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

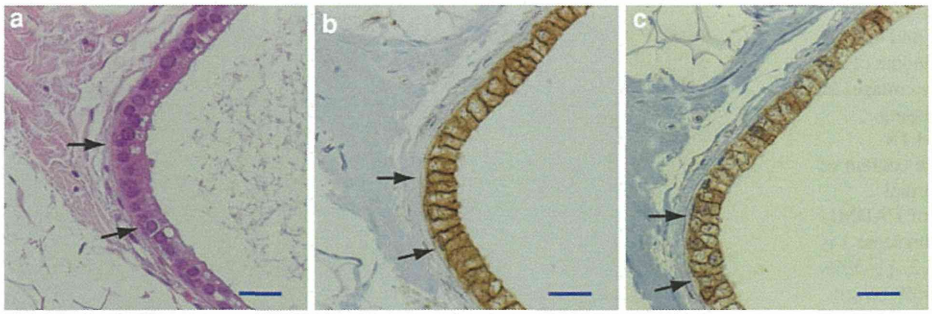

Fig. 1 Immunohistochemical analysis of CADM1 and 4.1B in normal mammary duct epithelial cells. **a** Hematoxylin and eosin (HE) staining. **b** and **c** Immunohistochemical staining of CADM1 protein by anti-CADM1 antibody (**b**) and anti-4.1B antibody (**c**). Normal membrane staining of CADM1 and 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

