determining the statistical significance of correlations between marker expression and histological chemotherapeutic effects. P values <0.05 were considered statistically significant.

Results

Patients

From January 2007 to September 2011, 45 patients with locally advanced gastric cancer were enrolled in the study. Two patients did not start chemotherapy for the following reasons: reassessment as inoperable ($n = 1$), and patient request ($n = 1$). Thus, 43 patients were eligible and received chemotherapy. A flow diagram from chemotherapy to surgery is shown in Fig. 1. Patient characteristics are summarized in Table 1. The subjects included 32 men and 11 women, with a median age of 65 years (range 31–78 years). Most of these patients were in good general condition (74.4 % with a performance status of 0). Histologically, 17 (39.5 %) patients had well-differentiated adenocarcinomas and 26 (60.5 %) had undifferentiated adenocarcinomas. On baseline EUS and CT, 7 patients (16.3 %) had T4 tumors and 39 patients (90.7 %) had N+ disease including N2 bulky mass (6.9 %, 3/43) or N3 paraaortic nodes metastases (9.3 %, 4/43).

Preoperative chemotherapy

Forty-three patients were administered a total of 108 courses, with a median of 2 courses (range 1–4). While all patients received course 1, 2 of them did not receive course 2 of preoperative chemotherapy but underwent surgery

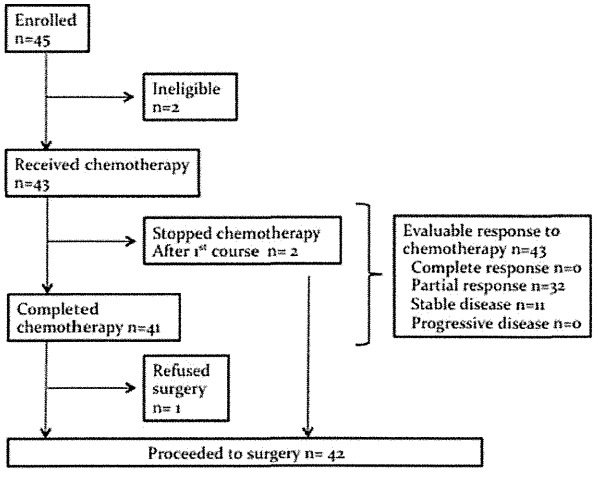

Fig. 1 Trial profile

Table 1 Patient characteristics at baseline ($N = 43$)

Characteristics	No. of patients	%
Age, years		
Median	65	
Range	31–78	
Sex		
Male	32	74.4
Female	11	25.6
Performance status		
0	32	74.4
1	11	25.6
Histology		
Intestinal type	17	39.5
Diffuse type	26	60.5
T stage (JGCA)		
T2	7	16.3
T3	29	67.4
T4	7	16.3
N stage (JGCA)		
N0	4	9.3
N1	8	18.6
N2	27	62.8
N3	4	9.3
Stage		
II	2	4.7
IIIA	15	34.9
IIIB	20	46.5
IV	6	13.9

(Fig. 1), due to physician's impression of poor tolerance and patient refusal (1 patient each). Thus, the completion rate of 2 courses was 95.3 % (41/43), and in the second course, 90 % delivery of the planned dose was achieved for S-1, docetaxel, and CDDP. Among patients who responded to treatment and were deemed by their physician after course 2 to be able to tolerate subsequent courses, 18 patients received a third course and 6 patients received a fourth course. A treatment delay of 7 or more days was noted in 13 of the courses. The clinical response rate (complete response + partial response) was 74.4 % (95 % CI, 61.4–87.4 %), and no patient had disease progression during pre-operative chemotherapy period. The incidence of hematological grade 4 adverse events was as follows: leukocytopenia, 37.2 %; neutropenia, 53.5 %; anemia, 2.3 %; febrile neutropenia, 2.3 %. Non-hematological grade 3 or higher adverse events were anorexia, 23.3 %; nausea, 14.0 %; vomiting, 7.0 %; and diarrhea, 23.3 % (Table 2). There were no chemotherapy-related deaths. All treatment-related toxicities resolved with appropriate care, and no treatment-related deaths were observed.

Table 2 Adverse events occurring during chemotherapy

Toxicity (NCI–CTC)	No. of patients (%)			
	Grade			
	1	2	3	4
Hematologic				
Leucopenia	1 (2.3)	1 (2.3)	18 (41.9)	16 (37.2)
Neutropenia	1 (2.3)	1 (2.3)	13 (30.2)	23 (53.5)
Anemia	4 (9.3)	8 (18.6)	2 (4.7)	1 (2.3)
Febrile neutropenia	–	–	6 (14.0)	1 (2.3)
Thrombocytopenia	5 (11.6)	4 (9.3)	1 (2.3)	0 (0.0)
Nonhemotological				
Anorexia	4 (9.3)	15 (34.9)	10 (23.3)	0 (0.0)
Nausea	5 (11.6)	9 (20.9)	6 (14.0)	0 (0.0)
Vomiting	4 (9.3)	3 (7.0)	3 (7.0)	0 (0.0)
Diarrhea	4 (9.3)	1 (2.3)	10 (23.3)	0 (0.0)
Stomatitis	6 (14.0)	4 (9.3)	1 (2.3)	0 (0.0)
Fatigue	8 (18.6)	6 (14.0)	0 (0.0)	0 (0.0)
AST/ALT elevation	3 (7.0)	3 (7.0)	0 (0.0)	0 (0.0)
Creatinine elevation	3 (7.0)	3 (7.0)	0 (0.0)	0 (0.0)

Surgical findings and surgical pathology

A total of 42 patients proceeded to surgery (Fig. 1; Table 3). Resection with curative intent was undertaken in only 41 patients because 1 patient underwent only gastrojejunostomy due to *localized peritoneal* metastases and *pancreatic invasion*. Of the 41 patients who had resection with curative intent, R0 resection was performed in 39, R1 in 1 (positive microscopic margin), and R2 in 1 with unresectable peritoneal metastases. Thus, the proportion of R0 resections in the 43 eligible patients was 90.7 % (95 % CI, 82.0–99.4 %). Among the 41 resected patients, 25 had D2 lymph node dissection, and the remaining 16 had D3 lymph node dissection. Postoperative complications were observed in 9 patients (21.4 %). The actual complications were as follows: delayed gastric emptying, wound infection, deep vein thrombosis, abdominal abscess, abdominal fluid collection, and ileus. Overall, there was no mortality and there were no serious complications. Of the 41 operated patients for whom data regarding surgical pathologic staging were available, 25 patients (61.0 %) had a decrease of at least 1 level in their T stage and 6 patients (15.4 %) with N+ disease had post-treatment N0 disease. Overall, gastric tumors were down staged in 28 patients (68.3 %), unchanged in 9 patients (21.9 %), and upstaged in 4 patients (9.8 %).

Survival analysis

The median follow-up time was 30.8 months. At the time of the analyses (April 1, 2012), 41 patients (95.3 %) were still alive; The median PFS and MST were not reached, the 3-year PFS was 85.9 % (95 % CI, 75.5–96.3), and the 3-year OS was 89.7 % (95 % CI, 80.6–98.8) (Fig. 2).

Table 3 Surgical and pathologic results

	No. of patients	%
Surgery results		
All	42	100
Types of surgery		
Total gastrectomy	34	80.9
Distal gastrectomy	7	16.7
Bypass surgery	1	2.4
Lymph node dissection		
No dissection	1	2.4
D2	25	59.5
D3	16	38.1
Extent of resection		
No resection	1	2.4
R0	39	92.8
R1	1	2.4
R2	1	2.4
Pathology results		
All	41	100
T stage (JGCA)		
T0	2	4.9
T1	4	9.8
T2	11	26.8
T3	21	51.2
T4	3	7.3
N stage (JGCA)		
N0	10	21.1
N1	12	31.6
N2	14	36.8
N3	5	10.5
M status (JGCA)		
M0	39	94.7
M1	2	5.3

JGCA Japanese Gastric Cancer Association

Chemotherapeutic effects and DDB2/ERCC1 expression in pretreatment biopsy specimens from gastric cancer patients treated with neoadjuvant DCS regimen

Excision repair cross-complementing 1 (ERCC1) is a key enzyme in the nucleotide excision repair (NER) pathway, and its expression is reported to be a useful predictor of the clinical outcome of advanced gastric cancer patients treated with platinum-based chemotherapy [17, 21, 22]. On the other hand, damage DNA binding protein complex subunit 2 (DDB2) was found to serve as the initial damage

