target genes of the Tbr-1-CASK complex. These observations suggest that CADM1 may function in the nucleus as a signal transducer via RIP.

CADM1 is known to act as a tumor suppressor in nude mice, and its expression is reduced in many types of human tumor. ADAM10 is overexpressed in colorectal cancer [25]. The hypermethylation of CADM1 and the reduced expression of CADM1 protein in colorectal cancer have also been reported [26]. ADAM10-mediated shedding may be a mechanism involved in the downregulation of CADM1 in colorectal cancer, which leads to disruption of CADM1-mediated tumor suppression. On the other hand, the functional significance of γ-cleavage is rather complicated. CADM1-ICD has two protein-binding domains, band 4.1 binding domain and PDZ binding domain [1]. These domains are indispensable for the tumor-suppressive functions of CADM1, including tumor suppression in mice, the induction of apoptosis, and the suppression of epithelial-mesenchymal transition [27–29], suggesting the functional significance of the CADM1 cytoplasmic domain.

In conclusion, our data demonstrated that CADM1 cleavage is mediated by ADAM10 and γ-secretase. This mechanism may be important for the downregulation of CADM1 by proteolytic degradation or for tumor-suppressive activity and other functions of CADM1, probably through the activation of CADM1-ICD and reduction of cell–cell adhesion.

Acknowledgments

We thank Dr. Bart De Strooper (Vlaams Instituut voor Biotechnologie) for Presenilin 1/2 double knockout cells and Dr. Philip C. Wong (The Johns Hopkins University) for Nicastrin$^{-/-}$ cells. Y.N. is supported by a JSPS Research Fellowship for Young Scientists.

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Breast Cancer (2012) 19:242–252
DOI 10.1007/s12282-011-0272-7

ORIGINAL ARTICLE

Aberrant expression of tumor suppressors CADM1 and 4.1B in invasive lesions of primary breast cancer

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Received: 25 November 2010 / Accepted: 4 April 2011 / Published online: 28 April 2011

Abstract
Background The tumor suppressor genes *CADM1/TSLC1* and *DAL-1/4.1B* are frequently inactivated by promoter methylation in non-small cell lung cancer. The proteins they encode, CADM1 and 4.1B, form a complex in human epithelial cells and are involved in cell–cell adhesion.
Methods Expression of CADM1 and 4.1B proteins was examined by immunohistochemistry in 67 primary breast cancer and adjacent noncancerous tissues. CADM1 and 4.1B messenger RNA (mRNA) was detected by reverse-transcription polymerase chain reaction (RT-PCR). The methylation status of the *CADM1* and *4.1B* promoters was determined quantitatively by bisulfite treatment followed by pyrosequencing.
Results CADM1 and 4.1B protein signals were detected along the cell membrane in normal mammary epithelia. By contrast, 47 (70%) and 49 (73%) of 67 primary breast cancers showed aberrant CADM1 and 4.1B staining, respectively. Aberrant CADM1 staining was more frequently observed in pT2 and pT3 tumors and for stages II and III ($P = 0.045$ and $P = 0.020$, respectively), while aberrant 4.1B staining was more often observed in tumors with lymph node metastasis, for pT2 and pT3 tumors, and for stages II and III ($P = 0.0058$, $P = 0.0098$, and $P = 0.0007$, respectively). Furthermore, aberrant CADM1 and 4.1B expression was preferentially observed in invasive relative to noninvasive lesions from the same specimen ($P = 0.036$ and $P = 0.0009$, respectively). Finally, hypermethylation of *CADM1* and *4.1B* genes was detected in 46% and 42% of primary breast cancers, respectively.
Conclusions Our findings suggest that aberrant CADM1 and 4.1B expression is involved in progression of breast cancer, especially in invasion into the stroma and metastasis.

Keywords CADM1 · 4.1B · Tumor suppressor protein · Breast cancer · Methylation

Introduction

Breast cancer is the most common malignancy in women, and its incidence has been increasing in recent years in Japan. Many clinical and pathological factors are routinely used to categorize patients with breast cancer in order to assess prognosis and determine the most appropriate therapy. Patient age, tumor size, lymph node metastasis, nuclear grade, lymphovascular invasion, expression status of hormone receptors including the estrogen receptor (ER) and progesterone receptor (PgR), and status of human epidermal growth factor receptor 2 (Her2) have been recognized as major prognostic factors. Although these factors are mostly useful, more effective indicators are required to determine prognosis precisely and to evaluate the risk of recurrence in patients with breast cancer. Recently,

Electronic supplementary material The online version of this article (doi:10.1007/s12282-011-0272-7) contains supplementary material, which is available to authorized users.

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additional new techniques, such as gene expression profiling [1], have been developed to improve subclassification of breast cancer and to determine the most appropriate therapy as well. However, these mRNA-based diagnostic approaches have several disadvantages in practical use.

The tumor suppressor gene *CADM1* (*Cell adhesion molecule 1*)/*TSLC1* (*Tumor suppressor in lung cancer 1*) was identified on chromosome 11q23.2 by functional complementation of tumorigenicity of non-small cell lung cancer (NSCLC) cells [2–5]. *CADM1* encodes an immunoglobulin superfamily molecule that is involved in cell–cell adhesion in a variety of human epithelia, including those of the mammary gland [5]. Previous studies have revealed that *CADM1* is frequently inactivated in various cancers, such as lung [2, 3], prostate [6], liver, pancreas [4], and breast cancer [7], especially in those with invasion and metastasis to lymph nodes and distant organs. Goto et al. [15] reported that CADM1 expression was preferentially lost in invasive lesions relative to noninvasive lesions of lung adenocarcinoma. Hypermethylation of the *CADM1* promoter has been shown to be one of the main mechanisms to inactivate the gene in these cancers [2–4, 6–8].

The tumor suppressor gene *DAL-1/4.1B* (*differentially expressed in adenocarcinoma of the lung*) was shown to be located on chromosomal fragment 18p11.3 and was inactivated in lung, breast, and brain tumors [9, 10]. The protein it encodes, 4.1B, belongs to the protein 4.1 superfamily of scaffold proteins. Yageta et al. [10, 11] reported that CADM1 interacts with the actin filament through 4.1B at the cell–cell attachment site, where complex formation of CADM1 and 4.1B is dependent on the integrity of the actin cytoskeleton, and that CADM1 and 4.1B are responsible for stable adhesion between adjacent cells. It has also been reported that loss of 4.1B expression and methylation of the *4.1B* promoter are involved in development and progression of NSCLC, providing a possible indicator of poor prognosis [12]. Thus, the CADM1–4.1B cascade appears to be involved in cell–cell attachment, while functional loss of CADM1 and/or 4.1B would play a role in invasion and metastasis of tumor cells in advanced stages.

In breast cancer, promoter methylation of *CADM1* was found in 33% of surgically resected tumors using bisulfate sequencing analysis [7]. Furthermore, loss of 4.1B expression was observed in 83% of breast cancer cell lines using Western blotting [13]. Previous studies have also shown that *CADM1* and/or *4.1B* methylation correlates with tumor grade and hormone receptor status in breast cancer [14].

