**CADM1 promotes adhesion to vascular endothelial cells and transendothelial migration in cultured GIST cells**

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**Abstract.** Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the human gastrointestinal tract. Small intestinal GISTs appear to be associated with poorer prognosis and higher metastasis rate than gastric GISTs of the same size and mitotic index. Recently, we reported that cell adhesion molecule 1 (CADM1) is expressed specifically in most small intestinal GISTs, but not in most gastric GISTs, suggesting that this difference in CADM1 expression between gastric GISTs and small intestinal GISTs might influence the difference in clinical behavior between them. The aim of the present study was to examine whether high CADM1 expression affected proliferation, migration, invasion, adhesion to endothelial cells and transendothelial migration of cultured GIST cells by comparing original GIST-T1 cells with very low CADM1 expression with GIST-T1 cells with high CADM1 expression induced by CADM1 cDNA transfection (GIST-T1-CAD cells). GIST-T1-CAD cells had reduced ability to proliferate, migrate and invade compared with the original GIST-T1 cells, but showed significantly higher ability to adhere to human umbilical vein endothelial cells and migrate through endothelial cell monolayers. Thus, CADM1 may contribute to higher metastasis rates in small intestinal GISTs facilitating tumor cell adhesion to vascular endothelial cell and transendothelial migration of tumor cells. CADM1 might serve as a potential target for inhibition of metastasis in small intestinal GISTs.

**Introduction**

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the human gastrointestinal (GI) tract (1). GISTs occur throughout the GI tract, but most of them arise in the stomach (60-70%) or small intestine (20-30%) (2). Activating mutations in the c-kit gene and PDGFRA gene, encoding KIT tyrosine kinase and platelet-derived growth factor receptor tyrosine kinase, respectively, are considered the main oncogenic drivers of GIST (3). The minority of GISTs harboring neither c-kit gene nor PDGFRA gene may have mutations in the NF1, BRAF, or SDH complex genes (4-6).

GISTs are neoplasms with malignant potential varying from virtual indolence to rapid progression. Up to 20% of GIST patients have overt metastases at diagnosis, and the metastases typically occur in the abdominal cavity or the liver. Small intestinal GISTs are considered to have a worse prognosis than gastric GISTs because of their higher risk of metastasis and tumor-related death (7). Recent research has shown that small intestinal GISTs exhibit more aggressive features such as high pathological grade and large size than gastric GISTs (8). Distinct transcription profiles related to the anatomical location of GISTs have also been reported previously (9). The results of hierarchical clustering analysis of the transcripts showing that GISTs, roughly divided into two groups such as gastric GISTs and small intestinal GISTs, may partially account for the more aggressive behavior of small intestinal GISTs than gastric GISTs of similar size and mitotic rate. Thus, the widely used risk classifications for GIST metastasis such as the Armed Forces Institute of Pathology (AFIP) classification and modified Fletcher classification include tumor location as a risk factor.

We have recently reported that cell adhesion molecule 1 (CADM1) is expressed specifically in most small intestinal GISTs but not in most gastric GISTs (10). CADM1 is a member of the immunoglobulin superfamily that was initially known as spermatogenic immunoglobulin superfamily (SgIGSF) and synaptic cell adhesion molecule (SynCAM) (11). CADM1 is also a tumor suppressor of lung cancer (TSLC1) (12). Loss of CADM1 expression probably due to methylation of the CADM1 gene promoter is frequently found in various types of epithelial cancer, such as gastric cancer, breast cancer, and esophageal squamous cell carcinoma (13-15). Therefore, CADM1 in those cancers is considered to act as a tumor suppressor. In contrast, CADM1 appears to be a tumor promoter in adult T cell leukemia/lymphoma (ATLL) (16) and acute myelocytic leukemia (17,18), which show high CADM1 expression with...
enhancement of tumor growth, tissue invasion by the tumor cells, and tumor cell adhesion to the vascular endothelium (18). Recent research also reported that CADM1 is highly expressed in ~80% of small cell lung cancers (SCLCs) and promotes malignant features of them (19,20).