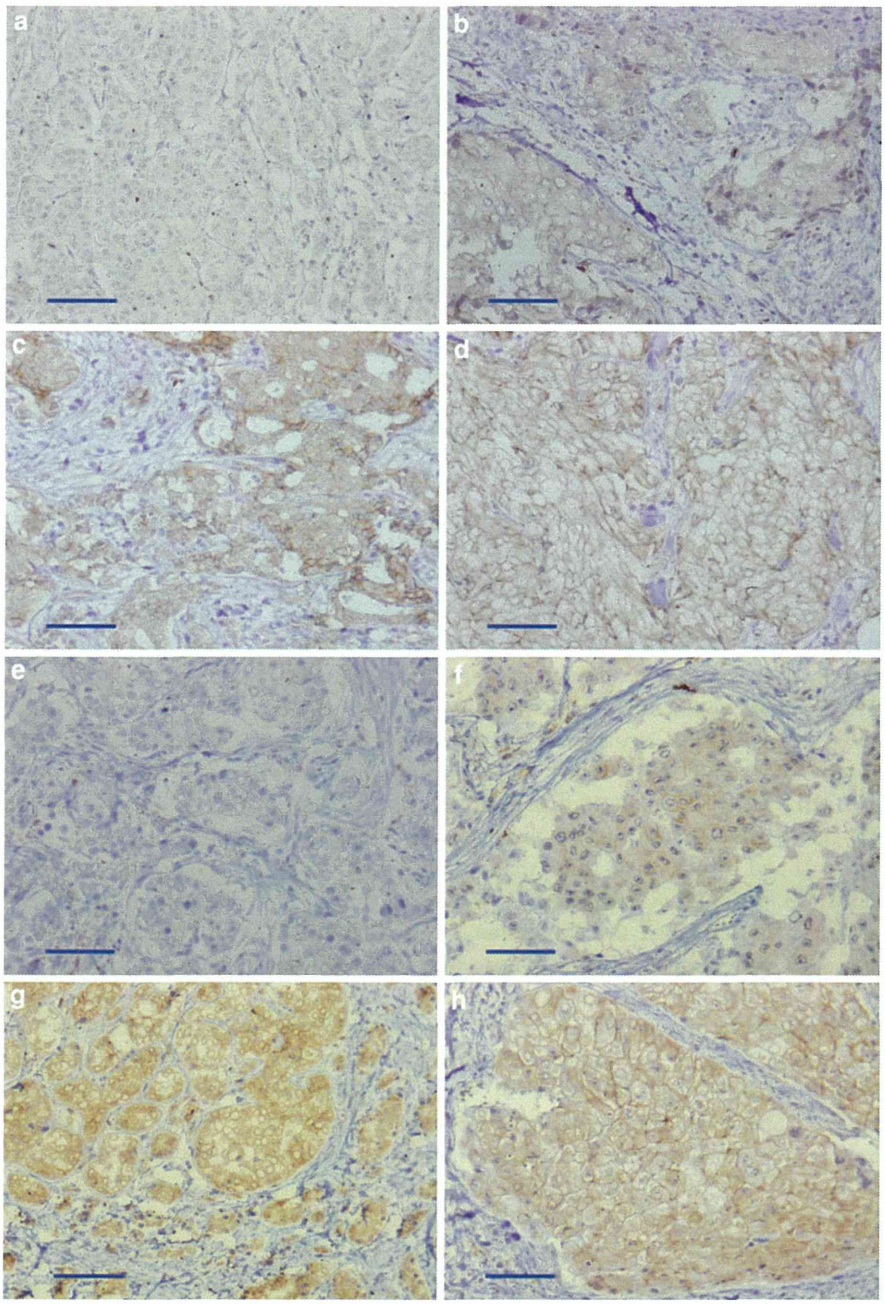

Fig. 2 Immunohistochemical analysis of CADM1 and 4.1B in invasive ductal carcinoma of the breast. Representative images of breast cancers expressing CADM1 (**a–d**) and 4.1B (**e–h**) are shown. The content of cancer cells with normal membrane staining of CADM1 or 4.1B in tumors with score 1 is 0–10% (**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