In the present study, we examined expression of CADM1 and 4.1B in 67 primary breast cancer specimens and the methylation status of *CADM1* and *4.1B* promoters in 6 breast cancer cell lines and 50 primary breast cancers. We then examined whether expression of CADM1 and 4.1B as well as their methylation status correlated with the clinicopathological factors of primary breast cancers in order to investigate the significance of CADM1 and 4.1B in breast tumorigenesis. Our results suggest that loss of CADM1 and 4.1B expression is involved in development and progression of breast cancer, especially in invasion and metastasis.

Materials and methods

Tissue samples and cell lines

A breast cancer cell line, MCF7, was obtained from the Human Science Research Resources Bank (Osaka, Japan). Four breast cancer cell lines (SK-BR-3, MDA-MB-361, MDA-MB-231, and BT474) were obtained from the American Type Culture Collection (Manassas, VA, USA). MDA-MB-453, another breast cancer cell line, was obtained from the RIKEN Cell Bank (Tsukuba, Japan). These cells were cultured according to the suppliers' recommendations. A series of 67 primary invasive breast cancers without systemic drug therapy before surgery were obtained from patients who underwent surgery at the Department of Breast and Endocrine Surgery of Juntendo University, Tokyo during the period 2006–2010. These included 65 invasive ductal carcinomas (8 papillotubular, 21 solid-tubular, and 36 scirrhous carcinomas) and 2 invasive lobular carcinomas. In 39 of 67 tumors, both invasive and noninvasive lesions were present within the same specimen. Informed consent was obtained from patients before surgery for specimens to be used for this research. As summarized in Table 1, the average age of the patients at diagnosis was 56.9 years, ranging from 32 to 82 years. Of 67 patients, 24 patients were under 50 years of age, while 43 patients were over 50 years of age at time of diagnosis. Twenty-one cases had lymph node metastasis. Seventeen cases had lymphovascular invasion. Fifty-six cases had positive ER and/or PgR hormone receptor expression. Ten cases had Her2 overexpression. Twenty-three, 37, and 7 cases were diagnosed as nuclear grade 1, 2, and 3, respectively, whereas 31, 33, and 3 cases were diagnosed as pT stage 1, 2, and 3, respectively. Twenty-three cases were categorized as pathological stage I, 30 cases as stage IIA, 10 cases as stage IIB, and 4 cases as stage III. Fifty of 67 tumors with diameter equal to or greater than 1.5 cm were obtained surgically, and tissue samples were frozen immediately and stored at −80°C. This study was approved by the ethics committees of Juntendo University and the Institute of Medical Science, The University of Tokyo. All samples were diagnosed and classified according to the World Health Organization (WHO) grading system and the General Rules for Clinical

Table 1 Clinicopathological characteristics of patients with primary breast cancer and expression of CADM1 and 4.1B

	n	CADM1 (%)	*P* value	4.1B (%)	*P* value
		Negative staining		Negative staining	
Total	67	47 (70)		49 (73)	
Age (years)					
<50	24	20 (80)	NS	19 (79)	NS
≥50	43	27 (63)		30 (70)	
Lymph node metastasis					
0	46	29 (63)	NS	29 (63)	
1–3	17	15 (88)		16 (94)	0.0058
≥4	4	3 (75)		4 (100)	
Lymphovascular invasion					
−	50	32 (64)	NS	33 (66)	NS
+	17	15 (88)		16 (94)	
Hormone receptor status					
+/+ or +/−	56	41 (73)	NS	40 (71)	NS
−/−	11	6 (55)		9 (82)	
Her2					
Negative	57	39 (68)	NS	41 (72)	NS
Positive	10	8 (80)		8 (80)	
NS					
1	23	15 (65)	NS	15 (65)	NS
2	37	26 (70)		29 (78)	
3	7	6 (86)		5 (71)	
pT stage[a]					
1	31	18 (58)		18 (58)	
2	33	27 (82)	0.045	28 (85)	0.0098
3	3	2 (67)		3 (100)	
Pathological stage[b]					
I	23	12 (52)		11 (48)	
II	40	32 (80)	0.020	34 (85)	0.0007
III	4	3 (75)		4 (100)	

P values calculated using the χ^2 test

NS not significant

[a] Size of tumor classified according to the tumor–node–metastasis (TNM) pathological classification

[b] According to the TNM pathological classification

and Pathological Recording of Breast Cancer established by the Japanese Breast Cancer Society [16].

Immunohistochemical (IHC) study

Antibody against CADM1 was generated by immunizing rabbits with the C-terminal peptide of CADM1 as described previously [17]. Antibody against 4.1B, sc-10046, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Formalin-fixed, paraffin-embedded tissue sections of 8 μm were examined. After deparaffinization and dehydration through graded alcohols and xylene, antigen unmasking was performed using Histofine pH 9 (NichireiI, Tokyo, Japan) in an autoclave for 20 min at 121°C, followed by cooling to room temperature. Endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 30 min. After rinsing and blocking with 5% normal donkey serum, the sections were incubated overnight at 4°C with primary antibodies, including anti-CADM1 (diluted at 1:500) and anti-4.1B (diluted at 1:300) and then washed and incubated for 1 h at room temperature with the secondary antibody [DAKO EnVision kit/HRP

(AEC); DAKO, Hamburg, Germany]. Diaminobenzidine [DAKO EnVision kit/HRP (DAB)] was used for detection. Finally, the sections were counterstained with hematoxylin.

The signals of CADM1 and 4.1B proteins were detected along the membrane in normal mammary epithelial cells. Cancer cells with membrane staining of CADM1 or 4.1B were defined as having membrane expression, while cells with no or greatly reduced signals were defined as having low expression. Cancer cells with cytoplasmic immunoreactivity of CADM1 and 4.1B but no membrane staining were defined as showing aberrant expression. We calculated the percentage of cancer cells with membrane expression in the entire area of invasive and noninvasive lesions and scored the tumors as 0 (0–10% cells with membrane expression), 1 (11–30%), 2 (31–60%), and 3 (61–100%). Finally, we defined tumors with scores 1, 2, and 3 as positive staining and tumors with score 0 as negative staining for CADM1 or 4.1B expression.

Reverse-transcription PCR

Genomic DNA and total cellular RNA were extracted from cell lines, frozen breast cancer specimens, and noncancerous breast tissues using an AllPrep DNA/RNA/Protein Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Expression of CADM1 and 4.1B mRNA in breast cancer cell lines was detected by reverse-transcription PCR (RT-PCR). An aliquot of total cellular RNA (1 μg) was reverse-transcribed using the Transcriptor first-strand complementary DNA (cDNA) synthesis kit (Roche, Switzerland). PCR was carried out using KOD FX (TOYOBO Life Science, Osaka, Japan). Primer sequences used for RT-PCR are shown in Supplementary Table 1. The expression of *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was examined as an internal control to confirm RNA integrity.

