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An antibody–drug conjugate for endometrioid carcinoma based on the expression of cell adhesion molecule 1

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ABSTRACT

Cell adhesion molecule 1 (CADM1), an immunoglobulin superfamily member, is expressed in endometrial glandular cells highly during the proliferative phase but lowly during the secretory phase. Previously, a CADM1–targeting antibody–drug conjugate (ADC) was generated, in which a humanized anti-CADM1 ectodomain antibody h3E1 was linked with monomethyl auristatin E (h3E1–MMAE ADC). The present study aimed at probing whether this ADC could be useful for the treatment of endometrial neoplasm. Firstly, immunohistochemistry for CADM1 was conducted on proliferative-phase endometrium ($n = 13$), endometrial hyperplasia ($n = 35$), and endometrioid carcinoma at various stages ($n = 166$). CADM1 immunostaining intensity was highest in atypical endometrial hyperplasia and endometrioid carcinoma confined within the endometrium and was decreased stepwise as the carcinoma stage progressed. Next, h3E1–MMAE ADC was examined for its cytotoxicity in vitro using human endometrial adenocarcinoma cell lines expressing CADM1; HEC-1B, HEC-50B, JHUM-3, and OMC-2. The ADC killed these cells in a dose-dependent manner with half maximal inhibitory concentration (IC50) of 12.02 nM for HEC-1B and 2.04 nM for HEC-50B. Collectively, h3E1–MMAE ADC may serve as a noninvasive alternative to simple hysterectomy in the treatment of endometrioid carcinoma confined within the endometrium.

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Introduction

Endometrial carcinoma is the most commonly occurring gynecologic malignancy in the United States and Japan.[1,2] The major precursor lesion is atypical endometrial hyperplasia,[3] and one of the major risk factors is endogenous and exogenous estrogen.[4,5] The standard treatment for endometrial carcinoma is simple hysterectomy or more invasive surgery.[6–8] Although this standard treatment assures favorable prognosis, these surgical procedures may be undesirable in a considerable proportion of patients. This issue is raised mainly from the fact that endometrial carcinoma is predominantly a disease of postmenopausal women, but more than a quarter of cases occur in premenopausal women.[3] For patients who wish to maintain their fertility, instead of surgical treatment, hormone therapies with progestins have often been applied in early-stage endometrial carcinoma, yielding fairly good outcomes.[8]

Antibody–drug conjugates (ADCs) are an emerging tumor treatment modality that uses antibodies to deliver cytotoxic drugs into tumor cells selectively and effectively.[9] The antibodies target tumor-specific or tumor-associated cell surface molecules. Generally speaking, higher expression levels of these molecules on tumor cells are considered to help ADCs exert greater therapeutic effects on tumor cells. There are some ADCs approved by FDA for gynecologic malignancies; for example, mirvetuximab soravtansine for platinum-resistant ovarian cancer, and tisotumab vedotin for recurrent or metastatic cervical cancer with disease progression on or after chemotherapy.[10,11]

Cell adhesion molecule 1 (CADM1) is a cell membrane-spanning glycoprotein belonging to the immunoglobulin superfamily.[12,13] This molecule is distributed in various types of cells, including neurons,[14] adrenal cortical cells,[15] pancreatic islet cells,[16] and a part of epithelial cells.[17–19] Recently, we reported that human endometrial glandular cells express CADM1 clearly on their cell membrane.[20] Interestingly, the CADM1 protein expression was markedly increased during the proliferative phase and markedly decreased during the secretion phase, in conjunction with estrogen receptor expression,[20] suggesting that CADM1 might be involved in endometrial glandular cell proliferation and carcinogenesis.

Besides, we previously generated an ADC, which is based on a humanized anti-CADM1 monoclonal antibody named h3E1, and has monomethyl auristatin E (MMAE), a tubulin polymerization inhibitor, as a payload, aiming to target CADM1 expressed on pleural malignant mesothelioma.[21] This ADC was effective in killing pleural malignant mesothelioma cells in vitro with half maximal inhibitory concentration (IC50) of approximately 3 μg/mL.[21]

The present study aimed at probing whether our ADC could be useful for the treatment of endometrial cancers. We

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first performed immunohistochemical staining for CADM1 in various endometrial carcinomas and precursors, and analyzed the association of the immunohistochemical staining intensity with the types and stages of the carcinomas and precursors. Next, we examined whether our CADM1-targeting ADC was effective in killing endometrial adenocarcinoma cells in a CADM1 expression-dependent manner. In addition, we conducted an in vitro invasion assay to assess whether CADM1 might be involved in acquisition of the capacity of endometrial adenocarcinoma cells to invade the uterine myometrium.