In the present study, we examined whether CADM1 affects proliferation, migration, invasion, adhesion to endothelial cells, and transendothelial migration of GIST cells. GIST-T1 cells with high CADM1 expression induced by CADM1 cDNA transfection, when compared to the original GIST-T1 cells with very low CADM1 expression, had decreased ability to grow, migrate, and invade but increased ability to adhere to endothelial cells and emigrate by transendothelial migration. CADM1 might play a role in tumor metastasis by facilitating adhesion between vascular endothelial cells and GIST cells and subsequent transendothelial migration of GIST cells with high CADM1 expression. There is a possibility that the higher expression of CADM1 in small intestinal GISTs than in gastric GISTs could contribute to the higher metastatic rate of small intestinal GISTs. CADM1 might provide a new strategy for inhibiting metastasis of small intestinal GISTs.

Materials and methods

Tumor tissue samples. Fresh tissue samples of a representative gastric GIST and a representative small intestinal GIST were collected intraoperatively, frozen, and stored at -80°C until use. RNA and protein were extracted from the samples. The RNA and protein in the present experiments have been used in previous experiments (10).

Cell lines. A human GIST cell line, GIST-T1, which is derived from a metastatic pleural tumor from gastric GIST in a Japanese woman, was purchased from Cosmo Bio. It harbors a heterozygous c-kit gene mutation at exon 11 (an in-frame deletion of 19 amino acids from Val560 to Tyr578). The GIST-T1 cell line was maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal bovine serum (FBS) (BioWest), 100 U/ml of penicillin G (Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal bovine serum (FBS) (BioWest), and 100 µg/ml of streptomycin (Invitrogen; Thermo Fisher Scientific). To verify product authenticity, the obtained vector with full-length CADM1 cDNA in 100 µl of Cell Line Nucleofector kit V solution (Lonza) and electroporated using the Amaxa Nucleofector II machine (program T-030) (Lonza) according to the manufacturer's protocol. GIST-T1 cells stably expressing CADM1 (GIST-T1-CAD) were selected in 250 µg/ml of Zeocin (Thermo Fisher Scientific), and a monoclonal cell population was isolated by limiting dilution. All experiments using recombinant DNA were approved by the Committee for Recombinant DNA Experiments of Hyogo College of Medicine (no. 24015).

Western blotting. GIST-T1 cells, GIST-T1-CAD cells, a representative gastric GIST tissue, and a representative small intestinal GIST tissue were lysed in Celllytic M Cell Lysis Reagent (Sigma-Aldrich; Merck KGaA) containing 5 mM NAF; 1 mM Na2VO4, and proteinase inhibitor cocktail (Roche). As described previously (10), almost all gastric GISTs express a very low level of CADM1 protein and almost all small intestinal GISTs apparently express CADM1 protein. Western blot analysis was performed as previously reported (10). Briefly, anti-CADM1 chicken monoclonal antibody (clone. 3E1, MBL International), anti-KIT rabbit polyclonal antibody (A4502, Dako) or anti-β-actin mouse monoclonal antibody (ab8226, Abcam) were used for primary antibodies after electrophoresis and membrane transfer. Then, membranes were incubated either with horse radish peroxidase (HRP)-conjugated donkey anti-chicken IgY antibody (EMD Millipore, Sigma-Aldrich; Merck KGaA), HRP-conjugated goat anti-rabbit IgG antibody, or HRP-conjugated goat anti-mouse IgG antibody (Dako) after the electrophoresis and membrane transfer. Proteins of interest were then visualized by incubation with enhanced chemiluminescence (ECL) reagent (Promega).

Cell proliferation assay. GIST-T1 and GIST-T1-CAD cells were plated in 24-well plates (Corning Incorporated) at 2x10⁴ cells per well in growth medium. After incubation for 1, 3, 5, and 7 days, cells were trypsinized, resuspended in Accumax (Innovative Cell Technologies), and counted by hemocytometer (Z1, Beckman Coulter). Six wells were used for each cell type in each experiment. The cell proliferation assay was repeated three times.