Pyrosequencing analysis

For the methylation analysis, 600 ng genomic DNA was subjected to bisulfite conversion using a MethylCode bisulfite conversion kit (Invitrogen, CA, USA) according to the manufacturer's instructions. The purified bisulfate-converted samples were eluted in a 10 μl volume and stored at −20°C. An aliquot of bisulfate-treated DNA (60 ng) was amplified by PCR with biotinylated primers and Platinum Taq DNA polymerase (Invitrogen). The sequences of all primers used for PCR as well as the reaction conditions are presented in Supplementary Table 1. The PCR products were purified using a Qiaquick PCR purification kit (QIAGEN), and single-strand DNA was prepared using Dynabeads M280 streptavidin (Invitrogen). Pyrosequencing was performed with single-strand DNA as a template, the Exo-Klenow fragment (Ambion, USA), and the single-strand binding protein (New England Biolabs, Ipswich, MA, USA) using a small DNA analyzer (Hitachi, Ltd., Central Research Laboratory, Tokyo, Japan). The methylation rate of the cytosine residue was quantified as (peak of the signals of methylated C)/(peak of methylated C + peak of unmethylated C) using the software provided for the Handylumi analyzer (Hitachi, Ltd.). The average methylation rates of 5 CpG sites that are located at −497, −480, −467, −440, and −433 bp from the first nucleotide at the translational start site of the *CADM1* gene and 8 CpG sites that are located at −158, −154, −152, −150, −139, −128, −117, and −115 bp from the first nucleotide in exon 1 of the *4.1B* gene were calculated as the methylation rate of each gene. For *CADM1*, methylation rates of more than 20%, between 5% and 20%, and less than 5% were defined as hypermethylation, partial methylation, and nonmethylation, respectively. For *4.1B*, methylation rates of more than 20%, between 10 and 20%, and less than 10% were defined as hypermethylation, partial methylation, and nonmethylation, respectively. Methylation rates of *CADM1* and *4.1B* were less than 5% and 10%, respectively.

Statistical analysis

All statistical analyses were carried out using Fisher's exact tests or the Pearson χ^2 test. Results were considered significant at P value <0.05.

Results

Loss of CADM1 and 4.1B protein expression in primary breast cancer

Expression of CADM1 and 4.1B proteins was examined by immunohistochemistry (IHC) in 67 primary breast cancers and 39 corresponding noncancerous breast tissues. Signals of CADM1 and 4.1B proteins were detected on the cell membrane at the cell–cell attachment sites in normal luminal epithelial cells but not in normal myoepithelial cells or interstitial cells (Fig. 1). On the other hand, 47 of 67 (70%) tumors showed negative staining with more than 90% of cancer cells presenting low or aberrant expression of CADM1 protein when examined by IHC (Fig. 2). Clinicopathological examination of the tumors showed that the incidence of negative CADM1 staining was significantly higher in tumors with diameter greater than 2 cm (pT2 and pT3; 29 of 36, 81%) than in those with diameter less than 2 cm (pT1; 18 of 31, 58%) ($P = 0.045$) (Table 1). Negative CADM1 staining was also observed at significantly higher incidence in tumors with pathological

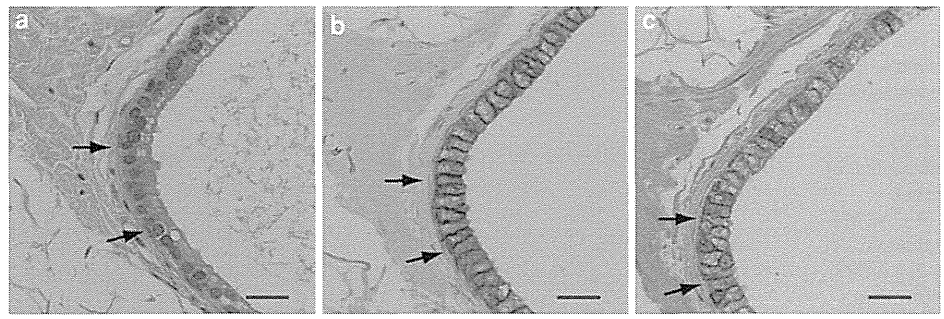

Fig. 1 Immunohistochemical analysis of CADM1 and 4.1B in normal mammary duct epithelial cells. **a** Hematoxylin and eosin (HE) staining. **b** and **c** Immunohistochemical staining of CADM1 protein by anti-CADM1 antibody (**b**) and anti-4.1B antibody (**c**). Normal membrane staining of CADM1 and 4.1B proteins is detected in mammary duct epithelia, whereas no staining is observed in normal myoepithelial cells, indicated by *arrows*. *Bars* 20 μm

stages II and III (35 of 44, 80%) than in those with stage I (12 of 23, 52%) ($P = 0.020$) (Table 1). IHC also revealed that expression of 4.1B protein was mostly lost or aberrant in at least 49 of 67 (73%) primary breast cancers. Negative staining of 4.1B protein was preferentially observed in tumors with lymph node metastasis (20 of 21, 95%) relative to those without lymph node metastasis (29 of 46, 63%) ($P = 0.0058$). Negative staining of 4.1B was also observed at significantly higher incidence in tumors with pT2 and pT3 (31 of 36, 86%) than in those with pT1 (18 of 31, 58%) ($P = 0.0098$) or in tumors with pathological stages II and III (38 of 44, 86%) than in those with stage I (11 of 23, 48%) ($P = 0.0007$).

Next, we combined the expression status of CADM1 and 4.1B and divided the 67 tumors into three groups: group 1, tumors with positive staining of both CADM1 and 4.1B; group 2, tumors with negative staining of either CADM1 or 4.1B; group 3, tumors with aberrant expression of both CADM1 and 4.1B (Table 2). Then, we combined groups 2 and 3 with loss of at least one of CADM1 or 4.1B protein. As summarized in Table 2, the numbers of tumors in groups 1, 2, and 3 were 7, 24, and 36, respectively (Table 2). Among the 24 tumors in group 2, 13 tumors expressed CADM1 but not 4.1B, whereas 11 tumors expressed 4.1B but not CADM1. Clinicopathological comparison demonstrated that the tumors from patients aged 50 years or older ($P = 0.037$) and the tumors with advanced pathological stages, II and III ($P = 0.029$), were more preferentially observed in group 2 and 3 tumors relative to group 1 tumors. In addition, no tumors with lymph node metastasis, lymphovascular invasion, nuclear grade 3, and pT stage 3 were found in group 1 tumors, although these scores were not statistically significant. On the other hand, the expression status of the hormone receptors ER or PgR, or that of Her2, and nuclear grade were not associated with the combined expression status of CADM1 and 4.1B. Moreover, no significant difference was observed between group 2 and group 3 tumors in terms of any clinicopathological characters.