Materials and methods

Human samples

Patients with endometrial hyperplasia and endometrioid carcinoma (hormone therapy, none; pill medication, none) were selected according to a computerized search of the surgical pathology databases of Kindai University Hospital, Osaka, Japan (Supplementary Table S1, upper). Stages of endometrioid carcinoma cases were determined according to International Federation of Gynecology and Obstetrics (FIGO) 1988 staging system: 1a, tumor limited to endometrium; 1b, invasion to <1/2 myometrium; 1c, invasion to ≥1/2 myometrium; 2, spread from the uterus to the cervical stroma; 3, spread beyond the uterus.[22] Pathological grade was G1 for all cases. Another four patients were analyzed as cases of recurrent endometrioid carcinoma (Supplementary Table S1, lower). Surgical specimens were fixed in 10% natural phosphate-buffered formalin immediately after the resection, embedded in paraffin, and cut into sections, followed by staining with hematoxylin and eosin, and immunohistochemistry. All experiments were approved by the ethics committee of Kindai University Faculty of Medicine (#27-073). This study was conducted according to an opt-out consent procedure, and written informed consent was not required. The committee granted this. Tissue microarray slides (EM1021a, UT240, UT801a, and UT1501) were purchased from US Biomax (Rockville, MD, USA). Each of endometrioid carcinomas on these slides had a pathological grade and a pathological stage according to FIGO 1988 staging system.

Cells and antibodies

Human endometrial adenocarcinoma HEC-50B and HEC-1B cells, and JHUM-3, OMC-2 and HHUA cells were purchased from the JCRB Cell Bank (National Institutes of Biomedical Innovation, Health and Nutrition, Osaka, Japan) and from Riken Cell Bank (Tsukuba, Japan), respectively. HEC-1B and HEC-50B cells were maintained in Eagle's minimal essential medium (Fujifilm Wako, Osaka, Japan) supplemented with 10% and 15% fetal calf serum (FCS; Biological Industries, Kibbutz Beit-Haemek, Israel), respectively. JHUM-3 cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle medium and Ham's F-12 medium containing 10% FCS and 0.1 mM nonessential amino acids. OMC-2 and HHUA cells were in Ham's F-12 containing 10% and 15% FCS, respectively. All experimentation using these cell lines was conducted within 3 months or 5 passages after resuscitation.

A rabbit anti-CADM1 polyclonal antibody against the C-terminal peptide (EGGQNNSEEKKEYFI) was generated in our laboratory,[23] and was used for Western blot (1:1000) and immunohistochemical (1:200) analyses. An anti-GAPDH antibody (mAb-HRP-DirecT, M171–7) was purchased from Medical & Biological Laboratories (Nagoya, Japan) and was used for Western blot analyses (1:2000).

Immunohistochemistry

The immunohistochemical procedures were previously described in detail.[24] Briefly, surgical specimens were formalin-fixed, paraffin-embedded, cut into sections (4-μm thick), and air-dried overnight at 37°C. Surgical sections and tissue microarray slides were deparaffinized in xylene, and rehydrated in a descending ethanol series. After the sections were autoclaved for 20 min at 95°C in 10 mM citrate buffer solution (pH 6.0), they were blocked with 2% bovine serum albumin and incubated with the anti-CADM1 antibody overnight at 4°C, followed by incubation with a peroxidase-conjugated anti-rabbit antibody (Cytiva, Tokyo, Japan) for 2 h at 4°C. Secondary antibody staining was enhanced using the Histofine Simple Stain MAX-PO (R) kit (Nichirei Biosciences, Tokyo, Japan). The sections were incubated with ImmPACTTM AEC (Vector Laboratories, Burlingame, CA, USA) and were then counterstained with Mayer hematoxylin, dehydrated, and mounted. Negative controls were prepared by substituting control rabbit IgG for the specific primary antibody.

Immunohistochemical staining for CADM1 was quantified by the H-score as follows.[25,26] The immunostaining intensity was graded in four levels as 0 (no stain), 1 (weak), 2 (moderate), and 3 (strong), and the cell area stained at each intensity level was measured as % in the total stained cell area (the sum of the four, levels 0–3, areas should be 100). H-score was calculated according to the following formula.

H-score = (% of cell areas stained at intensity level 1 × 1)
+ (% of cell areas stained at intensity level 2 × 2)
+ (% of cell areas stained at intensity level 3 × 3)

The scores were calculated independently by two pathologists (TO and AI), and their mean values were used for statistical analyses.

Transfection

The retroviral plasmid vector pCX4bsr expressing human CADM1 442-amino-acid isoform (pCX4bsr-CADM1) was generated previously.[15] The retroviral packaging cell line PT67 (Clontech, Takara Bio, Kusatsu, Japan) was transfected with pCX4bsr-CADM1 using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). After 2 days, the cells were selected by their resistance to blasticidin S (5 μg/ml; Invitrogen), and then a stable subline with high viral titers was established. These cells were grown to confluence in a 6 cm dish and cultured overnight in RPMI1640 containing 15% FCS. From the culture medium, retroviral particles were collected by centrifugation and filtration through 0.45 μm pores. The virus-containing supernatant and 15% FCS-containing Ham's F-12 medium

were added to a 60% confluent culture of HHUA cells at a ratio of 1:2. This cell culture was continued for 8 days, during which the medium was replaced with the new one every 2 days. HHUA cells expressing CADM1 exogenously (HHUA-CADM1) were then selected by resistance to blasticidin S (4 μg/ml). The gene recombination experiments were approved by the ethics committee of Kindai University Faculty of Medicine (KDMS-2022-005).

