate in this study. The CLSS questionnaire addresses 10 symptoms: daytime frequency, nocturia, urgency, urgency incontinence, stress incontinence, slow stream, straining, incomplete emptying, bladder pain and urethral pain [7]. Voiding frequency was scored as follows: 0 (fewer than seven times), 1 (8–9 times), 2 (10–14 times), 3 (15 times or more) for the day time, and 0 (never), 1 (once), 2 (two or three times), and 3 (four or more times) for the night time. Other symptoms were scored according to the frequency of episode (0–3) (Table 2). These symptoms were chosen from 25 types of LUTS as the most influential three symptoms using 1000 symptomatic individuals. The CLSS questionnaire further inquires into the single symptom (the single core symptom or the chief complaint) that the patients considered to have the most significant impact on daily life.

Difference in the symptom scores by clinical characteristics was analysed using the Wilcoxon rank sum test or chi-squared test. Correlations between the scores were evaluated by Spearman rank correlation coefficients. A multivariate regression model was used to identify symptoms predicting the poor QoL (QOL index score 4 or more). We used STATVIEW 5.0 software (SAS Institute, Cary, NC, USA) and regarded $P < 0.05$ as statistically significant.

RESULTS

Men with any diseases/conditions had significantly higher scores than the controls for all the symptoms examined ($P < 0.05$). For example, positive (score ≥1) rates in patients with BPH and patients with prostate cancer were significantly higher than in controls for all symptoms of CLSS (Fig. 1; top panel). Urgency incontinence, stress incontinence, bladder pain and urethral pain, which are not addressed in the IPSS questionnaire, also had higher positive rates in BPH and prostate cancer. Notably, 23% of men with BPH (27/116),

16% of men with BPH with OAB (13/80), and 15% of men with prostate cancer (19/128) experienced bladder/urethral pain to some extent.

The correlation between the corresponding symptoms of IPSS and CLSS is shown in Table 2. Spearman rank correlation coefficients (r) were 0.58 to 0.85 for all IPSS symptoms (all, $P < 0.001$) excepting interruption, which is not included in the CLSS. The total score of CLSS was significantly related to the total of IPSS (0.81; $P < 0.001$).

The single core symptom, the most influential symptom, was incomplete emptying (25.2%) in BPH followed by nocturia (20.9%), slow stream (15.6%) and urgency (9.6%), and was nocturia (23.7%), followed by urgency incontinence (22.5%) and urgency (20%), in BPH with OAB (Fig. 1; bottom panel). Men with prostate cancer complained of urgency (17.1%) most often, followed by urgency incontinence (10.9%), nocturia (10.6%), slow stream (10.1%) and stress incontinence (5.4%).

The QoL Index was significantly higher in all disease groups than in controls ($P < 0.001$). The poor QoL, which was defined as QoL Index of 4 (mostly dissatisfied) and over, had no significant relationship with clinical parameters, such as age, prostate volume, serum PSA levels, voiding volume and residual urine (data not shown) except for peak flow rate; peak flow rate under the median (11.9 mL/s) was correlated with poor QoL (hazard ratio; 2.0, $P = 0.04$).

By contrast the poor QoL significantly correlated with all symptom scores of two questionnaires ($P < 0.001$) (Table 3). The multivariate analysis identified nine symptoms as the independent factor to predict poor QoL; that is, daytime frequency, nocturia, urgency, urgency incontinence, slow stream, straining, incomplete emptying, bladder pain and urethral pain (Table 3). The hazard ratio was highest for bladder pain (2.2) followed by urgency incontinence (2.0). All nine symptoms are addressed in CLSS. The IPSS questionnaire, however, dismisses urgency incontinence, bladder pain and urethral pain. Interruption, which is embraced in the IPSS questionnaire alone, was not an independent factor.