Comparative analysis of CADM1 and 4.1B expression in invasive and noninvasive lesions from the same tumor

Thirty-nine out of 67 breast cancer tissues examined in this study were histologically heterogeneous and contained both invasive and noninvasive lesions within the same specimen (Fig. 3a, e). Thus, we next examined the expression status of CADM1 and 4.1B proteins in these lesions. As representatively shown in Fig. 3b, c, f, and g, normal membrane staining of CADM1 and 4.1B was detected in most of the noninvasive lesions. By contrast, CADM1 and 4.1B expression was low or aberrant in invasive lesions (Fig. 3b, d). As summarized in Table 3, 25 out of 39 tumors retained CADM1 expression in noninvasive lesions. Among them, 18 (72%) tumors showed low or aberrant expression of CADM1 in their invasive lesions. On the other hand, 14 of 39 tumors already showed low or aberrant CADM1 expression in noninvasive lesions, and all of them lacked normal CADM1 expression in their invasive lesions. Similarly, 12 out of 24 (50%) tumors expressing 4.1B in noninvasive lesions showed low or aberrant 4.1B expression in their invasive lesions, whereas all 15 tumors showing low or aberrant 4.1B expression in noninvasive lesions lacked normal 4.1B expression in invasive lesions as well.

Promoter methylation of the *CADM1* and *4.1B* genes in primary breast cancers and cell lines

We next examined CADM1 and 4.1B mRNA expression in human breast cancer cell lines by RT-PCR analysis. Of 6 cell lines examined, loss of CADM1 and 4.1B mRNA was observed in 3 and 4 cell lines, respectively (Fig. 4a). Two

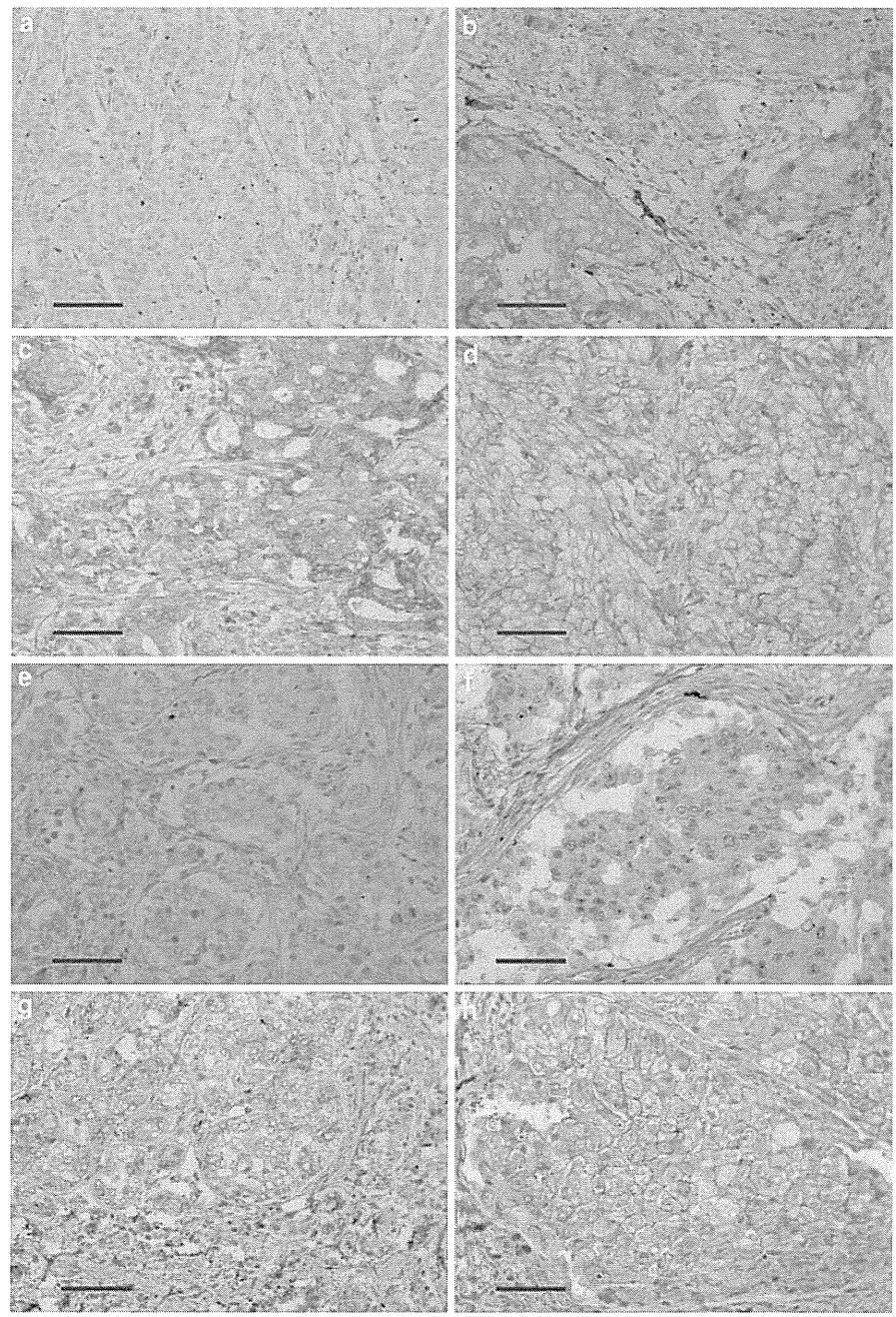

Fig. 2 Immunohistochemical analysis of CADM1 and 4.1B in invasive ductal carcinoma of the breast. Representative images of breast cancers expressing CADM1 (**a–d**) and 4.1B (**e–h**) are shown. The content of cancer cells with normal membrane staining of CADM1 or 4.1B in tumors with score 1 is 0–10% (**a**, **e**); score 2, 11–30% (**b**, **f**); score 3, 31–60% (**c**, **g**); and score 4, 61–100% (**d**, **h**)

cell lines, MDA-MB-361 and MDA-MB-453, showed loss of expression of both genes, while SK-BR-3 cells retained expression of both genes. Then, using pyrosequencing analysis, we performed quantitative analysis of DNA methylation at 5 and 8 CpG sites with the *CADM1* and *4.1B* gene promoter, respectively (Fig. 4b, c). Significant level of methylation of *CADM1* was detected in two cell lines, MDA-MB-453 and MDA-MB-231, showing loss of CADM1 mRNA (Fig. 4a, b). On the other hand, significant methylation of *4.1B* was observed in five cell lines: BT474, MCF7, MDA-MB-453, MDA-MB-361, and MDA-MB-231 (Fig. 4c). Among these, 4 cell lines, except for MDA-MB-231, lost 4.1B mRNA expression (Fig. 4a).