Western blotting analysis

Cells were grown to confluence in above-described medium. After washing with phosphate-buffered saline, cells were lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride, and after removal of impurities by centrifugation, the cells were subjected to Western blotting analyses as described in our previous report.[27,28] Immunoreactive band intensities were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA), as described previously.[29]

Generation of ADC

A precursor of ADC containing a maleimidocaproyl (mc) spacer, a cathepsin-sensitive valine–citrulline dipeptide, a p-amino-benzyloxy carbonyl linker and an MMAE payload (mc-vc-PAB-MMAE) was purchased from MedChemExpress (Monmouth Junction, NJ, USA). This precursor was conjugated with human IgG (hIgG–MMAE) or human 3E1 (h3E1–MMAE) as described in detail previously.[21] The drug antibody ratio (DAR) of the ADCs was accomplished by the standard ultraviolet-visible (UV-Vis) absorption spectroscopy.[30] The average DAR was 2.86 for hIgG–MMAE, and 2.57 for h3E1–MMAE.

Water-soluble tetrazolium-8 assay

Cell viability was assessed with the water-soluble tetrazolium-8 (WST-8)-based colorimetric assay using Cell Counting Kit 8 (Dojindo, Kumamoto, Japan).[20,21] Endometrial adenocarcinoma cell lines (3×10^3 cells in 100 μl) were seeded in a 96-well plate in triplicate. Next day, when cells grew to 30% confluency, either hIgG–MMAE ADC or h3E1–MMAE ADC was added to each well at indicated concentrations. After 5 days, cells were incubated with WST-8 for 40 min, and the absorbance at 450 nm was measured using an automated microplate reader. Measurement of mitochondrial dehydrogenase cleavage of WST-8 to formazan dye provided an indication of cell viability. To calculate half maximal inhibitory concentration (IC50) values, a 4-parameter logistic curve was drawn for each experimental group using ImageJ software (https://imagej.nih.gov/ij/). Then, IC50 was calculated according to the following equation: $IC50 = 10^{[\mathrm{Log}(A/B) \times (50 - C)/(D - C) + \mathrm{Log}(B)]}$, in which A, a higher concentration of two values that sandwich IC50; B, a lower concentration of two values that sandwich IC50; C, cell viability (%) at B; and D, cell viability (%) at A.

In vitro cell migration assay

Two-chamber culture plates (Corning, Durham, NC, USA) were used. 2.5×10^5 of HHUA or HHUA-CADM1 cells were suspended in 0.5 ml of Ham's F-12 medium containing 10 nM 17β-estradiol and were seeded in a 12-well culture insert with a collagen-coated polyethylene terephthalate membrane with multiple pores (8.0 μm in diameter) placed at its bottom. The lower well was filled with 1.0 ml of Ham's F-12 medium containing 15% FCS. After 2 days, cells were stained with phalloidin (Molecular Probes, Carlsbad, CA, USA) and the fluorescent images were captured through a 40× objective lens and analyzed on the Nikon C2+ computer system (Tokyo, Japan), and fluorescent intensity (arbitrary unit per unit area) was measured at five randomly selected high-power field views for each membrane using Analysis Controls tools. The mean and standard deviation of fluorescent intensities were calculated from triplicate wells for each experimental group using ROI Statistics. The fluorescent intensity was considered to be proportional to the number of cells present on the underside of the membrane. Assays were repeated 3 times using HHUA-CADM1 cells transfected individually prior to each assay, with essentially similar results.

Statistical analysis

H-scores were analyzed using one-way ANOVA among three or more groups, and the Dunnett contrasts were applied to particular two groups. Comparisons in experiments composed of two groups were done with two-tailed, paired Student's *t*-test. *P*-values ≤0.05 were considered statistically significant.

Results

CADM1 expression in endometrial hyperplasia and adenocarcinoma

We conducted CADM1 immunohistochemistry on sections of our in-house specimens of atypical endometrial hyperplasia ($n = 6$) and endometrioid carcinoma (pathological grade G1; $n = 16$). Representative results are shown in Figure 1. CADM1 proteins were clearly detected on the cell membrane of neoplastic cells aligning in a glandular epithelial arrangement, similar to their localization in the proliferative-phase endometrium in our previous report.[20] We scored the staining intensity using the H-score system. H-scores were higher in atypical endometrial hyperplasia than in endometrioid carcinoma (Table 1). As the carcinoma stage progressed, H-scores decreased gradually (Table 1).

For mass screening, we conducted CADM1 immunohistochemistry on a commercially available tissue microarray slide containing 13 cases of proliferative-phase endometrium, 35 cases of endometrial hyperplasia, 166 cases of endometrioid carcinoma, and 11 cases of the adenosquamous carcinoma component in endometrioid carcinoma with squamous differentiation. Each case of endometrioid carcinoma was given one of five FIGO 1988 stages, i.e., 1a, 1b, 1c, 2, and 3, and also given one of three pathological grades, i.e., G1, G2, and G3. Atypical endometrial hyperplasia and stage 1a endometrioid carcinoma nearly tied for the lead H-score, followed by proliferative-phase