Parameter	Spearman's correlation coefficient
Daytime frequency	0.58*
Nocturia	0.85*
Urgency	0.61*
Urgency incontinence†	–
Stress incontinence†	–
Slow stream	0.72*
Straining	0.67*
Interruption‡	–
Incomplete emptying	0.79*
Bladder pain†	–
Urethral pain†	–
Total score	0.81*

*CLSS, Core lower urinary tract symptom score; IPSS, International Prostate symptom score. *Spearman's correlation coefficient with CLSS: P < 0.001 for all correlations. †Not addressed in IPSS. ‡Not addressed in CLSS.*

TABLE 2
Correlation between CLSS and IPSS of corresponding symptom scores (N = 515)

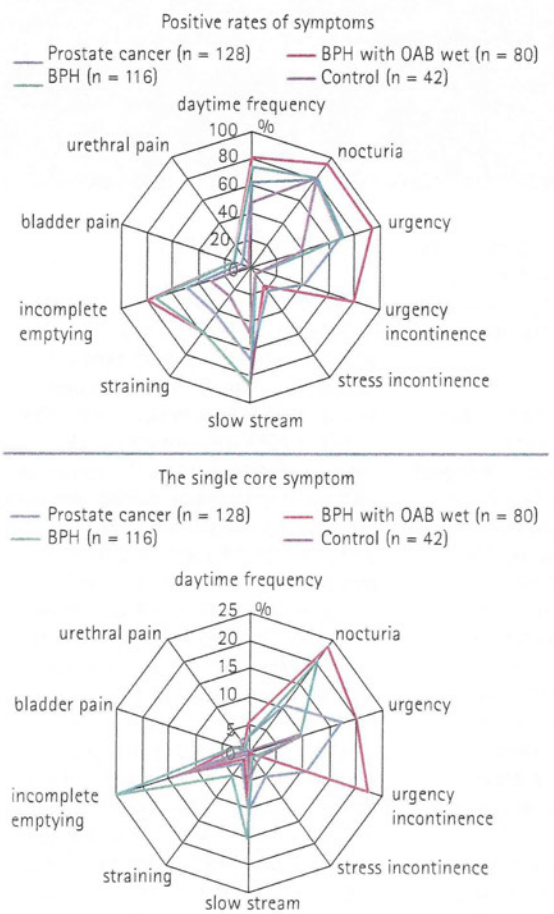

FIG. 1.
Positive rates of symptoms (top) and the single core symptom (bottom) in men with benign prostate hyperplasia (BPH) and prostate cancer. Top panel: positive (score ≥ 1) rates were significantly higher in men with BPH and prostate cancer than controls for all the symptoms addressed by the Core LUTS Score (CLSS). Urgency incontinence, stress incontinence, bladder pain, and urethral pain, which are not included in the International Prostate Symptom Score (IPSS) questionnaire, also showed higher positive rates. Lower panel: the single core symptom, the most influential symptom, was incomplete emptying (25.2%) in BPH, nocturia (23.7%) in BPH with overactive bladder (OAB) and urgency (17.1%) in prostate cancer.

DISCUSSION

Assessment of LUTS is indispensable for physicians to make the accurate diagnosis, treatment selection and efficacy evaluation in lower urinary tract disorders. For men complaining of LUTS the IPSS questionnaire has often been used as the symptom

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.