Migration assay. Migration ability was assessed by the wound-healing assay. GIST-T1 and GIST-T1-CAD cells were...
seeded in 6-well plates at 3x10^5 cells and allowed to grow to 90% confluence. The cell monolayer was scratched with a sterile micropipette tip, and then serum-free medium was added into plates after washing the cells thrice with PBS. Photographs of images captured at x200 magnification were taken at the same six selected locations for each well under a phase contrast microscope (All-in-One Microscope; Keyence). The area that remained clear after 0, 1, 2, 3, and 4 days was quantified with ImageJ (National Institutes of Health) and the covered area was calculated by comparing to the area of the wound at day 0. This assay was repeated three times.

Migration and invasion assays. The migration assay was performed using Falcon cell culture inserts (Corning Incorporated) without Matrigel and the invasion assay was performed using 24-well BD Bio-Coat Matrigel Invasion Chambers (BD Biosciences) according to the manufacturer's protocol. GIST-T1 and GIST-T1-CAD cells were resuspended at a density of 5x10^5 cells/l in 0.5 ml of the serum-free medium and added into the upper chamber of the insert. DMEM supplemented with 10% FBS (0.75 ml) was added to the lower chambers. After incubation for 2 days, non-migrated or non-invaded cells were removed from the upper surface of the membranes using a cotton tipped swab. The cells adhering to the bottom surface of the membrane were fixed and permeabilized in 10% neutral formalin and 100% methanol, respectively. Migrated or invaded cells were stained by Giemsa staining and counted in nine selected microscope fields per membrane. The experiments were conducted three times.

Tumor-endothelial cell adhesion assay. Static adhesion assay using fluorescence-labeled tumor cells was performed. HUVECs (2.5x10^5 cells/well) pretreated with or without 10 ng/ml TNF-α (Invitrogen; Thermo Fisher Scientific) were cultured in 96-well plates overnight. TNF-α has the potential to stimulate endothelial cell adhesion. GIST-T1 and GIST-T1-CAD cells were labeled with 2 μg/ml Calcein-AM (Dōjindo Laboratories) at 37°C for 30 min, washed thrice with PBS, and resuspended at 2.5x10^6 cells/ml with serum-free DMEM, and followed by pipetting onto confluent HUVECs monolayers. After coculturing for 2 h, medium and unbound tumor cells were removed and discarded. Adherent tumor cells and endothelial cells were washed three times with PBS. Then the amount of Calcein-AM fluorescence was measured using a fluorescence microplate reader (2030 ARVO X4, PerkinElmer Life and Analytical Sciences), at an excitation wavelength of 485 nm and emission wavelength of 530 nm.

Transendothelial migration assay. HUVECs (2x10^5) pretreated with 10 ng/ml TNF-α were seeded onto 24-well Transwell Inserts and cultured overnight. After formation of a confluent HUVEC monolayer, tumor cells labeled with Calcien-AM were added to the upper chamber, and cells were cocultured for 48 h. After incubation, the non-migrated cells which were present on the upper side of the membrane were removed with a cotton tipped swab, and the transmigrated cells on the bottom side of the membrane were fixed with 10% neutral formalin. Transmigrated cells were visualized using a fluorescence microscope and counted from 10 random fields under x200 magnification. Experiments were performed in triplicate and repeated three times.

Statistical analysis. Statistical analysis of proliferation assay, wound-healing assay was performed by two-way mixed ANOVA followed by Bonferroni's multiple comparison test. The significance of cell migration and Matrigel invasion in transwell assay, adhesion assay and transendothelial migration assay was analyzed by unpaired Student t-test. P<0.01 was considered statistically significant.

Results

GIST-T1-CAD cells show high expression of CADM1. GIST-T1 cells are a cell line of GIST cells originally derived from the stomach. Consistent with our previous report (10) showing that CADM1 expression was much weaker in GISTs of gastric origin than in GISTs of small intestinal origin, no CADM1 protein and CADM1 mRNA were detected in original GIST-T1 cells by western blotting and RT-qPCR, respectively (Fig. 1A and B). Using transfection of full length of CADM1 cDNA into GIST-T1 cells, we tried to establish GIST-T1 cells stably expressing CADM1 (GIST-T1-CAD cells). Western blotting and RT-qPCR, respectively, revealed high expression of CADM1 protein and CADM1 mRNA in the obtained GIST-T1-CAD cells (Fig. 1A and B).