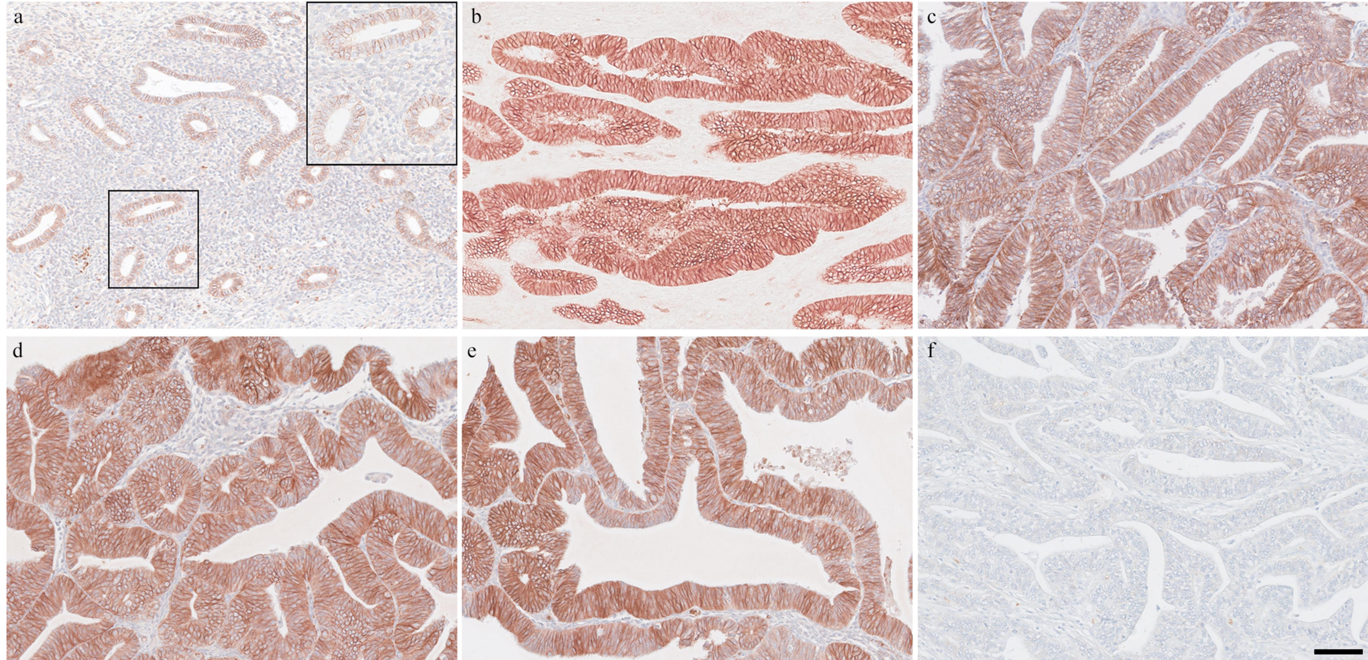

Figure 1. Immunohistochemistry of CADM1 on endometrium and endometrial neoplastic lesions. (a) Endometrium, proliferative phase (UT240; H-score 150). A boxed area is enlarged in the inset. (b) Atypical endometrial hyperplasia (case no. 3; H-score 300). (c–f) Endometrioid carcinoma. (c) UT1501; G1, stage 1a, H-score 300. (d) UT1501; G2, stage 1b, H-score 300. (e) UT1501; G1, stage 1b, H-score 300. (f) UT240; G1, stage 1b, H-score 0. Bar = 100 μm.

Table 1. CADM1 immunohistochemistry of surgical specimens at Kindai University Hospital.

Pathological diagnosis	n	Age	H-score
Atypical endometrial hyperplasia	6	39-51	232 ± 85
Endometrioid carcinoma	16	45-78	129 ± 91*
Stage 1a	2	50, 57	175 ± 106
1b	5	47-68	146 ± 102
1c	6	45-73	125 ± 105
2	3	60-78	80 ± 35

One way ANOVA: $p = .195$.
*$p = .026$ by Student's *t*-test (vs atypical endometrial hyperplasia).

endometrium (Table 2). Among endometrioid carcinoma cases, H-scores greatly decreased as the stage progressed from 1a to 1b, and gradually decreased as the stage progressed more (Table 2). In adenosquamous carcinoma components, the intensity was low in either stage 1 or 2. H-scores were not correlated with the pathological grade of endometrioid carcinoma in any stages (Table 3 and Supplementary Figure S1).

We conducted CADM1 immunohistochemistry on sections of our in-house specimens from four patients who had recurrent endometrioid carcinoma (Supplementary Table S1). H-scores were low in all four cases for both primary and recurrent lesions (Suplementary Figure S2).

h3E1–MMAE ADC effectively reduces endometrial adenocarcinoma cell viability

We performed Western blot analyses of five endometrial adenocarcinoma cell lines and CADM1-transfected cells, HHUA-CADM1. The full-length form of CADM1 was detected in four cell lines, HEC-1B, HEC-50B, JHUM-3, and OMC-2, and HHUA-CADM1 cells (Figure 2a), with a large variation in the expression levels, while HHUA cells were negative for CADM1. The C-terminal fragments generated by α- and β-ectodomain shedding, termed αCTF and βCTF, respectively,[29,31] were also detected faintly in two CADM1-positive lines, HEC-50B and HHUA-CADM1 (depicted by arrowheads in Figure 2a). Normalized expression levels of the full-length CADM1 were shown in the bottom of Figure 2b.