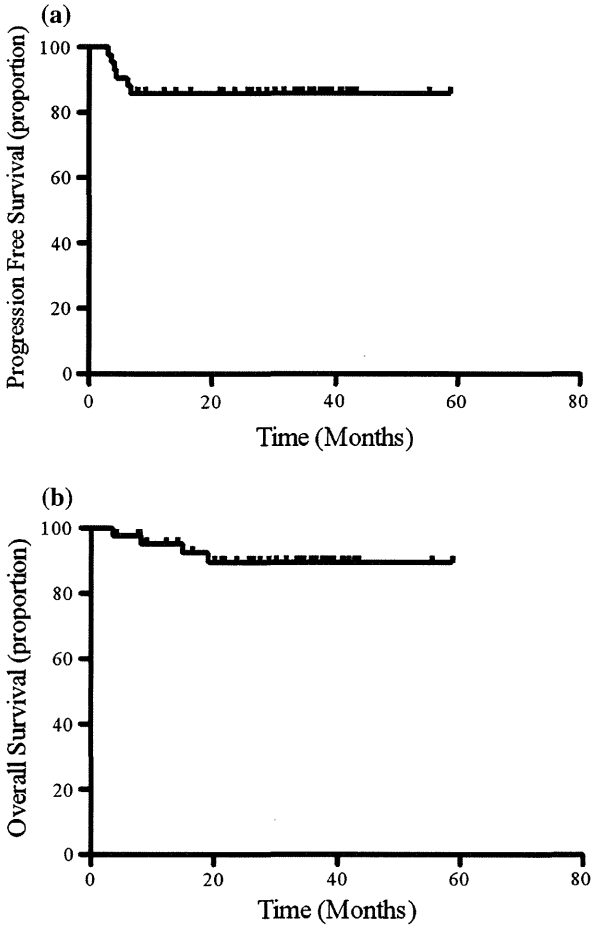

Fig. 2 Kaplan–Meier curves for **a** progression-free survival and **b** overall survival

recognition factor during NER, and we reported that loss of DDB2 repair function contribute to cancer susceptibility and cellular sensitivity to DNA damage [23]. We therefore examined the possible correlation between anti-tumor effect of DCS therapy (pathological response) and the expression of DDB2 and/or ERCC1 in pretreated tumor tissues by immunohistochemical staining. Table 4 shows the relationship between chemotherapeutic effects and marker expression in the pretreatment biopsy specimens. Histological chemotherapeutic responders consisted of 27 (65.9 %) out of 41 resected cases; grade 1b, 10 patients (24.4 %), grade 2, 15 patients (36.6 %); and grade 3, 2 patients (4.9 %). The remaining 14 patients (34.1 %) were categorized as nonresponders; grade 0, 1 patient (2.4 %); and grade 1a, 13 patients (31.7 %).

Adequate biopsy material was available in 40 out of the 43 cases prior to receiving neoadjuvant chemotherapy. High DDB2 expression was observed in 4 lesions (14.8 %) of 27 responders and in 8 lesions (61.5 %) of 13 nonresponders: statistical significance was noted between responders and nonresponders ($P = 0.0065$). The accuracy of DDB2 expression for predicting chemoresistance was 77.5 %; that is, 31 (23 responders and 8 nonresponders) out of 40 patients treated with neoadjuvant chemotherapy.

High expression of ERCC1 was observed in 8 lesions (61.5 %) of the nonresponders and in 5 lesions (18.5 %) of the responders: there was a significant difference between responders and nonresponders ($P = 0.029$). The accuracy of ERCC1 expression for predicting chemoresistance was 75.0 %; that is, 30 (22 responders and 8 nonresponders) out of 40 patients treated with neoadjuvant chemotherapy.

The DDB2- and/or ERCC1-high phenotype was observed in 13 lesions (100 %) of the nonresponders and in 7 lesions (25.9 %) of the responders: The difference between responders and nonresponders was statistically significant ($P < 0.0001$). The accuracy of the combination of DDB2 and ERCC1 expression for predicting chemoresistance was 82.5 %; that is, 33 (20 responders and 13 nonresponders) out of 40 patients treated with neoadjuvant chemotherapy.

Discussion

New chemotherapeutic regimens for advanced gastric cancer including taxanes, oral pyrimidine, and CPT-11

Table 4 Relationship between expression of DDB2 and ERCC1, and effects of neoadjuvant DCS chemotherapy in pretreatment biopsy specimens

Marker expression	Pathological responders (%) $N = 27$[a]	Pathological nonresponders (%) $N = 13$[a]	P value	Accuracy (%)
DDB2				
Positive	4 (14.8)	8 (61.5)	0.0065	77.5
Negative	23 (84)	5 (33)		
ERCC1				
Positive	5 (18.5)	8 (61.5)	0.029	75.0
Negative	22 (81.5)	5 (42)		
DDB2 and/or ERCC1-positive	7 (25.9)	13 (100)	<0.0001	82.5
DDB2 and ERCC1-negative	20 (72)	0 (0)		

[a] Pretreatment biopsy specimens were available for analysis in 40 out of 43 patients with neoadjuvant chemotherapy

have been developed and have proven to be highly effective [7]. Consequently, neoadjuvant chemotherapy using these new drugs is expected to improve the prognosis of advanced gastric cancer. We therefore evaluated the efficacy of a triple regimen including docetaxel, S-1, and CDDP (DCS) employed as neoadjuvant chemotherapy in patients with clinically resectable locally advanced gastric cancer. With this regimen, we achieved a high R0 resection rate, as expected, without an increase of operative morbidity and operative mortality in patients with relatively high-risk backgrounds.

It is generally assumed that low resectability is responsible for the poor prognosis of advanced gastric cancer patients. A number of clinical trials have shown that preoperative chemotherapy is feasible and able to increase the rate of R0 resection [24]. The response rate in previous neoadjuvant chemotherapy trials showed modest to moderate activity (40–60 % response rate) and R0 resection rates up to 83 % [25, 26]. Accordingly, there is a need to improve the response rate to achieve a further increase in R0 resection rates with treatment for advanced gastric cancer.

The high activity of the DCS combination (ORR 74.4 %; 95 % CI, 61.4–87.4 %, disease control rate; 100 %) in this study is in accordance with our previous trial for first-line treatment in unresectable metastatic gastric cancer [11, 12] and compares favorably with other active chemotherapy regimens reported in this setting [19, 25, 26]. This indicates that the DCS regimen may be an effective treatment option in the neoadjuvant setting, where high anti-tumor activity resulting in a high down-staging rate, and no progressive disease cases are required. In fact, downstaging was observed in 68.3 % of patients, and the R0 resection rate achieved in the present study (39/43, 90.7 %; 95 % CI, 82.0–99.4 %) was among the highest R0 rates reported [26]. It may not be justified to simply compare our results with those of other studies, since R0 resection rates are influenced by the patients' backgrounds and the operational definition of unresectability. Our patients' backgrounds were, however, relatively dominated by marginally resectable gastric cancers: para-aortic nodal metastases were seen in 9.3 % and bulky N2 in 6.9 % of the cases. Para-aortic lymph node (JGCA-N3) enlargement is regarded as unresectable distant metastases (M1) in the UICC TNM staging system, and usually patients with JGCA-bulky N2 rarely survive for more than 3 years when treated by chemotherapy alone or by surgery followed by postoperative chemotherapy [19, 27].

In this study, the R0 resection rate was nevertheless as high as 90.7 % (100 % in N3 and 67 % in T4 cases). Therefore, preoperative DCS chemotherapy might strongly promote tumor regression, eradicate nodal or possible peritoneal metastases, and improve resectability in patients with marginally resectable gastric cancer.

This regimen's effectiveness was also indicated by the fact that the pathological response rate was as high as 65.9 %. Although similar criteria for histopathological regression have been used in several studies, these criteria are not standardized and may be investigator dependent. Several studies of neoadjuvant chemotherapy employing the same Japanese criteria that were used in the present study reported pathological response rates of 51 and 48 % for the JCOG0405 [28] and JCOG0210 [29] trials, respectively, using the S-1/CDDP regimen, and 15 % for the JCOG0001 trial using the CDDP/CPT-11 regimen [19]. Hence, DCS neoadjuvant chemotherapy showed a much better therapeutic effect than other CDDP-based regimens.

There is a correlation between increased pathologic response to therapy and survival in retrospective studies [30]. Therefore, our regimen, which induced a high pathological response rate, is expected to bring about a good prognosis. Despite a short follow-up period, the 3-year OS of 89.7 % and 3-year PFS of 85.9 % in this study are also encouraging.

The degree of toxicity of neoadjuvant chemotherapy is a critical problem because of its potential adverse effects on operative morbidity and operative mortality. Like other docetaxel-containing triple regimens in which hematological toxicity was the major adverse effect [8], the DCS regimen was associated with a high incidence of severe neutropenia, which occurred in 53.5 % of patients in the neoadjuvant setting.However, febrile neutropenia occurred in only 16.3 % (grade 4; 2.3 %) of patients; all of these cases were transient and manageable with G-CSF administration and had dose reductions that prevented the recurrence of toxicity. Obviously, DCS treatment necessitates careful observation of these toxicity patterns to prevent treatment-associated toxicities. In fact, in our trial, 95.3 % of patients were able to receive the 2 planned courses of preoperative chemotherapy. Moreover, there was no increase in operative morbidity and no operative mortality as compared with patients who underwent identical surgery for gastric cancer at our institution during the same time period but who did not receive preoperative therapy.