High CADM1 expression suppresses the proliferation, migration, and invasion of cultured GIST cells. To investigate whether CADM1 is involved in the proliferation of GIST cells, we compared proliferative ability between GIST-T1 cells and GIST-T1-CAD cells. Cell number was counted at days 0, 1, 3, 5, and 7 after seeding with 2x10^4 of both cells. After day 3, the number of GIST-T1-CAD cells was significantly smaller than the number of GIST-T1 cells (Fig. 2) (P<0.001). To analyze the effect of CADM1 on the ability of GIST cells to migrate, we performed a wound healing assay. Closure of scratches in GIST-T1 and GIST-T1-CAD cell monolayers was measured at 0, 1, 2, 3, and 4 days. Closure of scratches in GIST-T1-CAD cell monolayers was significantly slower than closure of scratches in GIST-T1 cell monolayers at 1, 2, 3, and 4 days (Fig. 3A and B) (P<0.001). We also examined the effect of CADM1 on migration of GIST cells through transwell membranes without Matrigel and invasion of GIST cells through transwell membranes coated with Matrigel. Fewer GIST-T1-CAD cells than GIST-T1 cells appeared to migrate through the transwell membrane (Fig. 4A and B), but the difference was not statistically significant (P=0.1416). On the other hand, statistically significantly fewer GIST-T1-CAD cells than GIST-T1 cells invaded the Matrigel-coated transwell membrane (Fig. 4C and D) (P<0.001).
monolayers. We firstly examined the ability of tumor cells to adhere to TNF-α-unstimulated endothelium. GIST-T1-CAD cell-HUVEC adherence was 16.6 times greater than GIST-T1 cell-HUVEC adherence (Fig. 5A) (P<0.001). After overnight stimulation of HUVECs by TNF-α (10 ng/ml for 12 h), the number of GIST-T1-CAD cells adhering to HUVECs and GIST-T1 cells adhering to HUVECs was augmented. Even after stimulation of HUVECs by TNF-α, the number of GIST-T1-CAD cells adhering to HUVECs was 3 times greater than that of GIST-T1 cells adhering to HUVECs (Fig. 5B) (P<0.0001). To determine the effect of CADM1 on GIST cell transmigration across endothelium, the transendothelial migration assay was performed using TNF-α-stimulated HUVECs, GIST-T1 cells, and GIST-T1-CAD cells. Number of migrated Calcein-AM-labeled GIST-T1-CAD cells was significantly larger than the number of migrated Calcein-AM-labeled GIST-T1 cells (Fig. 6A and B) (P<0.001).

Discussion

We have recently reported that CADM1 is expressed specifically in most small intestinal GISTs but not in most gastric GISTs (10). Patients with small intestinal GISTs are...
considered to have a worse prognosis than patients with gastric GISTs because of their higher risk of metastases and tumor-related death (7). Therefore, we tried to clarify whether high CADM1 expression in small intestinal GISTs affects the biological behavior of GISTs. In the present study, proliferation, migration, invasion, adhesion to endothelial cells, and transendothelial migration were compared between

Figure 4. CADM1 expression suppresses GIST-T1 cell migration and invasion in vitro. Representative images of cell (A) migration and (C) Matrigel invasion in Transwell assays using the original GIST-T1 cells and GIST-T1-CAD cells. Scale bar, 200 µm. There were more migrating and invading original GIST-T1 cells than migrating and invading GIST-T1-CAD cells. The means of nine random microscopic fields per membrane in the cell (B) migration and (D) Matrigel invasion assays were calculated. Statistical significance (*P<0.001, n=3, unpaired Student’s t-test) is shown. GIST, gastrointestinal stromal tumor; GIST-T1-CAD, GIST-T1 cells with high CADM1 expression induced by CADM1 cDNA transfection; CADM1, cell adhesion molecule 1.