Table 2. CADM1 immunohistochemistry of tissue microarray slides.

Histology	Phase/Stage	n	H-score	*p* value*
Endometrium	Proliferative phase	13	185 ± 77	.985
Endometrial hyperplasia	Simple	22	134 ± 102	.147
	Atypical	13	225 ± 74	1
Endometrioid carcinoma	1a	14	212 ± 119	
	1b	91	106 ± 107	.003
	1c	43	76 ± 103	<.001
	2	11	73 ± 113	.006
	3	7	14 ± 38	<.001
Adenosquamous carcinoma	1	7	82 ± 81	.041
	2	4	38 ± 75	.019

One way ANOVA: $p < .001$.
*ANOVA Dunnett contrasts (vs endometrioid carcinoma stage 1a).

Table 3. H-score of endometrioid carcinoma according to stage and grade.

Stage	Grade G1	Grade G2	Grade G3	p value by ANOVA
1a	211 ± 124 (9)	194 ± 133 (4)	300 (1)	.758
1b	115 ± 94 (30)	103 ± 110 (41)	100 ± 123 (20)	.861
1c	75 ± 121 (9)	88 ± 100 (18)	64 ± 102 (16)	.810
2	50 ± 71 (2)	113 ± 144 (4)	50 ± 112 (5)	.721
3	0 (1)	50 ± 71 (9)	0 ± 0 (4)	.340

Shown are mean ± standard deviation of CADM1 immunohistochemistry H-scores, with case numbers in parentheses.

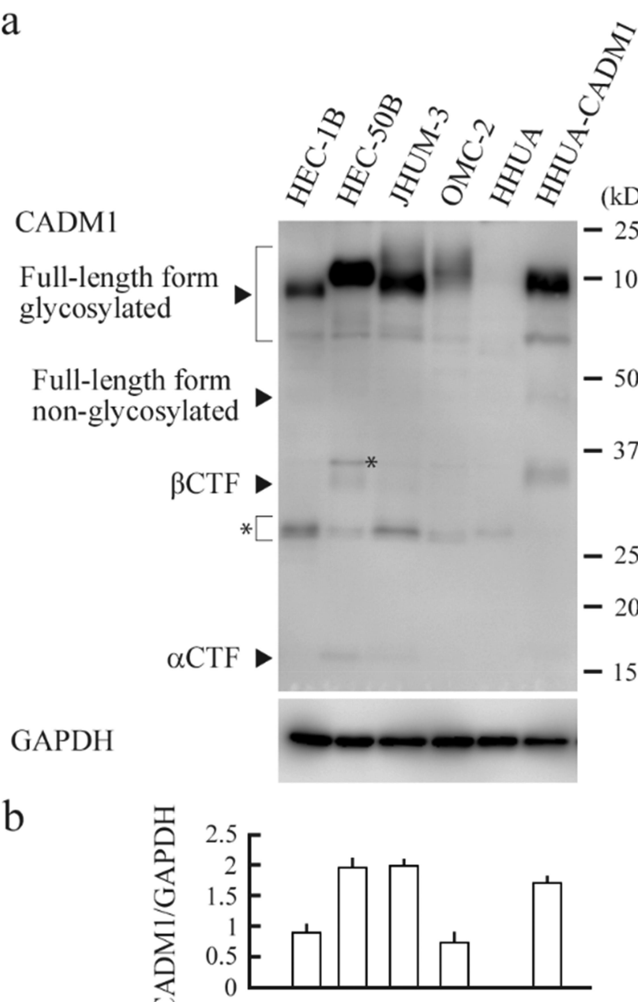

Figure 2. Detection of CADM1 protein in endometrial cell lines by Western blot analyses. (a) Cell lysates were blotted with an anti-CADM1 C-terminal antibody (upper). Arrowheads depict various forms of CADM1; full-length forms glycosylated and non-glycosylated, and C-terminal fragments generated by α- and β-ectodomain shedding (αCTF and βCTF, respectively). Asterisks indicate nonspecific bands caused by the secondary antibody. After stripping, the blot was reprobed with an anti-GAPDH antibody to indicate the amount of protein loading per lane (lower). (b) Expression levels of the glycosylated full-length form relative to GAPDH are plotted in a bar graph at the bottom. For each cell line, the mean value of three independent experiments is plotted with a thin line indicating the standard deviation.

Five endometrial adenocarcinoma cell lines were seeded in a 96-well plate and were cultured in the presence of either h3E1–MMAE ADC or control hIgG–MMAE ADC at various concentrations, i.e., 0.1, 0.5, 1, and 5 µg/mL for HEC-50B, JHUM-3 and HHUA, 0.5, 2.5, 5, and 25 µg/mL for HEC-1B, OMC-2. After 5 days, the cell viability was assessed by WST-8 assays. h3E1–MMAE ADC reduced the cell viability of CADM1-positive endometrial carcinoma cells by approximately 50–80% in a dose-dependent manner, with IC50 = 12.02 nM for HEC-1B, 2.04 nM for HEC-50B, 24.32 nM for JHUM-3, and 13.03 nM for OMC-2 (Figure 3). The cytotoxic effect was strongest on HEC-50B, probably because of the highest CADM1 levels in this cell line, and was substantially undetectable on HHUA, consistent with the cells being CADM1-negative (Figure 3).