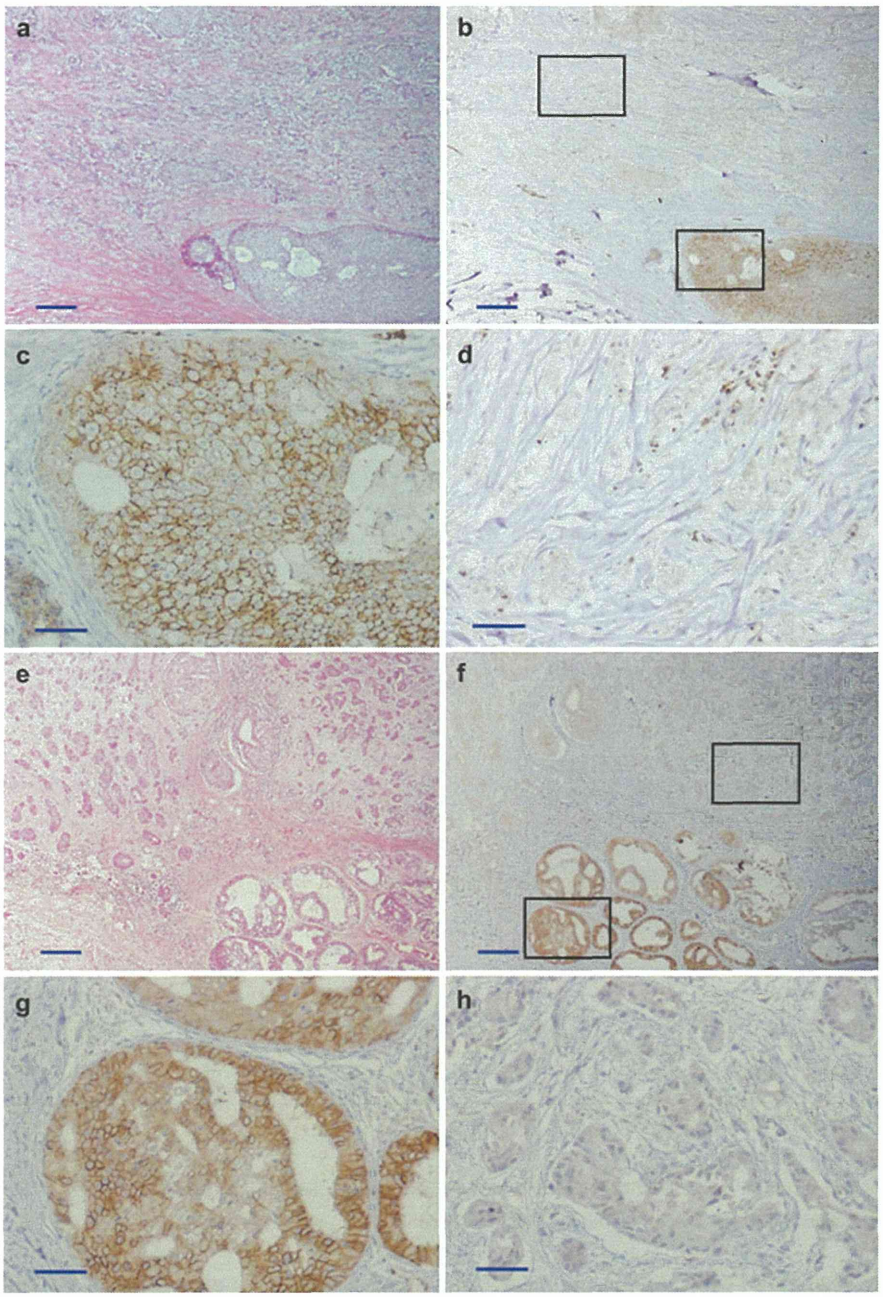

Fig. 3 Immunohistochemical analysis of CADM1 and 4.1B in invasive and noninvasive lesions of primary breast cancer. Representative images with HE staining (**a** and **e**) and stained with anti-CADM1 antibody (**b–d**) and with anti-4.1B antibody (**f–h**) are shown. Noninvasive lesions (**c** and **g**) 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

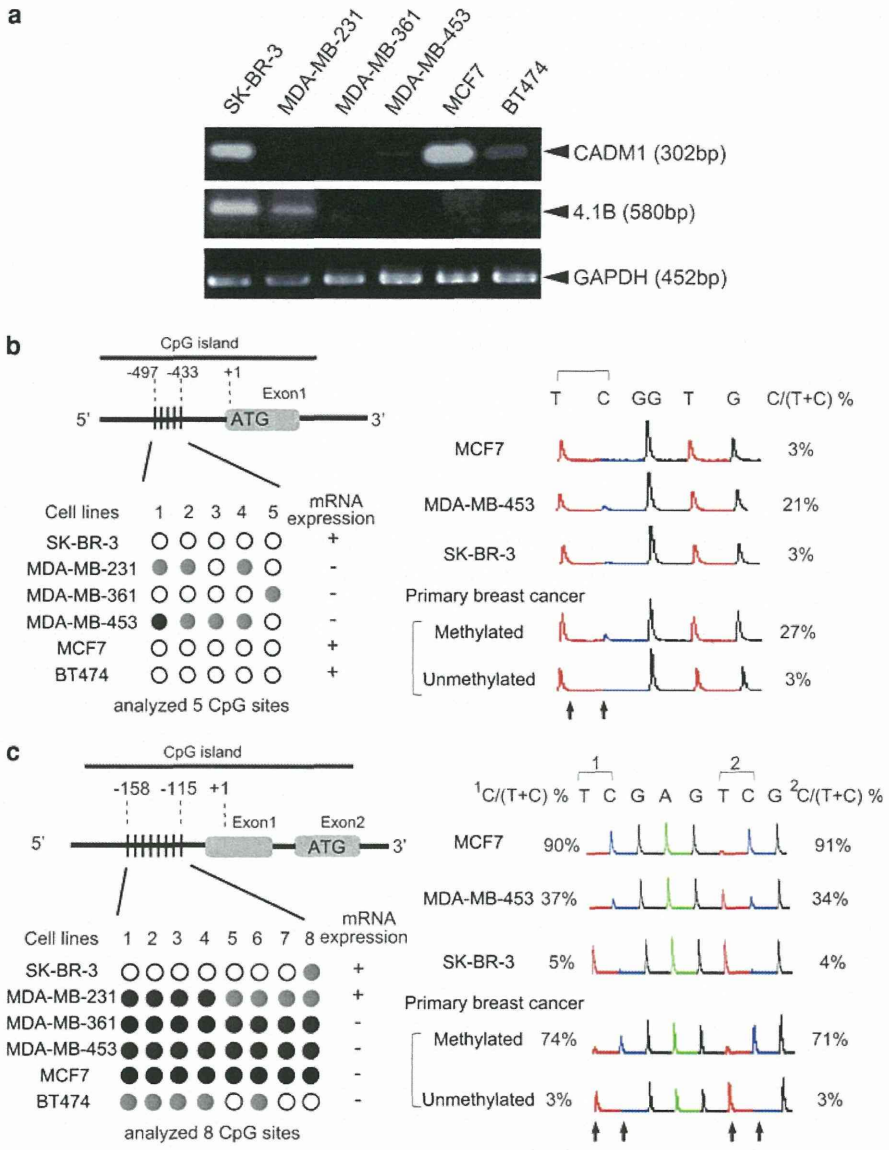

Fig. 4 mRNA expression and promoter methylation analyses of *CADM1* and *4.1B*. **a** RT-PCR analyses of CADM1 and 4.1B mRNA in six breast cancer cell lines. GAPDH serves as an internal control. **b**, **c** *left*, a schematic representation of the CpG island of the *CADM1* (**b**) and *4.1B* (**c**) 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.

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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