Abstract. Invasion into the matrix is one of hallmarks of malignant diseases and is the first step for tumor metastasis. Thus, analysis of the molecular mechanisms of invasion is essential to overcome tumor cell invasion. In the present study, we screened for colon carcinoma-specific genes using a cDNA microarray database of colon carcinoma tissues and normal colon tissues, and we found that fermitin family member-1 (FERMT1) is overexpressed in colon carcinoma cells. FRRMT1, FERMT2 and FERMT3 expression was investigated in colon carcinoma cells. Reverse transcription polymerase chain reaction (RT-PCR) analysis revealed that only FERMT1 had cancer cell-specific expression. Protein expression of FERMT1 was confirmed by western blotting and immunohistochemical staining. To address the molecular functions of FERMT genes in colon carcinoma cells. Reverse transcription polymerase chain reaction (RT-PCR) analysis revealed that only FERMT1 had cancer cell-specific expression. Protein expression of FERMT1 was confirmed by western blotting and immunohistochemical staining. To address the molecular functions of FERMT genes in colon carcinoma cells. Reverse transcription polymerase chain reaction (RT-PCR) analysis revealed that only FERMT1 had cancer cell-specific expression. Protein expression of FERMT1 was confirmed by western blotting and immunohistochemical staining. To address the molecular functions of FERMT genes in colon carcinoma cells.

Expression and Function of FERMT Genes in Colon Carcinoma Cells

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Materials and Methods

Cell lines, culture, cell growth assay and gene transfer. Colon adenocarcinoma cell lines HCT116, HCT15, Colo205, SW480, CaCO2, RTK, SW48, LoVo, DLD1, HT29 and Colo320 were kind gifts from Dr. K. Imai (Sapporo, Japan), and the KM12LM cell line was a kind gift from Dr. K. Itoh (Kurume, Japan). All cell lines were

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cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Life Technologies Japan, Tokyo, Japan).

For cell growth assay, 1×10^5 cells were seeded in a 6-well plate, and total cell numbers were counted every day by using Countess™ (Life Technologies).

A retrovirus system was used for gene transfer, as described previously (7). Briefly, a pMXs-puro retroviral vector was transfected into PLAT-A amphotropic packaging cells (kind gift from Dr. T. Kitamura), and then HCT116 and SW480 cells were infected with the retrovirus. Puromycin was added at 5 μg/ml for establishment of stable transformants.

Reverse transcription polymerase chain reaction (RT-PCR) analysis of FERMT genes in normal tissues and colon carcinoma cells. RT-PCR analysis was performed as described previously (8). Primer pairs used for RT-PCR analysis were 5'-GTCTGTCGAAACACAGGATT-3' and 5'-GTTTTTCTAGTGGTCTCCTT-3' for FERMT1, with an expected PCR product size of 272 base pairs (bps); 5'-CATGACATCGAGAATCATTT-3' and 5'-ACGGTATTCTCCCTTGGCTC-3' for FERMT2, with an expected PCR product size of 256 bps; 5'-AAAGTTCAAGGGAACAGAC-3' and 5'-GAAGGGCCAATTAGTTGTTGTA-3' for FERMT3, with an expected PCR product size of 452 bps. GAPDH was used as an internal control. The PCR products were visualized with ethidium bromide staining under UV light after electrophoresis on 1.2% agarose gel. Nucleotide sequences of the PCR products were confirmed by direct sequencing.

Construction of plasmids and transfection. Full-length FERMT1, FRERMT2 and FERMT3 cDNAs were amplified from cDNA of LoVo cells with PCR using KOD-Plus DNA polymerase (Toyobo, Osaka, Japan). The primer pairs were 5'-CGGGGCTACCATGCTCATCC-3' and 5'-ACTGACTTT-3' as a forward primer and 5'-CGCTGACATGGCCCGGTCAATTT-3' as a reverse primer (underlines indicating restriction enzyme recognition sites, respectively) for FERMT1, 5'-CGGGCCTACCGCCACCATGCTCTGAGGGAACAGAC-3' as a forward primer and 5'-CCGGCTGCAGCCCGCAGAC-3' as a reverse primer for FERMT2, and 5'-GGGAGGAAGGCCGCTCAATGACCACCCCGGAT-3' as a reverse primer for FERMT3. The PCR product was inserted into the pcDNA3.1 expression vector (Life Technologies) fused with a FLAG-tag. The cDNA sequences were confirmed by direct sequencing, and proved to be identical as reported previously (4). The inserts were then sub-cloned into a pMXs-puro retrovirus vector (kind gift from Dr. T. Kitamura, Tokyo, Japan). For the construction of protein expression, a BglII and Xhol-digested deletion mutant of FERMT1 cDNA that was overexpressed in normal organ tissues and about 4000 carcinoma tissues using the Affymetrix GeneChip Human Genome U133 Array Set that contains approximately 39,000 genes. One of the genes that was overexpressed in colon carcinoma tissues was shown to be FERMT1, a member of the FERMT gene family. In a previous study, FERMT1 was shown to be overexpressed in lung carcinoma cells and colon carcinoma cells (4). FERMT1 is a member of a family of highly homologous gene products including FERMT2 and FERMT3 (Figure 1A). FERMT1, FERMT2 and FERMT3 share a FERM domain and a Pleckstrin homology domain (PH) domain, which are a cytoskeletal-associated domain and phosphatidylinositol
lipids association domain, respectively (Figure 1B). Since FERMT1, FERMT2 and FERMT3 show high homology with each other, we evaluated the expressions of these genes in colon carcinoma cells and also in normal organ tissues by RT-PCR. FERMT1 was expressed in 9 (75%) out of 12 colon carcinoma line cells, and FERMT3 was expressed in 9 (75%) out of 12 colon carcinoma line cells and FERMT2 was expressed in 3 (25%) out of 12 colon carcinoma line cells.