Exogenous CADM1 suppresses invasive capacity of HHUA cells

Original HHUA and CADM1-transfected HHUA (HHUA-CADM1) cells were examined for their invasive capacity using two-chamber transmigration assays. Cells were plated on collagen-coated, 8-µm porous membranes and induced to transmigrate across the membrane through the pores with 17β-estradiol. After 2 days, the number of cells present on the underside of the porous membrane was compared between two types of cells using phalloidin staining and fluorescent intensity measurement (Figure 4). The number of transmigrated cells was markedly smaller in HHUA-CADM1 cell cultures than in HHUA cell cultures (Figure 4).

Discussion

In the present study, we found that the CADM1 protein level was significantly higher in atypical endometrial hyperplasia and FIGO 1988 stage 1a endometrioid carcinoma, and markedly decreased as the tumor stage progressed. This expression profile is quite similar to that in lung adenocarcinoma development/progression.[32] We previously revealed that the CADM1 gene was transcriptionally upregulated through two, estrogen- and cell density-dependent, mechanisms in endometrial glandular cells.[20] These mechanisms are likely to underlie the high CADM1 expression in early-stage carcinomas and precursors, since these neoplastic cells often have estrogen receptors and are highly crowded in the epithelial lining.[3] The decrease in CADM1 expression at the advanced stage is probably explained by the CADM1 gene methylation, as is the case with lung adenocarcinoma and ovarian endometrioid carcinoma.[33,34] Consistent with this speculation, CADM1 expression was low in recurrent endometrioid carcinoma lesions. However, in these cases, CADM1 expression was also low in the primary lesions. Perhaps, this is because these primary lesions were already in a potentially advanced stage at the time of the initial surgery. Further investigation is needed to determine whether methylation progresses during the course of recurrence.