Resistance to chemotherapy would be a serious problem in the successful treatment of gastric cancers especially in a neoadjuvant setting. In particular, for those patients who had achieved little or no response to preoperative chemotherapy, the use of alternative forms of adjuvant therapy could be considered to improve outcomes. Therefore, to identify chemoresistance markers, we focused on key DNA repair and damage signaling factors, since the antitumor activity of platinum-based chemotherapy is largely dependent on the DNA repair capacity of cancer cells. We showed that nuclear expression of ERCC1 is significantly associated with resistance to chemotherapy, consistent with

reports of other CDDP-based regimen such as 5-FU/oxaliplatin [17], ECF/ECX chemotherapy [21], and CDDP/S-1 or irinotecan [22]. In addition, we have provided the first evidence that DDB2, also as important NER factor, protein expression in pretreatment biopsy specimens is predictive of gastric cancer chemosensitivity. Moreover, we have shown that the accuracy for predicting chemoresistance to DCS was 82.5 % when DDB2 expression was combined with ERCC1 expression, whereas the predictive accuracy was only 77.5 % for DDB2 expression and 75.0 % for ERCC1 expression. These results indicate that the DDB2- and/or ERCC1-high phenotype as determined, by immunohistochemistry, is a strong predictor of resistance to DCS chemotherapy.

In summary, the results of the current study indicate that the DCS regimen is feasible and highly effective as neoadjuvant chemotherapy for locally advanced gastric cancer patients. These results warrant further large-scale investigation of the DCS regimen in a neoadjuvant setting especially for the treatment of marginally resectable gastric cancer.

Conflict of interest The authors have no conflict of interest.

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ANTICANCER RESEARCH *33*: 167-174 (2013)

Expression and Function of *FERMT* Genes in Colon Carcinoma Cells

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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, we established FERMT1-, FERMT2- and FERMT3-overexpressing colon carcinoma cells. FERMT1-overexpressing cells exhibited greater invasive ability than did FERMT2- and FERMT3-overexpressing cells. On the other hand, FERMT1-, FERMT2- and FERMT3-overexpressing cells exhibited enhancement of cell growth. Taken together, the results of this study indicate that FERMT1 is expressed specifically in colon carcinoma cells, and has roles in matrix invasion and cell growth. These findings indicate that FERMT1 is a potential molecular target for cancer therapy.*

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Key Words: Colon carcinoma, invasion, *FERMT1*, DNA microarray, fermitin family.

Colon carcinoma is a major malignancy, with a high mortality rate. In the process of tumorigenesis, tumor cells undergo multiple steps of genetic events (1), and multiple steps are also required for the cells to obtain several different phenotypes. Tissue invasion and metastasis are hallmarks that distinguish malignant from benign diseases (2). Several classes of proteins are involved in the process of tissue invasion; however, the exact molecular mechanisms of invasion remain unclear.

Fermitin family member (*FERMT*) genes include *FERMT1*, *FERMT2* and *FERMT3*, and these genes have been reported to be mammalian homologs of the *Caenorhabditis elegans* gene (3,4). The *unc-112* gene mutant had a phenotype similar to that of *unc-52* (perlecan), *pat-2* (α-integrin) and *pat-3* (β-integrin) mutants, and *unc-112* has been described as a novel matrix-associated protein (3). In subsequent studies, *FERMT2* was found to be related to invasion in MCF-7 breast carcinoma cells (5). *FERMT1* has been reported to be overexpressed in lung carcinoma cells and colon carcinoma cells (4), and has been reported to be related to invasion of breast carcinoma cells (6). However, the molecular functions of *FERMT1* in colon carcinoma cells remain elusive.

In this study, we screened a gene expression database of carcinoma tissues to analyze the molecular mechanisms of colon carcinoma, and we isolated *FERMT1* as a gene overexpressed in colon carcinoma tissues. We then analyzed the molecular functions of *FERMT* genes in colon carcinoma cells.

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

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'-GTCTGCTGAAACACAGGATTT-3' and 5'-GTTTTTCTAGTGGTTCTCCTT-3' for *FERMT1*, with an expected PCR product size of 272 base pairs (bps); 5'-CATGACATCAGAGAATCATTT-3' and 5'-ACTGGATTCTTCTTT GCTCTT-3' for *FERMT2*, with an expected PCR product size of 256 bps; 5'-AAAGTTCAAGGCCAAGCAGCT-3' and 5'-TGAAGGCCA CATTGATGTGTT-3' for *FERMT3* with an expected PCR product size of 326 bps; and 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3' for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) with an expected 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'-CGGGGTACCATGCTGTCATCC ACTGACTTT-3' as a forward primer and 5'-CCGCTCGAGATCCTG ACCGCCGGTCAATTT-3' as a reverse primer (underlines indicating *KpnI* and *XhoI* recognition sites, respectively) for *FERMT1*, 5'-CGGGGTACCGCCACCATGGCTCTGGACGGGATAAGG-3' as a forward primer and 5'-CCGCTCGAGCACCCAACCACTGGTA AGTTT-3' as a reverse primer for *FERMT2*, and 5'-CGGGGTACC GCCACCATGGCGGGGATGAAGACAGCC-3' as a forward primer and 5'-CCGCTCGAGGAAGGCCTCATGGCCCCCGGT-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 construct of protein expression, a *BglII* and *XhoI*-digested deletion mutant of *FERMT1* cDNA that was amplified by PCR using the primer pair 5'-GAAGATCTATGCT GTCATCCACTGACTTT-3' and 5'-CCGCTCGAGATCCTGACCGC CGGTCAATTT-3' (underlines indicating *BglII* and *XhoI* recognition sites, respectively) was inserted into a *BamHI* and *XhoI*-digested pQE30 (Qiagen Japan, Tokyo, Japan) vector.

FERMT1 recombinant protein production and establishment of a monoclonal antibody (mAb). A pQE30-*FERMT1* deletion mutant construct was transformed into Escherichia coli strain M15 (Qiagane Japan, Tokyo, Japan), and His6 tag-fused FERMT1 protein was induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h at 30°C. Cells were lysed in lysis buffer [6 M guanidine hydrochloride, 20 mM HEPES (pH 8.0), 50 mM NaCl], and recombinant FERMT1 protein was purified using Ni-NTA resin (Qiagen Japan).

The FERMT1 recombinant protein (100 μg) was used for immunization of BALB/c mice (CHARLES RIVER LABORATORIES JAPAN, INC., Yokohama, Japan) by intraperitoneal (*i.p.*) injection four times at two-week intervals. One week after the last injection, splenic cells were collected and fused with the NS-1 mouse myeloma cell line (ATCC, Manassas, VA, USA) at a 4:1 ratio. FERMT1 protein-specific hybrydomas were screened with enzyme-linked immunosorbent assay (ELISA) and western blotting using recombinant FERMT1 protein.

Immunohistochemical staining and western blotting. Immunohistochemical staining was performed with a colon carcinoma tissue microarray established from formalin-fixed surgically-resected tumor specimens of colon carcinoma at Sapporo the Medical University Hospital, as described previously (8). Anti-FERMT1 antibody was used at a 10-fold dilution with the anti-FERMT1-specific hybridoma culture supernatant. Western blotting of colon carcinoma tissues and colon carcinoma cells was performed as described previously (8). Anti-FERMT1 antibody was used at a 10-fold dilution with hybridoma culture supernatant.

Matrigel invasion assay. BD BioCoat Matrigel Invasion Chambers (Discovery Labware, Bedford, MA, USA) and polyethylene terephthalate (PET) track-etched membranes with pore sizes of 8.0 μm (Becton Dickinson, San Diego, CA, USA) were used for the invasion assay, according to the protocol of the manufacturer. HCT116- and SW480-transformant cells (2.5×10^4 cells/500 ml) were plated in the top chamber in DMEM, and culture medium with 10% FBS was used in the bottom chamber as a chemoattractant. Twenty-four hours later, cells were fixed and stained using a HEMA 3 STAT Pack (Fisher Scientific Japan, Tokyo, Japan). Cell numbers were counted on microphotographs taken in ten areas of the membrane.

Statistical analysis. In cell growth assays and invasion assays, samples were analyzed using Student's *t*-test, with $p<0.05$ conferring statistical significance.

Results

Isolation of the colon carcinoma-related gene FERMT1. We screened a gene expression database of approximately 700 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 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