The methylation status of the *CADM1* and *4.1B* was subsequently analyzed in 50 primary breast cancers using pyrosequencing. *CADM1* and *4.1B* methylation was observed in 23 (46%) and 21 (42%) of 50 tumors, respectively (Table 4). Among them, most of the tumors (18 of 23 and 19 of 21) showed loss or greatly reduced expression of CADM1 and 4.1B proteins by IHC,

Table 2 Clinicopathological characteristics of patients with primary breast cancer and combined state of CADM1 and 4.1B expression

	n	Group 1 CADM1 (+) 4.1B (+)	Groups 2 + 3	*P* value	Group 2 CADM1 (−) or 4.1B (−)	Group 3 CADM1 (−) 4.1B (−)
	67	7 (10)	60 (90)		24 (36)	36 (54)
Age (years)						
<50	24	0	24 (100)		9 (38)	15 (62)
≥50	43	7 (16)	36 (84)	0.037	15 (35)	21 (49)
Lymph node metastasis						
0	46	7 (15)	39 (85)		20 (43)	19 (42)
1–3	17	0	17 (100)	NS	3 (18)	14 (82)
≥4	4	0	4 (100)		1 (25)	3 (75)
Lymphovascular invasion						
−	50	7 (14)	43 (86)		21 (42)	22 (44)
+	17	0	17 (100)	NS	3 (18)	14 (82)
Hormone receptor status						
+/+ or +/−	56	6 (11)	50 (89)		19 (34)	31 (55)
−/−	11	1 (10)	10 (90)	NS	5 (45)	5 (45)
Her2						
Negative	57	6 (11)	51 (89)		22 (39)	29 (50)
Positive	10	1 (10)	9 (90)	NS	2 (20)	7 (70)
Nuclear grade						
1	23	3 (13)	20 (87)		10 (43)	10 (43)
2	37	4 (11)	33 (89)	NS	11 (30)	22 (59)
3	7	0	7 (100)		3 (43)	4 (57)
pT stage[a]						
1	31	5 (16)	26 (84)		16 (52)	10 (32)
2	33	2 (6)	31 (94)	NS	7 (21)	24 (73)
3	3	0	3 (100)		1 (33)	2 (67)
Pathological stage[b]						
I	23	5 (22)	18 (78)		13 (56)	5 (22)
II	40	2 (5)	38 (95)	0.029	10 (25)	28 (70)
III	4	0	4 (100)		1 (25)	3 (75)

P values calculated using the χ^2 test

NS not significant

[a] Size of tumor was classified according to the TNM pathological classification

[b] According to the TNM pathological classification

respectively (Table 4). On the other hand, about a half of tumors with negative staining of CADM1 (19 of 37) or 4.1B (19 of 38) showed an unmethylated promoter in each gene.

Discussion

Expression of CADM1 and 4.1B proteins was investigated in 67 primary tumors by IHC analysis. Low or aberrant expression of CADM1 and 4.1B was observed in 70% and 73% of primary breast cancers, respectively (Table 1). Statistical analysis demonstrated that low or aberrant expression of CADM1 in tumors was significantly associated with advanced pT stages of pT2 and pT3 and advanced pathological stages of II and III (Table 1). On the other hand, low or aberrant expression of 4.1B in tumors was significantly associated with lymph node metastasis, advanced pT stages of pT2 and pT3, and advanced pathological stages of II and III (Table 1). In addition, when we combined the expression status of CADM1 and 4.1B, tumors lacking at least one of CADM1 or 4.1B expression tended to show more malignant pathological features than tumors expressing both CADM1 and 4.1B. These results suggest that dysfunction of the CADM1–4.1B cascade plays a role in progression of primary invasive breast

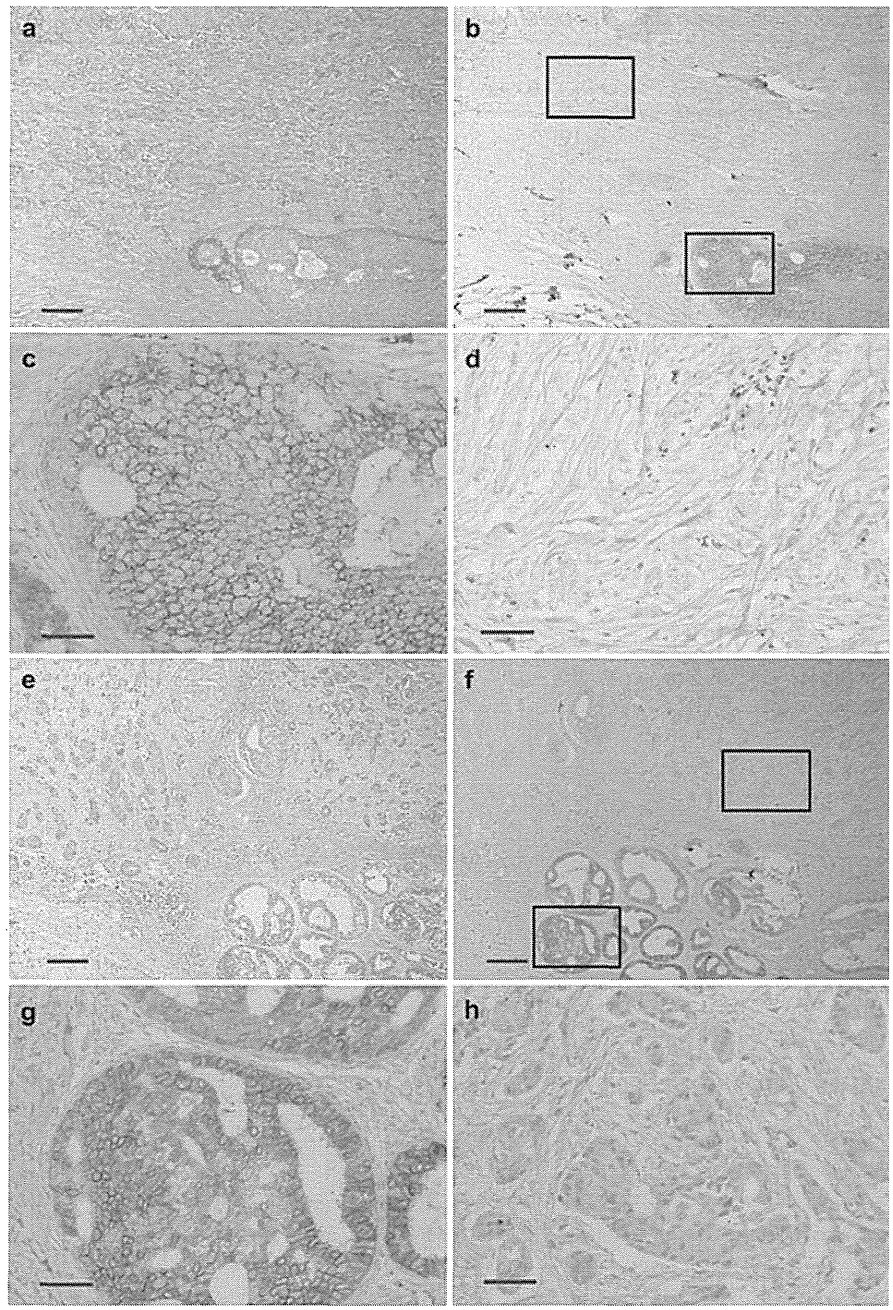

Fig. 3 Immunohistochemical analysis of CADM1 and 4.1B in invasive and noninvasive lesions of primary breast cancer. Representative images with HE staining (**a** and **e**) and stained with anti-CADM1 antibody (**b–d**) and with anti-4.1B antibody (**f–h**) are shown. Noninvasive lesions (**c** and **g**) and invasive lesions (**d** and **h**) are shown at high magnification from the same specimens (**b** and **f**). *Bars* 200 μm (**a**, **b**, **e**, and **f**) and 50 μm (**c**, **d**, **g**, and **h**)

cancer. On the other hand, no difference in clinicopathological features was detected between the tumors with low or aberrant expression of either CADM1 or 4.1B (group 2) versus both CADM1 and 4.1B (group 3). This could be consistent with previous findings that CADM1 and 4.1B act in the same cascade by interacting with each other [10]. In this connection, comparison of incidence in tumors with normal or aberrant expression of CADM1 and 4.1B proteins suggests that aberrant expression of CADM1 and 4.1B occurred independently.