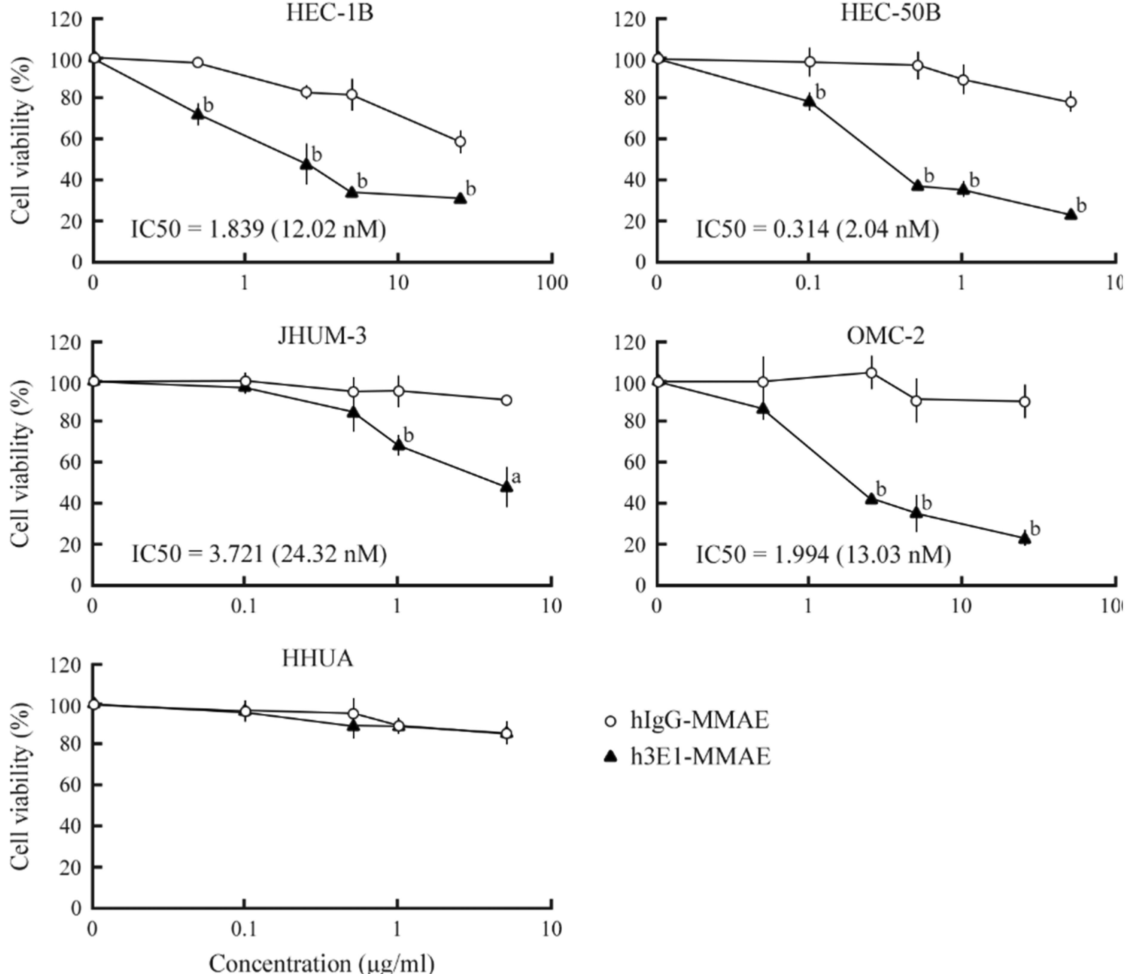

Figure 3. Killing of endometrial adenocarcinoma cells by h3E1–MMAE ADC. Endometrial adenocarcinoma cells were cultured in a 96-well plate at 30% confluence, and either hIgG–MMAE or h3E1–MMAE ADC was added at indicated concentrations. After 5 days, cell viability was calculated by WST-8 assays in triplicate. The mean is plotted in a line graph, with a thin vertical line indicating the standard deviation. a and b, *P*-value <.05 and .01, respectively, when compared with the value of h3E1–MMAE ADC at the identical concentration. IC50 (μg/ml and nM) is calculated.

What roles does the increased CADM1 play in early-stage endometrioid carcinoma and the precursor lesion? CADM1 was detected clearly on the cell membrane of endometrioid carcinoma and precursor cells in most cases positive for CADM1, suggesting that neoplastic cells with higher H-score have more CADM1 molecules that are functional. We previously showed that in epithelial lining, CADM1 downregulation resulted in induction of apoptosis in HEC-1B and OMC-2 endometrioid carcinoma cells.[27] The increased expression of CADM1 may serve as a mechanism by which early-stage endometrioid carcinoma and precursor cells can escape from anoikis, a type of apoptosis resulting from cell detachment,[35] simply by reinforcing cell–cell adhesion in the epithelial lining.[27] In other words, atypical endometrial hyperplasia and stage 1a endometrioid carcinoma cells may still retain the molecular system to induce anoikis, as well as noncancerous endometrial glandular cells.[20]

In the course of review of FIGO 2008 staging system, evidence has been presented that endometrioid carcinoma cells have biologically different characters between FIGO 1988 stages 1a and 1b, though these two stages were combined into one stage, IA, in FIGO 2008. For example, stage 1b cells are suggested to have a higher ability to recur and metastasize to lymph nodes than stage 1a cells.[36–38] This difference may be attributable at least partly to the expression profile of CADM1 that we found here, because CADM1 exerts its tumor-suppressor effects in breast, bladder, and ovary invasive cancer.[39–41,] Actually, exogenous CADM1 suppressed the ability of HHUA cells to transmigrate porous membranes in vitro. Downregulation of CADM1 expression may help stage 1a endometrioid carcinoma cells invade into the myometrium and progress to stage 1b.

Currently, there are no ADCs approved by the FDA for endometrial cancer, but several ADCs are under investigation. They are targeting folate receptor alpha (FRα), a cell-surface transmembrane glycoprotein that facilitates the unidirectional transport of folates into cells; human epidermal growth factor receptor 2 (HER2), a receptor tyrosine-protein kinase encoded by ERBB2; and trophoblast cell surface antigen-2 (Trop2), a tumor-associated calcium signal transducer.[10] These molecules are overexpressed in a considerable proportion of endometrial cancers. In terms of molecular characteristics, CADM1 is different from these molecules, and is categorized into the "adhesion molecule" family, which contains nectin-4.[42,43] An