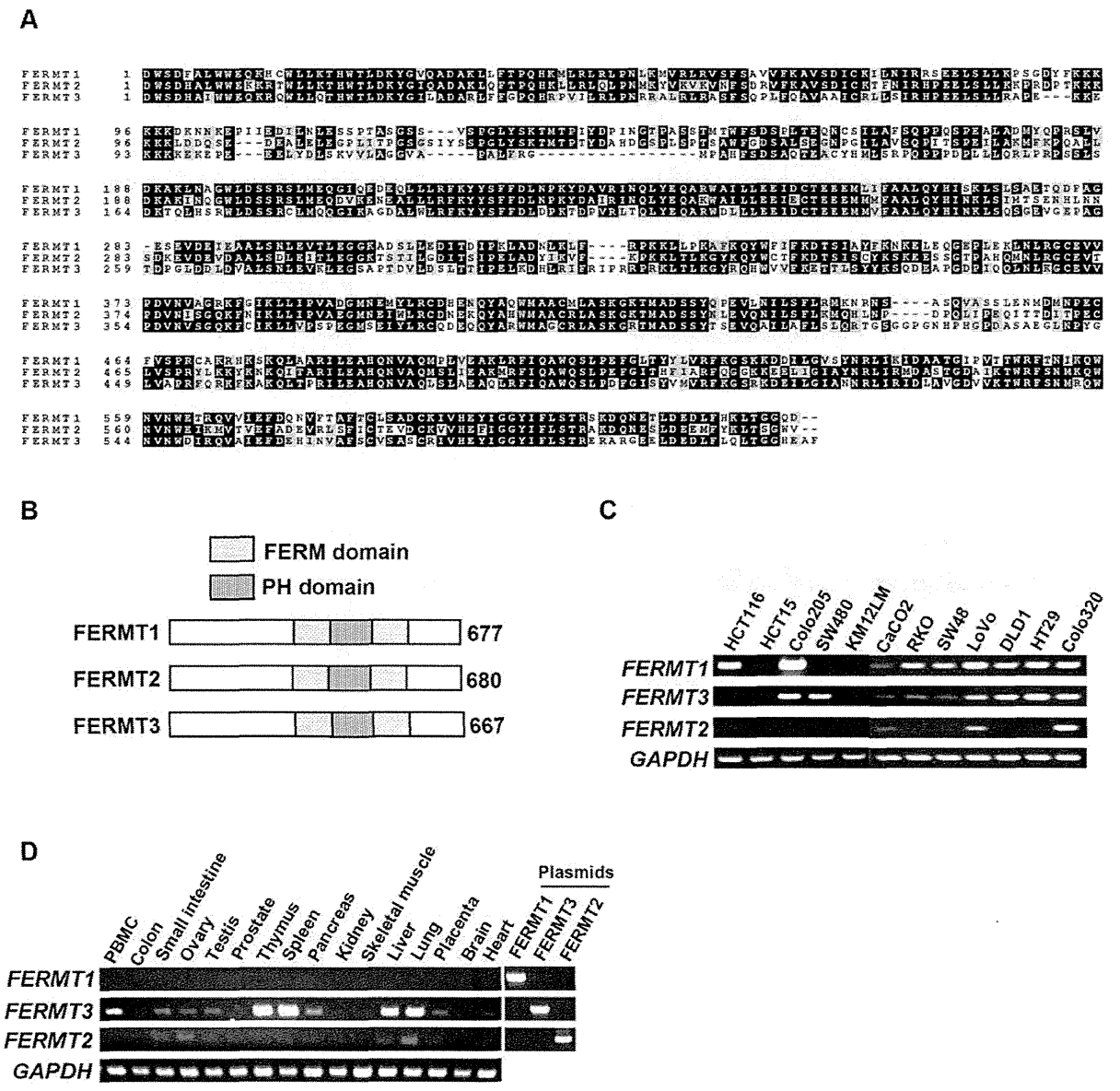

Figure 1. *Expression profiles of fermitin family member (FERMT) family genes. A: Sequence alignment of FERMT proteins. FERMT1, FERMT2 and FERMT3 amino acid sequences are shown. A black box indicates the same alignment, a gray box indicates similar alignment. B: Molecular structure of FERMT family proteins. 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.*

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

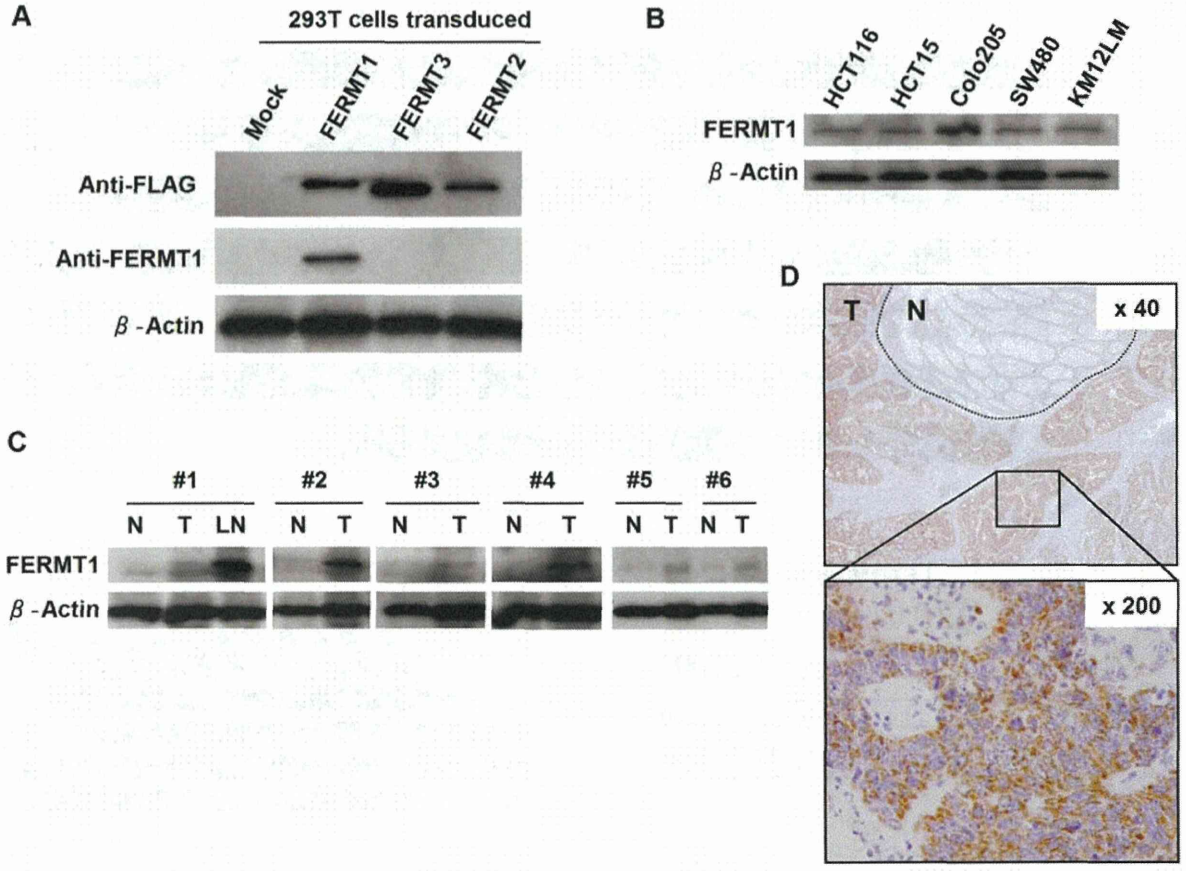

Figure 2. *Fermitin family member 1 (FERMT1) protein expression in colonic carcinomas. A: Western blotting using monoclonal antibody (mAb) against FERMT1. 293T cells were transfected with FERMT1, FERMT2 and FERMT3 plasmids. Western blotting using anti-FLAG mAb and anti-FERMT1 mAb was performed. Anti-FLAG mAb was used as a positive control. β-Actin was used as an internal positive control. B: Western blotting of colonic carcinoma cells. Western blotting using anti-FERMT1 mAb was performed. β-Actin was used as an internal positive control. C: Western blot of colon carcinoma tissues. Protein expression of FERMT1 in primary human colonic carcinoma cases (#1-#6) was evaluated by western blotting using an anti-FERMT1 mAb. T, Tumoral part of colonic carcinoma tissue; N, adjacent normal colonic mucosa tissue; LN, lymph node metastatic tissue of the corresponding case. β-Actin was used as an internal positive control. D: Immunohistochemical staining of FERMT1. Representative images of immunohistochemical staining of colonic carcinoma tissues using anti-FERMT1 mAb are shown. Brown indicates positive staining. Dotted line indicates normal colonic mucosa cells. N, Normal colon mucosa tissue; T, colonic carcinoma tissue.*

(Figure 1C). *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 colonic tumor tissue and normal colonic mucosa of the same case.