Involvement of dysfunction of the CADM1–4.1B cascade in breast cancer invasion was also supported by comparative analysis of CADM1 and 4.1B expression in invasive and noninvasive lesions within the same tumors from 39 breast cancer patients (Fig. 3). As shown in Table 3, 72% (18 of 25) of tumors expressing CADM1 and 50% (12 of 24) of tumors expressing 4.1B in noninvasive lesions showed selective loss of normal expression of these proteins in their invasive lesions. Inversely, no tumor was observed that showed aberrant expression of CADM1 or

Table 3 Preferential loss of CADM1 and 4.1B expression in invasive lesions of primary breast cancer

Non-Invasive lesions	Invasive lesions: CADM1 (+)	Invasive lesions: CADM1 (-)	total	*P*-value
CADM1 (+)	7	18	25	
CADM1 (-)	0	14	14	0.036
total	7	32	39	

	4.1B (+)	4.1B (-)		
4.1B (+)	12	12	24	
4.1B (-)	0	15	15	0.0009
total	12	7	39	

P values calculated using the χ^2test

4.1B in noninvasive lesions but retained normal membrane expression of these proteins in invasive lesions. Preferential loss of CADM1 expression in invasive lesions has been also reported in lung adenocarcinoma [15]. These results appear to be consistent with previously reported experimental evidence that CADM1 suppresses epithelial–mesenchymal transition (EMT) and oncogenic signaling [18, 19].

It should be noted that we could identify a special group of tumors retaining expression of both CADM1 and 4.1B by combinatorial expression analysis of these two proteins (group 1 in Table 2). Although only 10% (7 of 67) of tumors were subclassified into this group, these 7 tumors showed neither lymph node metastasis nor lymphovascular invasion, suggesting that this group of tumors shows better prognosis. Inversely, all 21 tumors with lymph node metastasis or all 17 tumors with lymphovascular invasion showed low or aberrant expression of either or both of the CADM1 and 4.1B proteins. It is also important to note that aberrant expression of CADM1 or 4.1B was observed in significant portions of noninvasive lesions [14 of 39 (36%) and 15 of 39 (38%), respectively] (Table 3) as well as in tumors without lymph node metastasis, those without lymphovascular invasion, those with pT1, and those with pathological stage I [39/46 (85%), 43/50 (86%), 26/31 (84%), 18/23 (78%), respectively; Table 2]. Therefore, it is tempting to speculate that tumors with low or aberrant expression of either or both of the CADM1 and 4.1B proteins might have some potential for breast cancer recurrence even if they do not show lymph node metastasis or lymphovascular invasion at time of surgery. Further studies on the prognosis of these cases would be required to prove this hypothesis. It is also noteworthy that all 7 tumors with normal expression of CADM1 and 4.1B proteins were from patients who were more than 50 years old. Considering the possible distinct molecular pathways underlying tumorigenesis between premenopausal and postmenopausal breast cancer, dysfunction of the CADM1–4.1B cascade appears to be a prerequisite for premenopausal breast cancer.

In the present study, promoter methylation of the *CADM1* and *4.1B* genes was also examined to elucidate the molecular mechanism of gene inactivation, because hypermethylation of *CADM1* and *4.1B* and the resultant gene silencing have been reported in various cancers [2, 3, 6–8, 12, 20]. To analyze the methylation status of the gene promoter, we carried out pyrosequencing of the specific fragments within the gene promoter containing several CpG residues after bisulfate treatment. Since pyrosequencing can detect the content of methylcytosine and unmethylated cytosine, which are converted to cytosine and thymine by bisulfite, respectively, it provides a significant advantage in quantifying the level of methylation at the specified CpG sites over various other methods, including bisulfate sequencing and methylation-specific PCR (MSP). Heller et al. [14] used MSP to examine the methylation status of the *CADM1* and *4.1B* genes in breast cancers. Although MSP is a convenient method for assessing methylation status, evaluation of the results of MSP, especially those obtained from surgical specimens, can be difficult and sometimes even misleading because MSP is not quantitative; it only detects the methylation status of a single CpG site per primer and inevitably includes false-positive and/or false-negative results.

Using bisulfate treatment coupled with pyrosequencing, we found strong correlation of promoter methylation with loss of mRNA expression in both *CADM1* and *4.1B* genes in breast cancer cell lines (Fig. 4). We also used this technique to examine the methylation status in primary breast cancer and found that 46% and 42% of tumors showed promoter methylation of the *CADM1* and *4.1B* genes, respectively. It is interesting that most of the tumors with a methylated promoter of *CADM1* (17 of 23) and *4.1B* (19 of 21) showed low or aberrant expression of each