Figure 1. Expression profiles of fermitin family member (FERMT) family genes. A: Sequence alignment of FERMT proteins. FERMT1, FERMT2 and FERMT3 amino acid sequences are shown. A black box indicates the same alignment, a gray box indicates similar alignment. B: Molecular structure of FERMT family proteins. A dotted box indicates the FERMT domain, cytoskeletal-associated domain, a lined box indicates the Pleckstrin homology domain (PH) domain, phosphatidylinositol lipid association domain. C: Reverse transcription-polymerase chain reaction (RT-PCR) of FERMT family in colon carcinoma cells. FERMT1, FERMT2 and FERMT3 expression in colon carcinoma cells was evaluated by RT-PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal positive control. D: RT-PCR of FERMT family genes in normal organ tissues. FERMT1, FERMT2 and FERMT3 expression in normal organ tissues was evaluated by RT-PCR. FERMT1, FERMT2 and FERMT3 plasmids were used as positive controls. GAPDH was used as an internal positive control.
FERMT1 was not expressed in normal organ tissues, whereas FERMT3 and FERMT2 were expressed ubiquitously in normal organ tissues. Only FERMT1 exhibits colon carcinoma cell-specific expression. We therefore focused on FERMT1 for further analysis.

Protein expression of FERMT1 in colon carcinoma cells and tissues. To address FERMT1 protein expression, we established a novel anti-FERMT1 mAb. Since FERMT1, FERMT2 and FERMT3 have similar protein structures, we evaluated the specificity of the mAb to FERMT1. FERMT1 mAb showed reactivity for 293T cells transfected with a FERMT1 expression vector, whereas it did not react to 293T cells transfected with a FERMT2 or FERMT3 vector, as shown in western blot analysis (Figure 2A), indicating that the mAb against FERMT1 mAb is specific for FERMT1. Western blot analysis revealed positive FERMT1 protein expression in all five colon carcinoma lines tested (Figure 2B).

Further evaluation of FERMT1 protein expression in primary colon carcinoma tissues was performed. Six colon carcinoma primary tumor tissues exhibited higher levels of FERMT1 protein expression than those in adjacent normal colonic mucosa tissues (Figure 2C). Of note, stronger FERMT1 protein expression was detected in tissue from lymph node metastasis of case #1 than in primary colon carcinoma tissue and normal colonic mucosa of the same case.
Figure 3. Molecular function of FERMT1 in colon carcinoma cells. A: Western blotting using monoclonal antibody (mAb) to FLAG-tag. HCT116 cells were transfected with FREMT1, FERMT3, FERMT2 plasmids, and analyzed by western blot using mAb to FLAG-tag. β-Actin was used as an internal positive control. B: Western blotting using a monoclonal antibody (mAb) to FLAG-tag. SW480 cells were transfected with FREMT1, FERMT3, FERMT2 plasmids, and analyzed by western blot using a mAb to FLAG-tag. β-Actin was used as an internal positive control. C: Invasion assay of FERMT family-overexpressing HCT116 cells. Representative images of invasion assay using FERMT family cDNA-overexpressing HCT116 cells. Purple cells indicate HCT116 cells that have invaded through the Matrigel. D: Invasion assay of FERMT family-overexpressing HCT116 cells. Invading cells were counted in 10 high power fields (HPFs). Data represent means±SD. Differences between FERMT family-overexpressing HCT116 cells and mock-transfected HCT116 cells were examined for statistical significance using the Student’s t-test. *p=0.03, **p=0.001, ***p<0.0001. E: Invasion assay of FERMT family-overexpressing SW480 cells. Representative images of invasion assay using FERMT family cDNA-overexpressing SW480 cells. Purple cells indicate SW480 cells that have invaded through the Matrigel. F: Invasion assay of FERMT family-overexpressing SW480 cells. Invaded cells were counted in 10 HPF. Data represent means±SD. Differences between FERMT family-overexpressing SW480 cells and mock-transfected SW480 cells were examined for statistical significance using Student’s t-test. *p=0.04.
Discussion