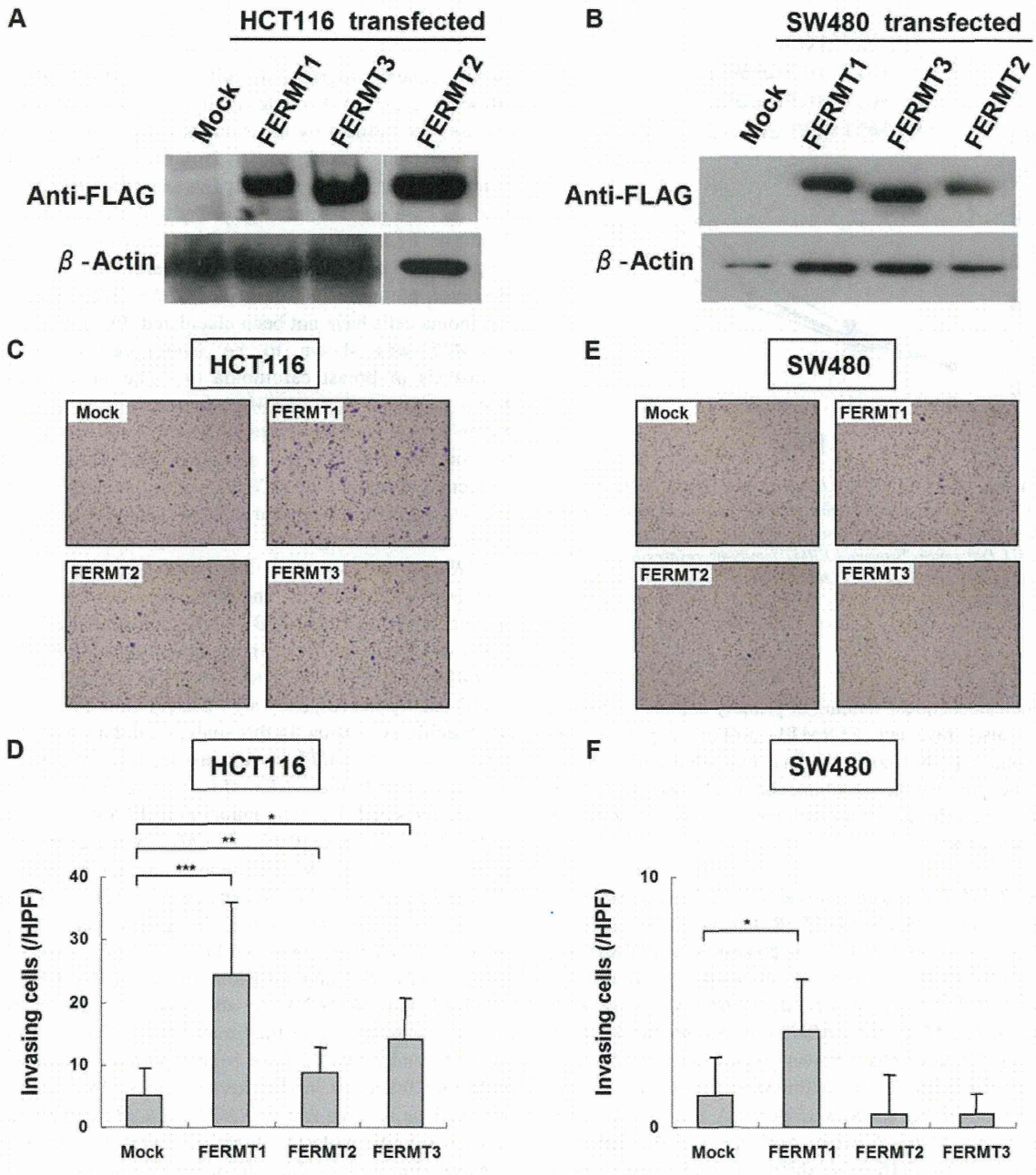

Figure 3. *Molecular function of FERMT1 in colon carcinoma cells. A: Western blotting using monoclonal antibody (mAb) to FLAG-tag. HCT116 cells were transfected with FREMT1, FERMT3, FERMT2 plasmids, and analyzed by western blot using mAb to FLAG-tag. β-Actin was used as an internal positive control. 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.*

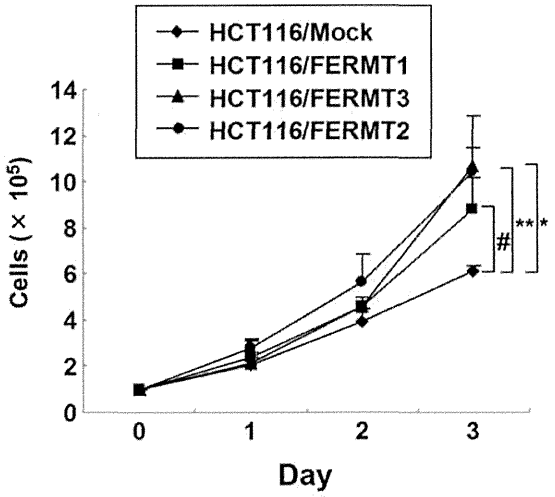

Figure 4. *Cell growth of FERMT famliy-overexpressing HCT116 cells. FERMT family cDNA-overexpressing HCT116 cells were seeded in a 6-well plate, and the cell growth rate was recorded daily. Data represent means±SD. Differences between FERMT family-overexpressing HCT116 cells and mock-transfected HCT116 cells were examined for statistical significance using Student's t-test. *p=0.015, #p=0.012, **p=0.001.*

Immunohistochemical staining of primary colonic 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 colonic 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).

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 skin 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 *FRMT3* 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* exhibites cancer cell-specific expression. FERMT1 also has a role in invasion and growth of colonic carcinoma cells. The results indicate that *FERMT1* is a possible target for cancer therapy.

Declaration of Financial Disclosure

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

Acknowledgements

The Authors are grateful to Drs K. Imai and K. Itoh for kindly providing cell lines, and to Dr. T. Kitamura for kindly providing a retrovirus system. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant no. 16209013, 17016061 and 15659097) for Practical Application Research from the Japan Science and Technology Agency, and for Cancer Research (15-17 and 19-14) from the Ministry of Health, Labor and Welfare of Japan, Ono Cancer Research Fund (to N. S.) and Takeda Science Foundation (to Y. H.). This work was supported in part by the National Cancer Center Research and Development Fund (23-A-44).

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Received October 29, 2012
Revised November 12, 2012
Accepted November 13, 2012

Cancer Science
The official journal of the Japanese Cancer Association

Immunotherapeutic benefit of α-interferon (IFNα) in survivin2B-derived peptide vaccination for advanced pancreatic cancer patients

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(Received July 3, 2012/Revised October 4, 2012/Accepted October 10, 2012/Accepted manuscript online October 19, 2012/Article first published online December 16, 2012)

Survivin, a member of the inhibitor of apoptosis protein (IAP) family containing a single baculovirus IAP repeat domain, is highly expressed in cancerous tissues but not in normal counterparts. Our group identified an HLA-A24-restricted antigenic peptide, survivin-2B80–88 (AYACNTSTL), that is recognized by CD8 + CTLs and functions as an immunogenic molecule in patients with cancers of various histological origins such as colon, breast, lung, oral, and urogenital malignancies. Subsequent clinical trials with this epitope peptide alone resulted in clinical and immunological responses. However, these were not strong enough for routine clinical use as a therapeutic cancer vaccine, and our previous study of colon cancer patients indicated that treatment with a vaccination protocol of survivin-2B80–88 plus incomplete Freund's adjuvant (IFA) and α-interferon (IFNα) conferred overt clinical improvement and enhanced the immunological responses of patients. In the current study, we further investigated whether this vaccination protocol could efficiently provide not only improved immune responses but also better clinical outcomes for advanced pancreatic cancers. Tetramer and enzyme-linked immunosorbent spot analysis data indicated that more than 50% of the patients had positive clinical and immunological responses. In contrast, assessment of treatment with IFNα only to another group of cancer patients resulted in no obvious increase in the frequency of survivin-2B80-88 peptide-specific CTLs. Taken together, our data clearly indicate that a vaccination protocol of survivin-2B80-88 plus IFA and IFNα is very effective and useful in immunotherapy for this type of poor-prognosis neoplasm. This trial was registered with the UMIN Clinical Trials Registry, no. UMIN000000905. (*Cancer Sci* 2013; 104: 124–129)

Recent progress in human tumor immunology research has presented us with the possibility that immunotherapy could be established as an effective cancer therapy in the very near future.[1–6] Indeed, since the first discovery of a human tumor antigen in 1992,[7] many clinical trials for cancer vaccines have been carried out, and these studies have suggested that active immunization using HLA class I restricted tumor antigenic peptides and the whole or part of the tumor antigenic protein could work as activators of antigen-specific CTLs, at least in some cancer patients.[8–16] However, even in effective cases, vaccination with these molecules alone is not sufficient to evoke a potent and stable immune response and subsequent strong clinical effect. Thus, it is crucial to develop various methods for enhancing the immunological efficacy of tumor antigens.

We have studied how tumor antigenicity can be efficiently enhanced in cancer patients since 2003. In our studies, the HLA-A24-restricted peptide survivin-2B80-88 was given s.c. to patients six times or more at biweekly intervals for colon, breast, lung, oral cavity, and urinary bladder cancers, and lymphomas. Clinically, certain patients with colon, lung, and urinary bladder cancers showed reductions in tumor markers and growth arrest as assessed by computed tomography (CT).[8–12] These effects, however, were not strong enough for the clinical requirements as decided by the criteria for cancer chemotherapy. When assessed with the Response Evaluation Criteria in Solid Tumors, which requires more than 30% regression of tumors on CT, only one patient each of 15 with colon cancers and three with urinary bladder cancers had a positive clinical response, indicating that the therapeutic potential was obviously not strong enough for routine clinical use as a cancer treatment.

In a previous study,[8] to determine if the immunogenicity of the survivin-2B80-88 peptide could be enhanced with other vaccination protocols, we carried out and compared clinical trials in advanced colon cancer patients with two vaccination protocols: (i) survivin-2B80-88 plus incomplete Freund's adjuvant (IFA); and (ii) survivin-2B80-88 plus IFA and a type-I interferon (IFN), IFNα. Our data clearly indicated that, although the effect of survivin-2B80-88 plus IFA was not significantly different from that with survivin-2B80-88 alone, treatment with survivin-2B80-88 plus IFA and IFNα resulted in clear clinical improvement and enhanced the immunological responses of patients. We also analyzed CTLs of these patients by single-cell sorting, and found that each CTL clone from vaccinated patients was indeed not only peptide-specific but also cytotoxic against human cancer cells in the context of the expression of both HLA-A24 and survivin molecules.