Figure 5. CADM1 expression enhances adhesion of GIST-T1 cells to endothelial cells. (A) Without TNF-α stimulation, significantly greater numbers of Calcein-AM labeled GIST-T1-CAD cells than Calcein-AM labeled original GIST-T1 cells adhered to HUVECs. (B) With TNF-α stimulation, significantly greater numbers of Calcein-AM labeled GIST-T1-CAD cells than Calcein-AM labeled original GIST-T1 cells also adhered to HUVECs. Statistical significance (*P<0.001, n=21, unpaired Student’s t-test) is shown. GIST, gastrointestinal stromal tumor; GIST-T1-CAD, GIST-T1 cells with high CADM1 expression induced by CADM1 cDNA transfection; CADM1, cell adhesion molecule 1; HUVECs, human umbilical vein endothelial cells.

Figure 6. CADM1 expression enhances transendothelial migration of GIST-T1 cells. (A) Representative images of transmigrated GIST-T1 cells and GIST-T1-CAD cells labeled by Calcein-AM green dye are shown. Scale bar, 200 µm. (B) The mean numbers of GIST-T1-CAD cells and GIST-T1 cells in 14 random microscopic fields per membrane are shown. The number of GIST-T1-CAD cells showing transendothelial migration was significantly higher than that of GIST-T1 cells showing transendothelial migration. Data are from three independent experiments. Statistical significance (*P<0.001, n=14, unpaired Student’s t-test) is shown. GIST, gastrointestinal stromal tumor; GIST-T1-CAD, GIST-T1 cells with high CADM1 expression induced by CADM1 cDNA transfection; CADM1, cell adhesion molecule 1.
original GIST-T1 cells with very low CADM1 expression and GIST-T1 cells with high CADM1 expression induced by CADM1 cDNA transfection (GIST-T1-CAD cells). GIST-T1-CAD cells showed lower ability to grow, migrate, and invade, but higher ability to adhere to endothelium and transmigrate across endothelium than the original GIST-T1 cells. These results suggested that CADM1 might facilitate the metastasis of GISTs by increasing tumor cell adherence to vascular endothelial cells and subsequent passage through the vascular endothelium but not by increasing tumor cell growth and motility.

CADM1 expression is frequently lost in numerous types of epithelial neoplasms (13-15), and CADM1 is considered to be a tumor suppressor in epithelial neoplasms. In contrast, upregulated CADM1 appears to promote ATLL (16) and AML (17) progression through enhancement of tumor cell growth, tissue invasion, and adhesion to the vascular endothelium (18). There is also recent research that high CADM1 expression in SCLCs might promote the malignant features of the cancer (19,20). In the present study, we showed that high CADM1 expression decreased tumor cell growth and motility but increased tumor cell adherence to vascular endothelial cells and subsequent transmigration across vascular endothelium. Thus, high CADM1 expression in GISTs appears to have two roles, as a tumor suppressor and a tumor promoter. Poorer prognosis in small intestinal GISTs might indicate that the metastasis promoter role through strong adherence to endothelial cells exceeds the tumor suppressor role through reduced tumor growth and motility.

Expression of CADM1 is extremely low in most gastric GISTs (10). The loss of CADM1 expression frequently found in a variety of cancers is considered to be due to aberrant hypermethylation of the CADM1 promoter. In our preliminary research, there was no methylation of CADM1 promoter in not only small intestinal GISTs but also gastric GISTs (unpublished data), and methylation is not considered to be the cause of low CADM1 expression in gastric GISTs. Therefore, we should clarify the cause of low CADM1 expression in gastric GISTs.

In the present study, expression of CADM1 in cultured GIST cells increased their ability to adhere to endothelium and transmigrate across endothelium. This is similar to previous reports showing CADM1 promotes ATLL cell infiltration of organs (18). CADM1 promotes an invasive phenotype of ATLL cells by activating the Rac pathway through PDZ-BM interaction with TIAM1 (21). A recent study reported that CADM1 recruits 4.1R to the cell-cell contact site and can enhance the malignant features of SCLC (20). In addition, 4.1R modulates the localization of several G-protein coupled receptors including Duffy/ACKR1 (22). Besides, it had been reported that CADM1 activates PI3K signaling by forming a tripartite protein complex with the p85 subunit of PI3K through the membrane-associated guanylate kinases (MAGuKs), membrane palmitoylated protein 3 (MPP3) and Drosophila tumor suppressor discs large (Dlg) (23), which play roles in the extension of epithelial cells. Further examination of CADM1 involvement in the mechanism of GIST cell adhesion to endothelium and transmigration of GIST cells across endothelium is required.