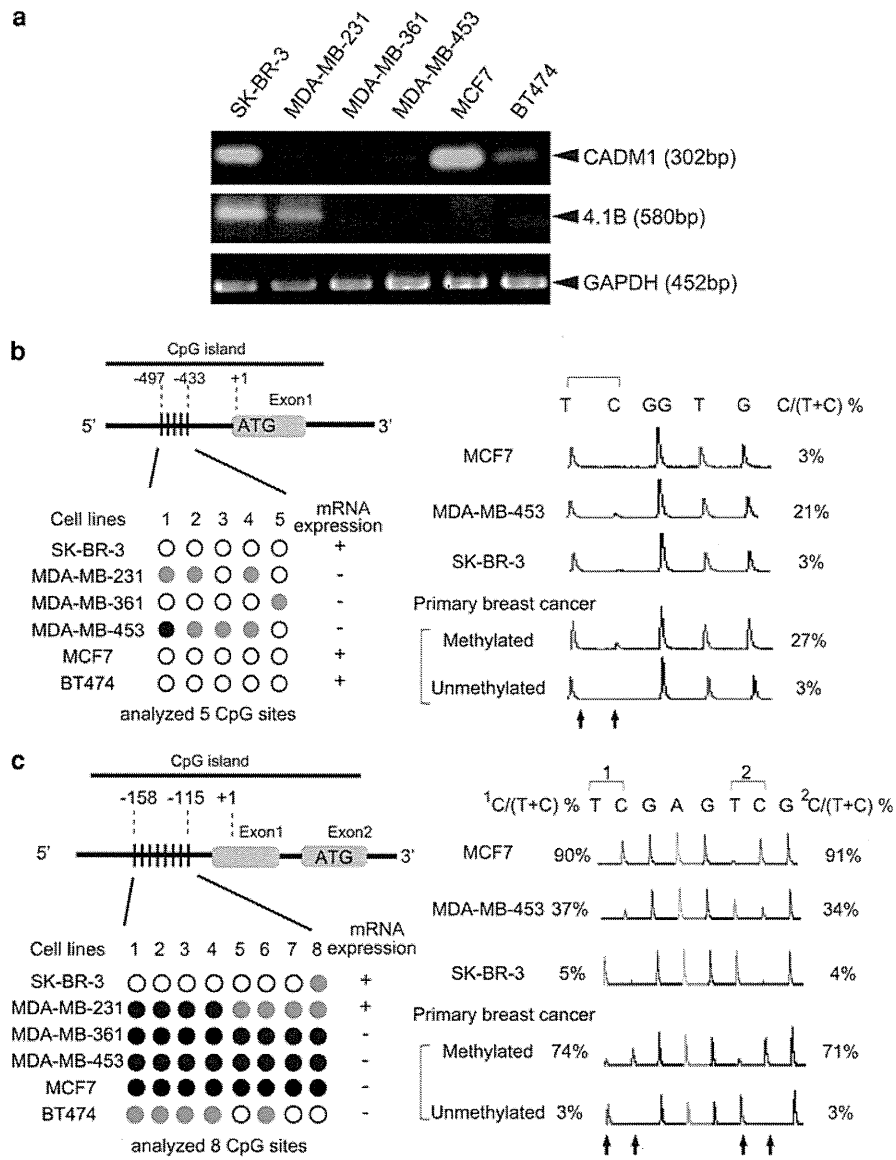

Fig. 4 mRNA expression and promoter methylation analyses of *CADM1* and *4.1B*. **a** RT-PCR analyses of CADM1 and 4.1B mRNA in six breast cancer cell lines. GAPDH serves as an internal control. **b**, **c** *left*, a schematic representation of the CpG island of the *CADM1* (**b**) and *4.1B* (**c**) genes and summary of methylation status. The *gray box* indicates exons. *Vertical bars* indicate CpG sites examined for methylation, while *black*, *gray*, and *white circles* represent hypermethylation, partial methylation, and unmethylation, respectively, as described in "Materials and methods." *Right*, representative results of bisulfate pyrosequencing of a cytosine residue(s) at −497 bp in the *CADM1* promoter (**b**) and at −158 and −154 bp in the *4.1B* promoter (**c**). *Arrows* indicate T and C residues converted by bisulfate from unmethylated C and methylated C residues, respectively

Table 4 Promoter methylation state and protein expression of CADM1 and 4.1B in primary breast cancer

	CADM1 methylation (%)			*4.1B* methylation (%)		
	Unmethylated	Methylated	Total	Unmethylated	Methylated	Total
Protein expression						
Positive	8	5	13	10	2	12
Negative	19	18	37	19	19	38
Total	27 (54)	23 (46)	50 (100)	29 (58)	21 (42)	50 (100)

protein, implying that promoter methylation would inactivate these genes in at least some breast cancers. However, approximately half of the tumors with low or aberrant expression of CADM1 or 4.1B showed no methylation of these genes. This observation may be attributable to the noncancerous cells or noninvasive cancer cells that are inevitably present in tumor tissues. Alternatively, some additional mechanisms other than promoter methylation, including loss of each chromosomal locus, transcriptional repressors, and microRNA (miRNA), may suppress the

expression of CADM1 or 4.1B. Since frequent loss of heterozygosity (LOH) at the chromosomal region, 11q23 and 18p11, on which *CADM1* and *4.1B* are located, has been reported in breast cancer [7, 21, 22], LOH would be one of the possible molecular mechanisms of the second hit to inactivate these genes. Further study will be necessary to clarify this issue.

In conclusion, the present study demonstrated strong correlation between low or aberrant expression of CADM1 and 4.1B proteins and local invasion, lymph node metastasis, and lymphovascular invasion in primary breast cancer. Expression status of CADM1 and 4.1B may serve as a novel significant biomarker predicting postoperative metastasis or recurrence of breast cancer when utilized in combination with other established diagnostic indicators.

Acknowledgments We thank Dr. H. Miura, Dr. K. Nakai, Dr. K. Senuma, Dr. I. Abe, Dr. T. Kosaka, Dr. H. Shimizu, and all members of the Breast and Endocrine Surgery Department at Juntendo University for collecting breast cancer samples. We also thank Dr. Y. Tsuboi and Dr. M. Nagata for fruitful discussion and Ms. Seiko Iwata and Ms. Takako Kohmoto for secretarial assistance. This work was supported by a Grant-in-Aid for Scientific Research (B) [22300336 to Y.M.]; a Grant-in-Aid for Young Scientists (B) [22700914 to M.I. and 21790309 to M.S.-Y.] from the Ministry of Education, Culture, Sports, Science, and Technology, Japan; and a Grant-in-Aid for the Third-Term Comprehensive Control Research for Cancer from the Ministry of Health, Labor, and Welfare, Japan (Y.M.).

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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.

Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology, Japan; **Grant numbers:** 22300336, 21790309; **Grant sponsor:** Ministry of Health, Labor and Welfare, Japan; **Grant sponsor:**National Institute of Biomedical Innovation; **Grant number:** ID 05-10

DOI: 10.1002/ijc.26160

History: Received 27 Aug 2010; Accepted 27 Jan 2011; Online 4 May 2011

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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.

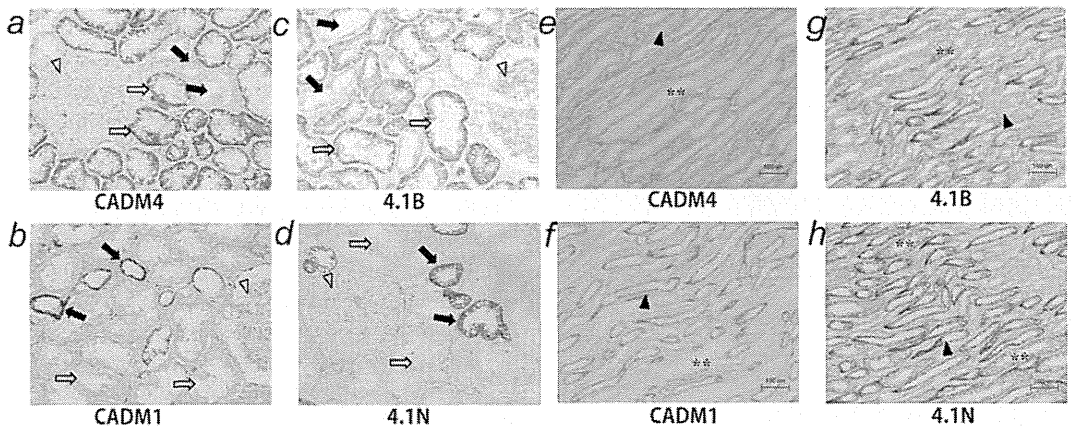

Figure 1. Immunohistochemical analyses of CADM4 (*a* and *e*), CADM1 (*b* and *f*), 4.1B (*c* and *g*) and 4.1N (*d* and *h*) proteins in normal human renal cortex (*a–d*) and medulla (*e–h*). Expression of CADM4 and 4.1B is detected in the proximal convoluted tubules (*a* and *c*), whereas that of CADM1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