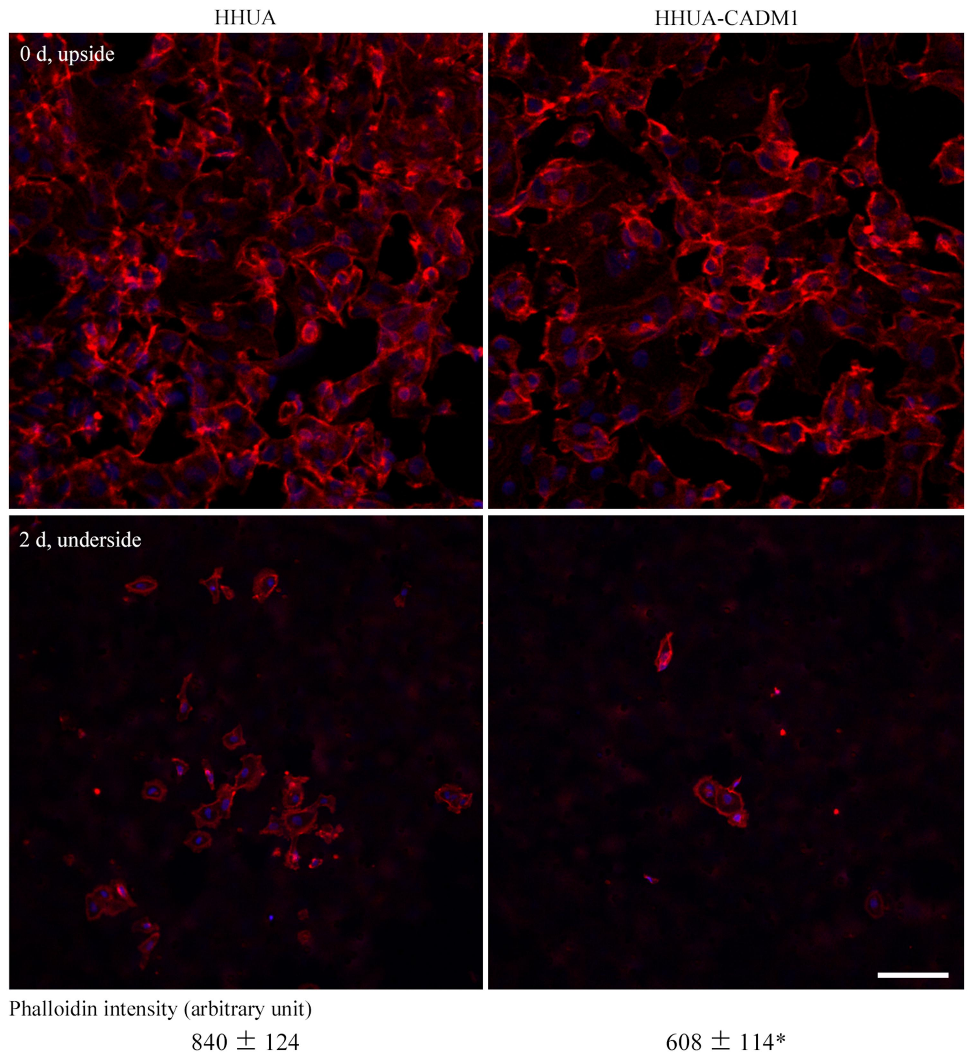

Figure 4. Invasion assay of HHUA cells in two-chamber culture plates. 2.5×10^5 of original HHUA or CADM1-transfected HHUA (HHUA-CADM1; right column) cells were seeded in triplicate onto a collagen-coated, 8 μm porous membrane of the 12-well insert placed in a well filled with 1.0 ml of Ham's F-12 medium (at 0 day). Then, the culture was continued for 2 days. At 0 and 2 days, cells on the membrane were stained with phalloidin. Representative photomicrographs of the upside and underside of the membrane are shown at 0 (upper) and 2 (lower) days, respectively. Phalloidin staining intensities at 2 days are shown at the bottom (mean ± standard deviation). Bar = 100 μm. * $p = .014$ by Student's *t*-test vs. original HHUA cells.

ADC targeting nectin-4, enfortumab vedotin,[44] has received a lot of attention in recent years, because it is very effective for patients with urothelial carcinoma.[45–47] Of note, h3E1–MMAE ADC not only has similarity with enfortumab vedotin in the target molecule, but the two ADCs are also structurally similar.[21,44] IC50 of h3E1–MMAE ADC (MW ~ 153,000) is calculated to be 2.04 nM for HEC-50B, which compares favorably with that of enfortumab vedotin (a humanized recombinant antibody-based version; 1.52 nM).[44] In parallel with the present study, we conducted animal experiments to test in vivo cytotoxic activity of h3E1–MMAE ADC.[48] While CADM1 is expressed in a variety of cells (Supplementary Table S2), significant toxicity of this ADC was detected as sperm hypoplasia in the testes, but not severe enough to threaten mouse survival.[48] Adverse effects do not appear to be of much concern, especially in women.

According to the overexpression profile of CADM1, h3E1–MMAE ADC is suggested to serve potentially as a drug for the treatment of stage 1a endometrioid carcinoma. For this stage carcinoma, the most commonly performed procedure is simple hysterectomy, but there is controversy regarding whether the surgery is excessive.[49] This is even more so for patients who wish to maintain their fertility.[50] Past studies demonstrate that early-stage endometrial cancers have a fairly good response to hormone therapy with progestins, such as medroxyprogesterone acetate, megestrol acetate, and levonorgestrel.[49] Generally, levonorgestrel is administered as an intrauterine device that releases the progestin slowly.[51] Considering that CADM1 is not a tumor-specific antigen, we are now trying to develop an intrauterine device containing h3E1–MMAE ADC so as to avoid its on-target side effects. This ADC-containing intrauterine device is expected to be welcomed by patients who have stage 1a endometrioid carcinoma and wish less aggressive management to preserve their fertility.

In conclusion, endometrioid carcinomas were found to express CADM1 abundantly at the early developmental stage, and to lose the expression as the invasive stage progresses. h3E1-MMAE ADC could potentially be an option in noninvasive treatment for early-stage endometrioid carcinoma.

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Disclosure Statement

No potential conflict of interest was reported by the author(s).

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Author contributions

A.Y., T.O. and F.T. performed immunohistochemistry, and T.O. and A. I. analyzed the staining intensity. M.H. and A.W. constructed expression vectors, performed transfection, and generated ADCs. M.H., A.Y. and T. I. conducted cell culture experiments and western blot analyses. M.H. and A.I. conducted the statistical analyses. M.H. and A.I. confirmed the authenticity of all the raw data. Y.S. and A.I. conceived and designed the study, and A.I. drafted the manuscript. All authors read and approved the final manuscript.

Data availability statement

Data is not publicly available due to ethical reasons. Further enquiries can be directed to the corresponding author.

Statement of ethics

An opt-out informed consent protocol was used for use of participant data for research purposes. This consent procedure was reviewed and approved by Ethical Committee of Kindai University Faculty of Medicine, approval number [#27-073], date of decision [April 13, 2020].

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