Pancreatic cancer is still one of most difficult malignant neoplasms to treat, so in the current study we investigated whether the most effective protocol for colon cancer patients, namely survivin-2B80-88 plus IFA and IFNα, could work similarly in pancreatic cancers as in colon cancers. Furthermore, we carried out frequency monitoring of survivin-2B80-88 peptide-specific CTL in cases of cancer patients treated with IFNα alone, and found no overt increase of these CTLs. Once the survivin-2B80-88 peptide was administered with IFNα, patients showed strong clinical and immunological responses as assessed by tetramer and enzyme-linked immunosorbent spot (ELISPOT) analyses. Taken together, our current data strongly suggest that vaccination using survivin-2B80-88 plus IFA and IFNα is actually very effective in patients with advanced pancreatic cancers from both the clinical and immunological points of view.

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doi: 10.1111/cas.12046

Materials and Methods

Patients. Patient selection was done as reported in our previously published work. The study protocol was approved by the Clinic Institutional Ethical Review Board of the Medical Institute of Bioregulation, Sapporo Medical University (Sapporo, Japan).[8–12] All patients gave informed consent before being enrolled. Patients who participated in this study were required to: (i) have histologically confirmed pancreatic cancer; (ii) be HLA-A*2402 positive; (iii) have survivin-positive carcinomatous lesions by immunohistochemistry; (iv) be between 20 and 85 years old; (v) have unresectable advanced cancer or recurrent cancer; and (vi) have Eastern Cooperative Oncology Group performance status between 0 and 2. Exclusion criteria included: (i) prior cancer therapy such as chemotherapy, radiation therapy, steroid therapy, or other immunotherapy within the past 4 weeks; (ii) the presence of other cancers that might influence the prognosis; (iii) immunodeficiency or a history of splenectomy; (iv) severe cardiac insufficiency, acute infection, or hematopoietic failure; (v) use of anticoagulants; and (vi) unsuitability for the trial based on clinical judgment. This study was carried out at the Department of Surgery, in the Sapporo Medical University Primary Hospital from December 2005 through to November 2010.

Peptide, IFA, and IFNα preparation. The peptide, survivin-2B80-88 with the sequence AYACNTSTL, was prepared under good manufacturing practice conditions by Multiple Peptide Systems (San Diego, CA, USA).[8–10,12] The identity of the peptide was confirmed by mass spectrometry analysis, and the purity was shown to be more than 98% as assessed by HPLC analysis. The peptide was supplied as a freeze-dried, sterile white powder. It was dissolved in 1.0 mL physiological saline (Otsuka Pharmaceutical, Tokyo, Japan) and stored at −80°C until just before use. Montanide ISA 51 (Seppic, Paris, France) was used as IFA. Human IFNα was purchased from Dainippon-Sumitomo Pharmaceutical (Osaka, Japan).

Patient treatment. In this clinical study, we used the protocol illustrated in Fig. 1, with the survivin-2B80-88 peptide plus IFA and IFNα. In this trial, the primary endpoint was safety. The second endpoint was investigation of the antitumor effects and clinical and immunological monitoring.

In this protocol, survivin-2B80-88 at a dose of 1 mg/1 mL plus IFA at a dose of 1 mL were mixed immediately before vaccination. The patients were then vaccinated s.c. four times at 14-day intervals. In addition, IFNα at a dose of 3 000 000 IU was given s.c. twice a week close to the site of vaccination. For this, IFNα was mixed with the peptide and IFA immediately before vaccination and given at the time of peptide and IFA biweekly vaccination (Fig. 1).

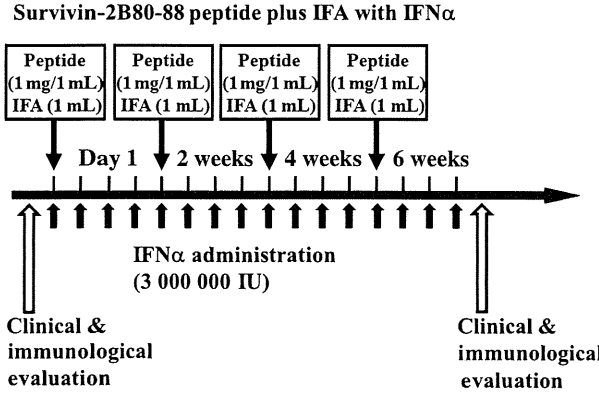

Fig. 1. Clinical protocol of study. Survivin-2B80-88 and incomplete Freund's adjuvant (IFA) were mixed immediately before vaccination. The patients were then vaccinated s.c. four times at 14-day intervals. In addition, α-interferon (IFNα) was given twice a week close to the site of vaccination. For this, IFNα was mixed with the peptide and IFA immediately before vaccination and given at the time of peptide and IFA biweekly vaccination.

Toxicity evaluation. Patients were examined closely for signs of toxicity during and after vaccination. Adverse events were recorded using the National Cancer Institute Common Toxicity Criteria.[8–10]

Clinical response evaluation. Physical examinations and hematological examinations were carried out before and after each vaccination.[8–10] A tumor marker (Ca19-9) was examined. Changes in the tumor marker levels were evaluated by comparison of the serum level before the first vaccination and that after the fourth vaccination. Immunohistochemical study of the HLA class I expression in patients' primary pancreatic cancer tissues was done with anti-HLA class I heavy chain mAb EMR-8-5[13] (Funakoshi, Tokyo, Japan).

Tumor size was evaluated by CT scans or MRI by comparing the size before the first vaccination with that after the fourth vaccination. A complete response (CR) was defined as complete disappearance of all measurable and evaluable disease. A partial response was defined as a $\geq 30\%$ decrease from the baseline in the size of all measurable lesions (sum of maximal diameters). Progressive disease (PD) was defined as an increase in the sum of maximal diameters by at least 20% or the appearance of new lesions. Stable disease (SD) was defined as the absence of criteria matching those for complete response, partial response, or PD.[8–10] Patients who received fewer than four vaccinations were excluded from all evaluations in this study.

***In vitro* stimulation of PBMC, tetramer staining, and ELISPOT assay.** The samples for tetramer analysis and ELISPOT analysis were simultaneously obtained at the time of the hematological examination before and after each vaccination. These experiments were carried out as in our previous report. The PBMCs were isolated from blood samples by Ficoll–Conray density gradient centrifugation. Then they were frozen and stored at −80°C. As needed, frozen PBMCs were thawed and incubated in the presence of 30 μg/mL survivin-2B80-88 in AIM V (Life Technologies Corp, Grand Island, NY, USA) medium containing 10% human serum at room temperature. Next, interleukin-2 was added at a final concentration of 50 U/mL 1 h, 2 days, 4 days, and 6 days after the addition of the peptide. On day 7 of culture, the PBMCs were analyzed by tetramer staining and ELISPOT assay.

The FITC-labeled HLA-A*2402-HIV peptide (RYLRDQQLL) and phycoerythrin (PE)-labeled HLA-A*2402-survivin-2B8-88 peptide tetramers were purchased from Medical and Biological Laboratories (MBL) Co., Ltd (Nagoya, Japan). For flow cytometric analysis, PBMCs, stimulated *in vitro* as above, were stained with the PE-labeled tetramer at 37°C for 20 min, followed by staining with a PE-Cy5-conjugated anti-CD8 mAb (BD Biosciences, San Jose, CA, USA) at 4°C for 30 min. Cells were washed twice with PBS before fixation in 1% formaldehyde. Flow cytometric analysis was carried out using FACSCalibur and CellQuest software (BD Biosciences). The frequency of CTL precursors was calculated as the number of tetramer-positive cells divided by the number of CD8-positive cells.[8,10,12]

The ELISPOT plates were coated overnight in a sterile environment with an IFNγ capture antibody (BD Biosciences) at 4°C. The plates were then washed once and blocked with AIM V medium containing 10% human serum for 2 h at room temperature. CD8-positive T cells separated from patients' PBMCs (5×10^3 cells/well) that were stimulated *in vitro* as above were then added to each well along with HLA-A24-transfected T2 cells (T2-A24) (5×10^4 cells/well) that had been preincubated with or without survivin-2B80-88 (10 mg/mL) or

with an HIV peptide as a negative control. After incubation in a 5% CO_2 humidified chamber at 37°C for 24 h, the wells were washed vigorously five times with PBS and incubated with a biotinylated anti-human IFNγ antibody and HRP-conjugated avidin. Spots were visualized and analyzed using KS ELISPOT (Carl Zeiss, Oberkochen, Germany). In this study, positive (+) ELISPOT represents a more than twofold increase of survivin-2B80-88 peptide-specific CD8 T cell IFNγ-postive spots as compared with HIV peptide-specific CD8 T cell spots, whereas negative (−) means a less than twofold increase.

Single-cell cloning and functional assessment of tetramer-positive CTLs. Survivin-2B80-88 peptide tetramer-positive CTLs were sorted and subsequently cloned to single cells using FACS (Aria II Special Order; BD Biosciences). The peptide-specific cytotoxicity of each of these CTLs was determined by pulsing T2A24 cells[8,17] with survivin-2B80-88 or HLA-A*2402 HIV (RYLRDQQLL) peptides, as previously described.