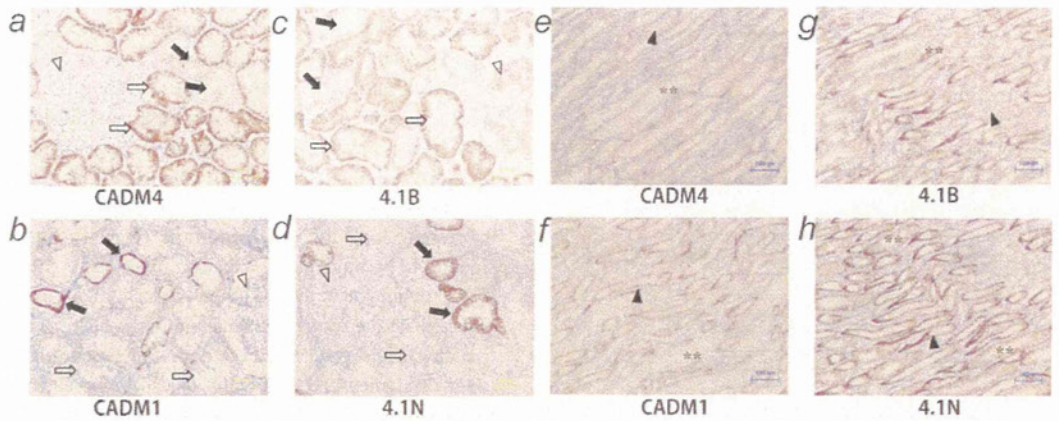

Figure 1. Immunohistochemical analyses of CADM4 (*a* and *e*), CADM1 (*b* and *f*), 4.1B (*c* and *g*) and 4.1N (*d* and *h*) proteins in normal human renal cortex (*a–d*) and medulla (*e–h*). Expression of CADM4 and 4.1B is detected in the proximal convoluted tubules (*a* and *c*), whereas that of CADM1 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

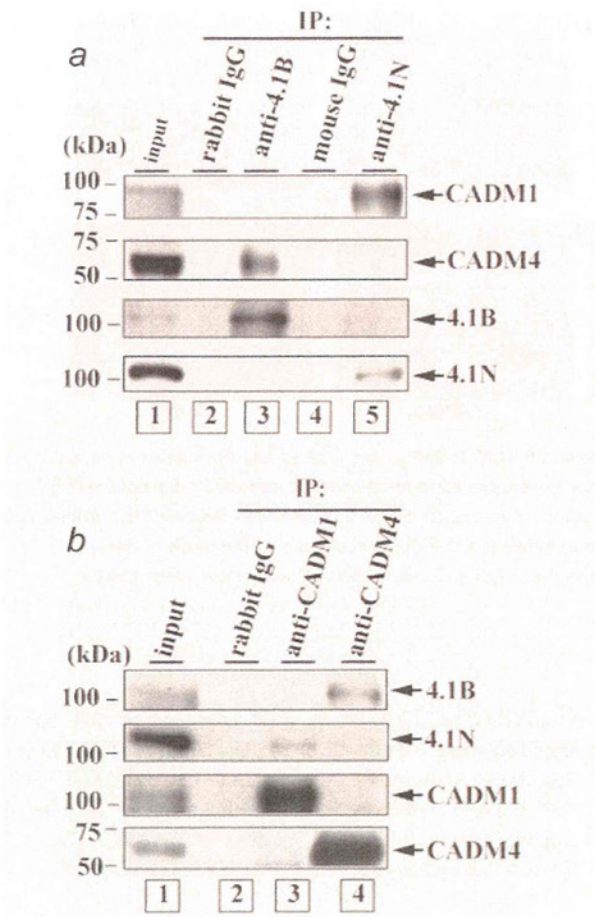

Figure 2. Interaction of CADM4 with 4.1B and CADM1 with 4.1N. (*a*) Total lysates of normal human kidney were immunoprecipitated with control rabbit IgG (lane 2), anti-4.1B pAb (lane 3), control mouse IgG (lane 4) and anti-4.1N pAb (lane 5), and binding proteins were detected by immunoblotting using 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