CADM1 is ubiquitously expressed in vascular endothelial cells (24). In our study, compared to the original GIST-T1 cells, GIST-T1-CAD cells showed a much higher ability to adhere to TNF-α unstimulated HUVECs, suggesting that CADM1-mediated homotypic contacts between GIST-T1-CAD cells and HUVECs is extremely important for their adhesion. The adhesion of the original GIST-T1 cells to HUVECs was significantly enhanced by pre-stimulation of the HUVECs with TNF-α. TNF-α can upregulate expression of the intercellular adhesion molecule type 1 (ICAM-1), E-selectin, and vascular cell adhesion molecule type 1 (VCAM-1) (25,26) in vascular endothelial cells. Promotion of original GIST-T1 cell adhesion to TNF-α-stimulated HUVECs might be significantly induced by increased expression of those adhesion molecules on TNF-α-stimulated HUVECs. On the other hand, improved GIST-T1-CAD cell adhesion to HUVECs after TNF-α-stimulation might derive from increased expression of not only those adhesion molecules but also CADM1 on TNF-α-stimulated HUVECs. Detailed mechanisms underlying the change in the ability of GIST-T1-CAD cells and original GIST-T1 cells to adhere to HUVECs before and after TNF-α stimulation should be clarified.

Recently, anti-CADM1 antibodies were developed as a promising candidate agent for reducing ATLL cell invasion via blocking cell adhesion. The antibodies appear to show a minimal cytotoxic effect on the growth of the ATLL cell line (27). Such antibodies are also expected to inhibit GIST cell adhesion to the endothelium and show inhibition of GIST metastasis. Moreover, anti-CADM1 antibodies could cause cell damage via antibody dependent-cellular cytotoxicity or complement-dependent cytotoxicity. The anti-tumor effect of antibody-drug conjugates using CADM1 antibodies might also be stronger. Anti-CADM1 antibodies could become a new treatment strategy for small intestinal GISTs.

There are some limitations in our study. First, we did not examine expression of surface adhesion molecules other than CADM1 that may affect both migration/invasion of GIST-T1 cells and adhesion between GIST-T1 cells and HUVECs. CADM1 cDNA transfection to GIST-T1 cells may also change the expression of such surface adhesion molecules. We are planning to examine the expression levels of those molecules in HUVECs and GIST-T1 cells before and after CADM1 cDNA transfection. Second, we did not examine that CADM1 expression in HUVECs was really augmented after TNF-α stimulation. We will examine whether expression levels of not only CADM1 but also other surface adhesion molecules increase in HUVECs after TNF-α stimulation in the near future. Third, we only carried out in vitro experiments concerning migration, invasion and adhesion of GIST-T1 and GIST-T1-CAD cells, but CADM1 contribution to metastatic activity in GIST should be evaluated by in vivo experiments. In vivo studies using mouse models of metastasis and GIST cells are being planned.

In summary, CADM1 in GISTs might act as a suppressor of tumor growth, migration, and matrix invasion, but stronger GIST cell-endothelial cell interaction induced by high CADM1 expression could serve as a potential target for the treatment of small intestinal GISTs, especially for inhibiting GIST metastasis.
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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JY, TK, NK, TY, MY, KI, AI and SH participated in data collection and discussion of the findings. JY, TK, NK, TY and MY carried out the experiments. JY and TK performed the statistical analysis. JY and SH confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Experiments using recombinant DNA were approved by the Committee for Recombinant DNA Experiments of Hyogo College of Medicine (approval no. 24015; Nishinomiya, Japan). The use of fresh human gastrointestinal stromal tumor (GIST) tissue samples for GIST assays, including gene analysis, was approved by the Ethical Committee of Hyogo College of Medicine (approval no. 28), and the patients/participants provided their written informed consent to participate in this study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


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