Results

Patient profiles, safety, and clinical responses. In the present protocol with the survivin-2B80-88 peptide plus IFA and IFNα, six patients were enrolled in the study (Table 1). None dropped out because of adverse events due to the vaccination. They consisted of three men and three women, whose age range was 50–80 years.

With respect to the safety, vaccination was well tolerated in all patients. Four patients had fever reaching nearly 39°C after the vaccination, possibly due to the action of IFNα. No other severe adverse events were observed during or after vaccination except for induration at the injection site, which was conduced by IFA.

The clinical outcomes for the six patients treated with survivin-2B80-88 plus IFA and IFNα are summarized in Table 1. In some patients, particularly No. 1, the postvaccination Ca19-9 value was clearly decreased as compared with prevaccination, and was within the normal limit. Other patients (Nos. 2, 4, and 6) also had decreased or stable postvaccination levels of Ca19-9, although not as large. As for tumor size evaluated by CT, four patients (Nos. 1, 2, 4, and 6) were considered to have SD, but the other two patients (Nos. 3 and 5) had PD. Consequently, it appeared that there was a close correlation between clinical SD outcomes and a reduced or stable Ca19-9 level.

Immune responses, single-cell cloning, and subsequent functional assessment of tetramer-positive CTLs. As in our previous study with colon cancer patients, we determined if the survivin-2B80-88 peptide vaccination could actually induce specific immune responses in the patients enrolled. The peptide-specific CTL frequency was analyzed using the HLA-A24/peptide tetramer. The CTL frequencies before the first vaccination (prevaccination) and after the last vaccination (postvaccination) were assessed with an HLA-A24-restricted survivin-2B80-88 (AYACNTSTL) peptide tetramer, compared with an HLA-A24-restricted HIV peptide (RYLRDQQLL) tetramer as a negative tetramer control. The number of survivin-2B80-88 peptide tetramer-positive but HIV peptide-negative CD8 T cells in 10^4 CD8 T cells was determined. In the current study, ELISPOT was also carried out using these peptides.

As summarized in Table 1, four of the six patients (Nos. 1, 2, 4, and 6) had enhanced frequency with a more than 200% increase. It was also interesting that all four of these patients were also positive in the ELISPOT study, and all four had SD by CT evaluation, suggesting that immune responses might appropriately reflect clinical responses with the current vaccination protocol.

As in our previous work, we also analyzed tetramer-positive CD8 T cells at the single-cell level, and determined whether these T cells had specificity for the survivin-2B80-88 peptide and cytotoxic potential against live survivin-2B-positive tumor cells in the context of HLA-A*2402. As shown in Fig. 2, patient No. 1 (62 years old, female) had a reduced serum Ca19-9 level, and obvious immune responses as assessed by the survivin-2B80-88 ELISPOT and tetramer analyses (Fig. 3) after vaccination.

Subsequently, CD8 T cells of the tetramer-positive fraction were sorted by FACS, then cultured with 1, 3, and 10 cells/well for 7–10 days. Almost all growing T cells were survivin-2B peptide-specific T cells (data not shown), and we next assessed peptide-specific cytotoxicity by using these T cells. As Fig. 4 clearly shows, all T cells had very high peptide-specific cytotoxic potential. Taken together, these data clearly indicated that the vaccination protocol with survivin-2B80-88 plus IFA and IFNα was capable of inducing a strong CTL response and for some pancreatic cancer patients might result in clinical effectiveness.

Assessment of treatment effect with IFNα alone. The above data strongly suggested that the current vaccination protocol

Table 1. Profiles of patients with advanced pancreatic cancer enrolled in the study and their clinical and immunological responses to vaccination with survivin-2B80-88 peptide, incomplete Freund's adjuvant and IFNα

Patient no.	Age/sex	Adverse effects	Tumor markers pre/post (CA19-9 U/mL)	CT eval.	Tetramer staining†		ELISPOT‡	
					Pre/post	% Increase	Pre/post	% Increase
1	62/F	Induration	136.5/31.4	SD	23/246	1069.6	27/294	1088.9
2	61/F	Induration Fever	63.6/60.6	SD	1/157	15700.0	25/71	284.0
3	56/M	Induration Fever Thrombopenia	171.4/978.8	PD	22/19	86.3	19/525	2763.2
4	80/F	Induration Fever	30.0/22.7	SD	9/1030	11444.4	1/101	10100.0
5	58/M	Induration Fever	436.0/2885.0	PD	3/0	0.0	34/20	58.8
6	50/M	Induration	4389.0/4295.0	SD	2/7	350.0	27/85	314.8

†Cytotoxic T-lymphocyte frequency of prevaccinated (pre) and postvaccinated (post) patients was assessed with an HLA-A24-restricted survivin-2B80-88 (AYACNTSTL) peptide tetramer. HLA-A24-restricted HIV peptide (RYLRDQQLL) tetramer was used as a negative control. The numbers of survivin-2B80-88 peptide tetramer-positive but HIV peptide-negative CTLs in 10^4 × CD8 T cells are shown. ‡γ-Interferon (IFNγ) secretion of pre- and postvaccinated patients' CD8 T cells was assesed with enzyme-linked immunosorbent spot (ELISPOT) assay using T2-A24 cells pulsed with survivin-2B80-88 peptide. The numbers of spots in 5 × 10^3 CD8 T cells are shown. CT eval., evaluation by computed tomography; IFNα, α-interferon; PD, progressive disease; SD, stable disease.

doi: 10.1111/cas.12046

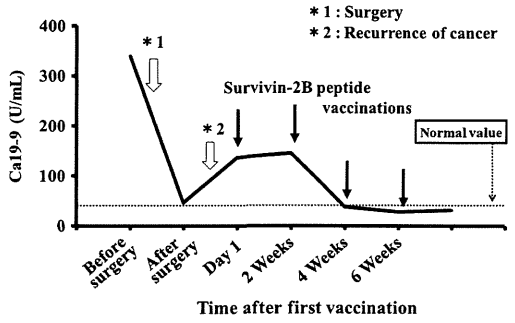

Fig. 2. Representative illustration of the clinical effect in patient No. 1 as assessed by the serum Ca19-9 level. Arrows indicate vaccinations with survivin-2B80-88 plus incomplete Freund's adjuvant with α-interferon (IFNα).

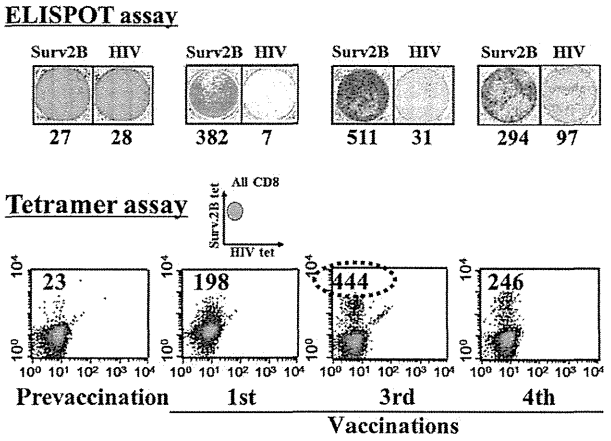

Fig. 3. Immunological analysis of CTL responses against HLA-A24 restricted survivin-2B80-88 peptide (surv2B) before and after vaccinations as assessed by enzyme-linked immunosorbent spot (ELISPOT) and tetramer (tet) analyses. Numbers in the ELISPOT assay indicate γ-interferon (IFNγ) secretion against survivin2B80-88 or HIV peptide pulsed T2-A24 cells in $10^4\times$ $CD8^+$ T cells. Numbers in tetramer analysis indicate survivin-2B80-88 peptide-specific $CD8^+$ T cells among $10^4\times$ $CD8^+$ T cells.

with the survivin-2B80-88 peptide plus IFA and IFNα could work as a potential therapeutic regimen in pancreatic cancers. However, it remained to be clarified if IFNα alone without the peptide could function in a similar manner, at least to some extent, as this cytokine is considered to be the most potent for the activation and maturation of dendritic cells (DCs) as well as upregulation of HLA class I in tumor cells. To this end, we studied this profile in three patients with colon cancer, not pancreatic cancer, whose condition was similar to those in this study, that is, patients with unresectable advanced or recurrent cancer. This was done because patients with the latter cancer had highly advanced clinical cases, making this type of study impossible. As shown Table 2, all three patients showed no obvious increases, but rather reductions, in the frequency of survivin-2B peptide-specific T cells as assessed by tetramer analysis before and after two to four treatments with IFNα alone. Furthermore, this was also true for ELISPOT analysis. These data supported the idea that IFNα alone did not actively participate in the activation of survivin-2B peptide-specific T cells.