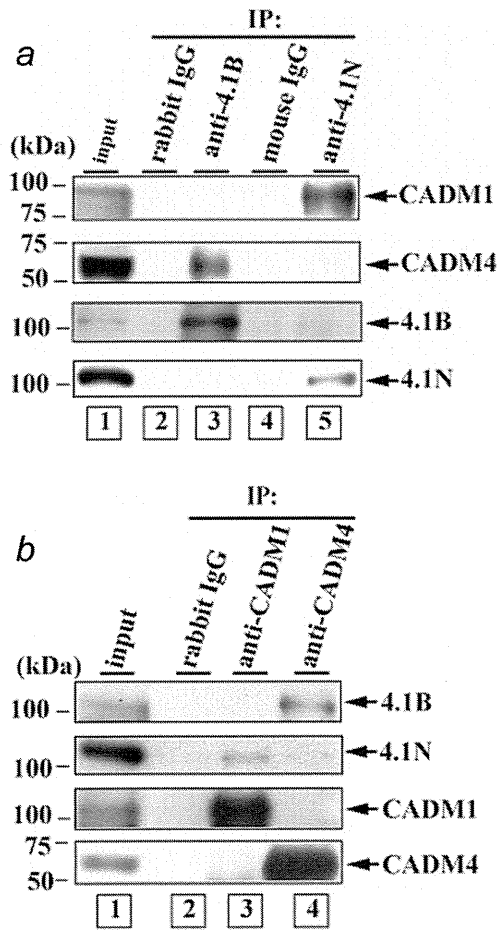

Figure 2. Interaction of CADM4 with 4.1B and CADM1 with 4.1N. (*a*) Total lysates of normal human kidney were immunoprecipitated with control rabbit IgG (lane 2), anti-4.1B pAb (lane 3), control mouse IgG (lane 4) and anti-4.1N pAb (lane 5), and binding proteins were detected by immunoblotting using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

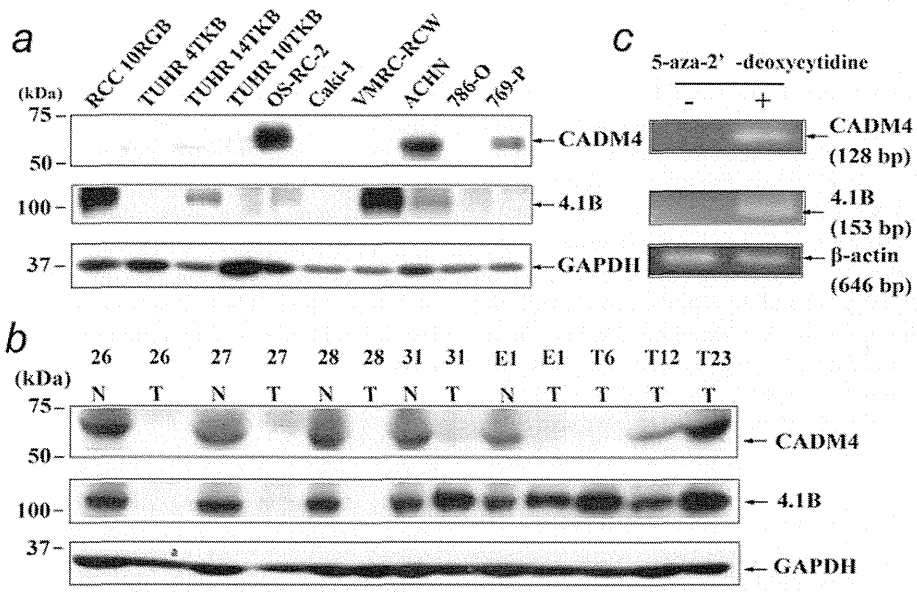

Figure 3. Loss of expression of CADM4 and 4.1B proteins in RCCC and restoration of CADM4 expression by 5-aza-2′-deoxycytidine. (*a* and *b*) Immunoblotting of CADM4 and 4.1B proteins in 10 RCC cell lines (*a*) and the primary tumors of RCCC (*b*). An aliquot of the tissue lysates (1 μg) was subjected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

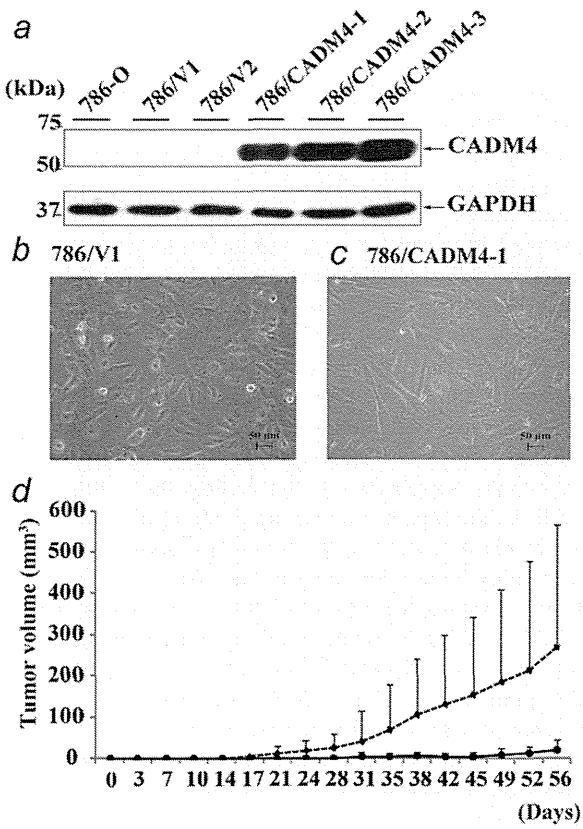

Figure 4. Suppression of tumorigenicity of 786-O cells in nude mice by CADM4. (*a*) Western blotting of CADM4 protein (upper) and control GAPDH protein (lower) in parental 786-O cells, 786-O cell clones transfected with a vector alone (786/V1, 786/V2) and 786-O cell clones transfected with CADM4 (786/CADM4-1,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

Acknowledgements

We are grateful to Ms. Hiromi Ichihara, Ms. Tomoko Masuda and Ms. Keiko Kimura for their technical assistance and to Ms. Seiko Iwata for her secretarial assistance. A Grant-in-Aid for Scientific Research (B) [for Y.M.] and a Grant-in-Aid for Young Scientists (B) [for M.SY.] from the Ministry of Education, Culture, Sports, Science, and Technology, Japan; a Grant-in-Aid for the Third Term Comprehensive Control Research for Cancer from the Ministry of Health, Labor, and Welfare, Japan (Y.M.); and a Grant for the Promotion of Fundamental Studies in Health Sciences from the National Institute of Biomedical Innovation (for Y.M.)

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