During cancer progression, cells gain multiple abilities allowing them to become malignant cells. Malignant diseases are defined by invasion into adjacent organs and distant metastasis, and invasion is thus a prominent ability of malignant cells. In this study, we identified FERMT1 as a colon carcinoma-related gene by screening of a gene database. FERMT1 was reported to be overexpressed in lung carcinoma cells and colonic carcinoma cells (4). However, the molecular functions of FERMT1 in colonic carcinoma cells have not been elucidated. In another study, FERMT1 was shown to be overexpressed in lung metastasis of breast carcinoma (9). The same research group reported that FERMT1 has a role in epithelial mesenchymal transition through activation of transforming growth factor-β (TGFβ) signaling (6). However, the molecular functions of FERMT1 have remained elusive, and we therefore analyzed FERMT1 function in colon carcinoma cells.

FERMT1 has 80% homology with FERMT2 and 72% homology with FERMT3. The three molecules have similar domain structures (Figure 1B), suggesting similar molecular functions. However, the expression profiles of FERMT1, FERMT2 and FERMT3 in normal organ tissues exhibited significant differences, and only FERMT1 showed carcinoma cell-specific expression. In this study, we did not address the expression of FERMT1 in skin tissue; however, previous studies showed that FERMT1 is expressed in keratinocytes and that gene mutation in FERMT1 is related to Kindler syndrome (10–12). FERMT2 was shown to have invasion ability in MCF7 breast carcinoma cells (5). FERMT3 was reported to be expressed in leukocytes and to have a role in the activation of integrin signals (13, 14); however, there has been no report describing the relationship between FERMT3 and invasion. In our study, FERMT1, FERMT2 and FERMT3 were all shown to have roles in invasion, indicating that they may have similar functions. FERMT1 and FERMT2 have been reported to share some molecular functions in skin keratinocytes (15, 16). These observations indicate that FERMT1, FERMT2 and FERMT3 may have similar molecular functions and that the difference in expression defines the role of each molecule. Of note, FERMT1 is ectopically and specifically overexpressed in carcinoma cells and FERMT1 is thus the most suitable target for future cancer therapy.

In summary, to our knowledge this is the first report on FERMT1 functions in colon carcinoma cells. While FERMT1, FERMT2 and FERMT3 are expressed in colon carcinoma cells, only FERMT1 exhibits cancer cell-specific expression. FERMT1 also has a role in invasion and growth of colon carcinoma cells. The results indicate that FERMT1 is a possible target for cancer therapy.

Immunohistochemical staining of primary colon carcinoma tissues also revealed FERMT1 protein expression in carcinoma cells but not in normal epithelial cells (Figure 2D). The positive immunohistochemical staining rate of FERMT1 protein in colon carcinoma tissues was 95% (38 out of 40 cases).

**Role of FERMT1 in invasion and cell growth.** Since western blot analysis revealed a high level of FERMT1 protein expression in lymph node metastasis tissue, we hypothesized that FERMT1 is related to the invasion of colon carcinoma cells. In order to analyze the functions of FERMT genes, we established FERMT1-, FERMT2- and FERMT3-overexpressing HCT116 cells and SW480 cells. Protein expression of FERMT1, FERMT2 and FERMT3 was confirmed by western blot analysis, using an anti-FLAG antibody (Figure 3A and 3B). Invasion assays using Matrigel were performed, and FERMT1-overexpressing HCT116 cells exhibited greater invasive ability than mock vector-transformed HCT116 cells (p<0.001) (Figure 3C and 3D). FERMT1-overexpressing SW480 cells also exhibited greater invasive ability than did mock-transfected SW480 cells (Figure 3E and 3F). FERMT2 and FERMT3 had the ability to enhance the invasion of HCT116 cells, whereas they had no effect on SW480 cells. Cell growth ability was evaluated by a cell growth assay. FERMT1-, FERMT2- and FERMT3-overexpressing HCT116 cells showed greater growth in vitro than non-transfected cells, indicating that FERMT1, FERMT2 and FERMT3 have roles in cell growth (Figure 4).

![Cell growth of FERMT family-overexpressing HCT116 cells.](image-url)
Declaration of Financial Disclosure

Hideo Takasu is an employee of Dainippon Sumitomo Pharma Co., Ltd.

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