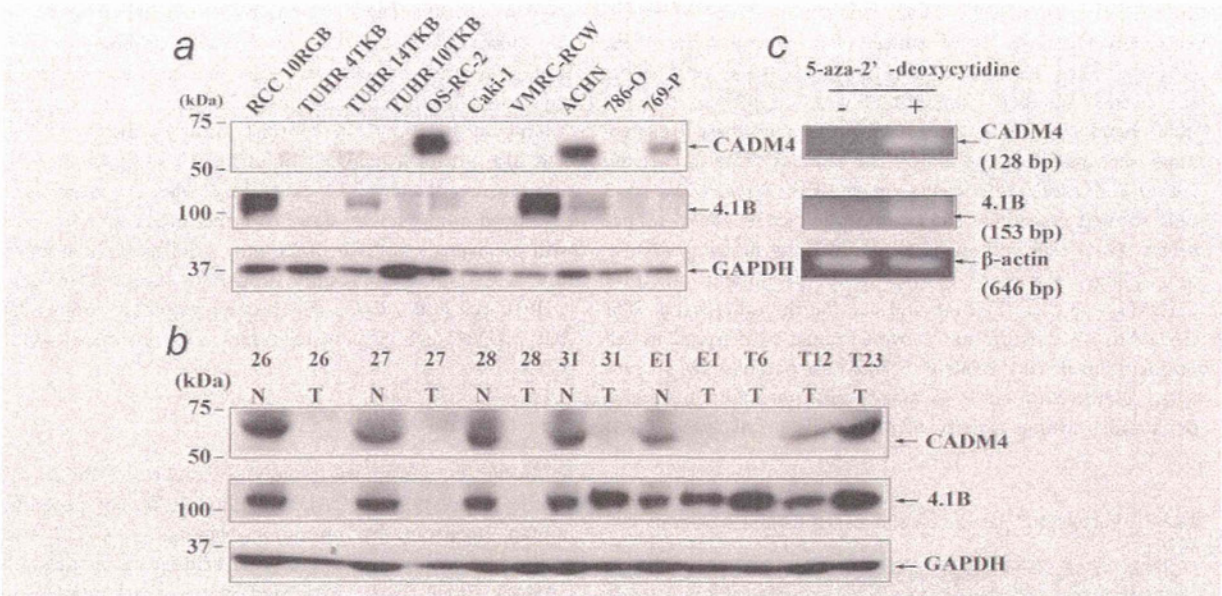

Figure 3. Loss of expression of CADM4 and 4.1B proteins in RCCC and restoration of CADM4 expression by 5-aza-2′-deoxycytidine. (*a* and *b*) Immunoblotting of CADM4 and 4.1B proteins in 10 RCC cell lines (*a*) and the primary tumors of RCCC (*b*). An aliquot of the tissue lysates (1 μg) was subjected 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		Vascular Infiltration		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.

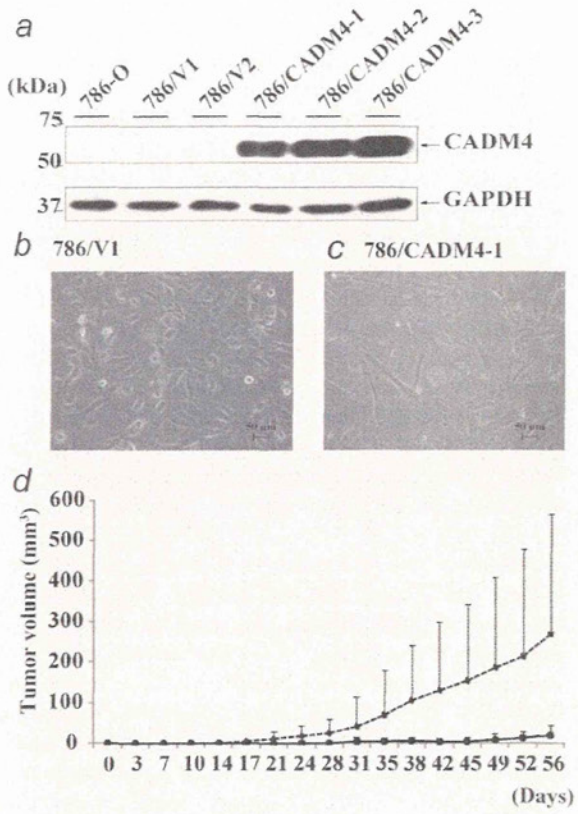

Figure 4. Suppression of tumorigenicity of 786-O cells in nude mice by CADM4. (*a*) Western blotting of CADM4 protein (upper) and control GAPDH protein (lower) in parental 786-O cells, 786-O cell clones transfected with a vector alone (786/V1, 786/V2) and 786-O cell clones transfected with CADM4 (786/CADM4-1, 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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