Discussion

Our group previously showed that the vaccination protocol of survivin-2B80-88 plus IFA and IFNα could work as a potent

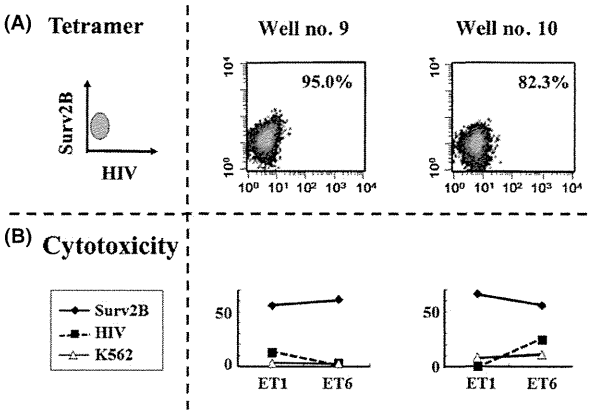

Fig. 4. Single-cell analysis of survivin-2B80-88 peptide tetramer-positive CD8 CTL cells). Survivin-2B80-88 peptide tetramer-positive CD8 T cells in Fig. 3 (circled) were sorted and cultured at 1, 3, and 10 cells /well for 7–10 days. Subsequently, clonal CTL cells were examined for their reactivity to the survivin-2B80-88 peptide tetramer (Surv2B) (A) and against T2A24 target cells pulsed with the survivin-2B80-88 peptide and HIV peptide and against control K562 cells (B). ET, effector/target ratio.

immunotherapeutic regimen in colon cancers.[8] In addition to colon cancer, survivin2B protein is expressed in most tumor cells of various tissue origins, such as those in the gastrointestinal and biliary tracts and pancreas, therefore, there is a possibility that the survivin2B peptide could work as a potential therapeutic tumor vaccine in cancer patients with these neoplasms.

In this present study, we assessed whether the vaccination protocol using survivin-2B80-88 plus IFA and IFNα could be effective in pancreatic cancer patients from immunological and clinical points of views. Consequently, our data strongly suggested that this protocol was very effective and useful in immunotherapy for advanced pancreatic cancers as in colon cancers. Actually it was shown that more than 50% of patients with pancreatic cancers showed positive clinical and immunological responses in tetramer and ELISPOT analyses. In some cases, the immunological response of survivin-2B80-88 peptide-specific CTLs was elucidated at the single-cell level. Taken together, the current data implied that our vaccination protocol was very useful in immunotherapy for pancreatic cancers.

As shown in Fig. 3, the number of tetramer-positive populations and IFNγ-positive spots in the ELISPOT assay was reduced from the third to the fourth vaccination. We speculate that there could be various reasons for this reduction. One might be immune escape by the downregulation of HLA expression, cytokines, or regulatory T cells. Another might be an activity of the stored samples, or differences between the environment of the peripheral circulation and the tumor. In other words, the peptide-specific CTL responses were reduced in immunological monitoring in the peripheral circulation, but maintained in the local cancer environment. In this case, the clinical responses, such as tumor marker (CA19-9) level and tumor size evaluated by CT, had been maintained also after that, even though the number of tetramer-positive populations and IFNγ-positive spots in the ELISPOT assay was reduced between the third and fourth vaccinations. Therefore, CA19-9 levels had been kept within normal limits and new cancer lesions had not appeared.

We evaluated immunological monitoring of this clinical protocol by tetramer staining and IFNγ ELISPOT assay. Tetramer staining recognizes the structure of the T cell receptor, and

Table 2. Frequency monitoring of the number of survivin-2B80-88 peptide tetramer-positive CTLs in cancer patients treated with IFNα alone

Patient no.	Tumor	Age/sex	Number of treatment	Tetramer staining†		ELISPOT‡	
				Pre/post	% Increase	Pre/post	% Increase
1	Colon	60/M	3	1/0	0.0	111/75	67.6
2	Colon	63/M	4	11/9	81.8	44/20	45.5
3	Colon	77/F	2	13/3	23.1	26/40	153.8

†CTL frequency before and after treatment with IFNα alone in patients was assessed with an HLA-A24-restricted survivin-2B80-88 (AYACNTSTL) peptide tetramer. An HLA-A24-restricted HIV peptide (RYLRDQQLL) tetramer was used as a negative control. The number of survivin-2B80-88 peptide tetramer-positive but HIV peptide-negative CTLs in 10^4 CD8 T cells is shown. ‡γ-Interferon (IFNγ) secretion of pre and post IFNα treatmnet were assesed with ELISPOT assay using T2-A24 cells pulsed with survivin2B80-88 peptide. The number of spots in 5×10^3 CD8 T cells are shown. IFNα, α-interferon.

detects naive T cells, memory T cells, and activated CTLs. The ELISPOT assay detects more the functional aspects of T cells by IFNγ release, therefore, ELISPOT detects memory T cells and CTLs. In this study, the tetramer-positive cases are also positive in the ELISPOT study. Therefore, these results indicate that memory T cells and CTLs can be effectively induced by this peptide vaccination protocol.

In this present study, we also assessed evidence concerning the extent to which peptide-specific CTL responses in pancreatic cancer patients treated with peptide vaccines could occur at the single-cell level. To assess this, CTLs of patients were sorted to the single-cell level, and we confirmed that each CTL obtained from vaccinated patients was indeed peptide-specific in the context of the expression of HLA-A24.

Type-I interferons such as IFNα are known to work in various immunological manners to activate T cell responses.[18–25] The maturation of DCs and their effect on the expression of HLA molecules seems to be the main action of this cytokine. Although we could not actually compare these features of patients' DCs and primary pancreatic tumor tissues before and after treatment with IFNα, the obvious enhancement of CTL responses and improvement of clinical responses in our previous and current studies favors the two main actions described above. These observations strongly suggest that the action of IFNα is remarkable from the aspect of being an immunogenic enhancer for human cancer peptide vaccines.

It is widely known that IFNα is involved in DC maturation and activation.[18,21] This particular cytokine is also potent for increasing the expression of MHC class I molecules.[26–29] Indeed, our previous study of the expression of HLA class I molecules in pancreatic cancer indicated that many tumor tissues heterogeneously expressed such molecules, with some tumor cells showing high expression, whereas others had only weak expression. Interferon-α is presumed to actually enhance their expression even in those tumor tissues with weak expression. Moreover, because tumor patients generally show overt expression of survivin protein in their tumor tissues and, although in small numbers, survivin-2B peptide-specific T cells in peripheral blood, it is considered that IFNα alone may increase the frequency of these T cells in peripheral blood as well. These features of this particular cytokine lead to the possibility that treatment with IFNα alone could result in, at least to some extent, certain immunological and clinical effects of survivin-2B peptide-specific T cells in tumor-bearing patients. However, we analyzed three colon cancer patients, and our data strongly suggested that there was no increase of these T cells as assessed by tetramer and ELISPOT analyses.

Taken together, our results highly suggest that the vaccination protocol with survivin-2B80-88 plus IFA and IFNα is very effective for pancreatic and colon cancers, and that this protocol might be useful as a standard, general immunotherapy modality for human cancers. However, further clinical studies involving many patients are necessary in order to consolidate the immunotherapeutic benefit of this vaccination protocol.

Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant nos 16209013, 17016061, and 15659097), and for Practical Application Research from the Japan Science and Technology Agency and for Cancer Research (15-17 and 19-14) from the Ministry of Health, Labor and Welfare of Japan. We are also very grateful for grants from the Akiyama Life Science Foundation and the Ono Foundation, and for continuous support providing study samples by the Hokkaido Red Cross Blood Center.

Disclosure Statement

The authors have no conflict of interest.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i45.6577

World J Gastroenterol 2012 December 7; 18(45): 6577-6586
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Alterations in the human epidermal growth factor receptor 2-phosphatidylinositol 3-kinase-v-Akt pathway in gastric cancer

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Supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, to Yamamoto H and Shinomura Y
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Received: June 18, 2012 Revised: August 21, 2012
Accepted: August 25, 2012
Published online: December 7, 2012

Abstract

AIM: To investigate human epidermal growth factor receptor 2 (HER2)-phosphatidylinositol 3-kinase (PI3K)-v-Akt murine thymoma viral oncogene homolog signaling pathway.

METHODS: We analyzed 231 formalin-fixed, paraffin-embedded gastric cancer tissue specimens from Japanese patients who had undergone surgical treatment. The patients' age, sex, tumor location, depth of invasion, pathological type, lymph node metastasis, and pathological stage were determined by a review of the medical records. Expression of HER2 was analyzed by immunohistochemistry (IHC) using the HercepTest™ kit. Standard criteria for HER2 positivity (0, 1+, 2+, and 3+) were used. Tumors that scored 3+ were considered HER2-positive. Expression of phospho Akt (pAkt) was also analyzed by IHC. Tumors were considered pAkt-positive when the percentage of positive tumor cells was 10% or more. PI3K, catalytic, alpha polypeptide (PIK3CA) mutations in exons 1, 9 and 20 were analyzed by pyrosequencing. Epstein-Barr virus (EBV) infection was analyzed by *in situ* hybridization targeting EBV-encoded small RNA (EBER) with an EBER-RNA probe. Microsatellite instability (MSI) was analyzed by polymerase chain reaction using the mononucleotide markers BAT25 and BAT26.

RESULTS: HER2 expression levels of 0, 1+, 2+ and 3+ were found in 167 (72%), 32 (14%), 12 (5%) and 20 (8.7%) samples, respectively. HER2 overexpression (IHC 3+) significantly correlated with intestinal histological type (15/20 *vs* 98 /205, $P = 0.05$). PIK3CA mutations were present in 20 cases (8.7%) and significantly correlated with MSI (10/20 *vs* 9/211, $P < 0.01$).