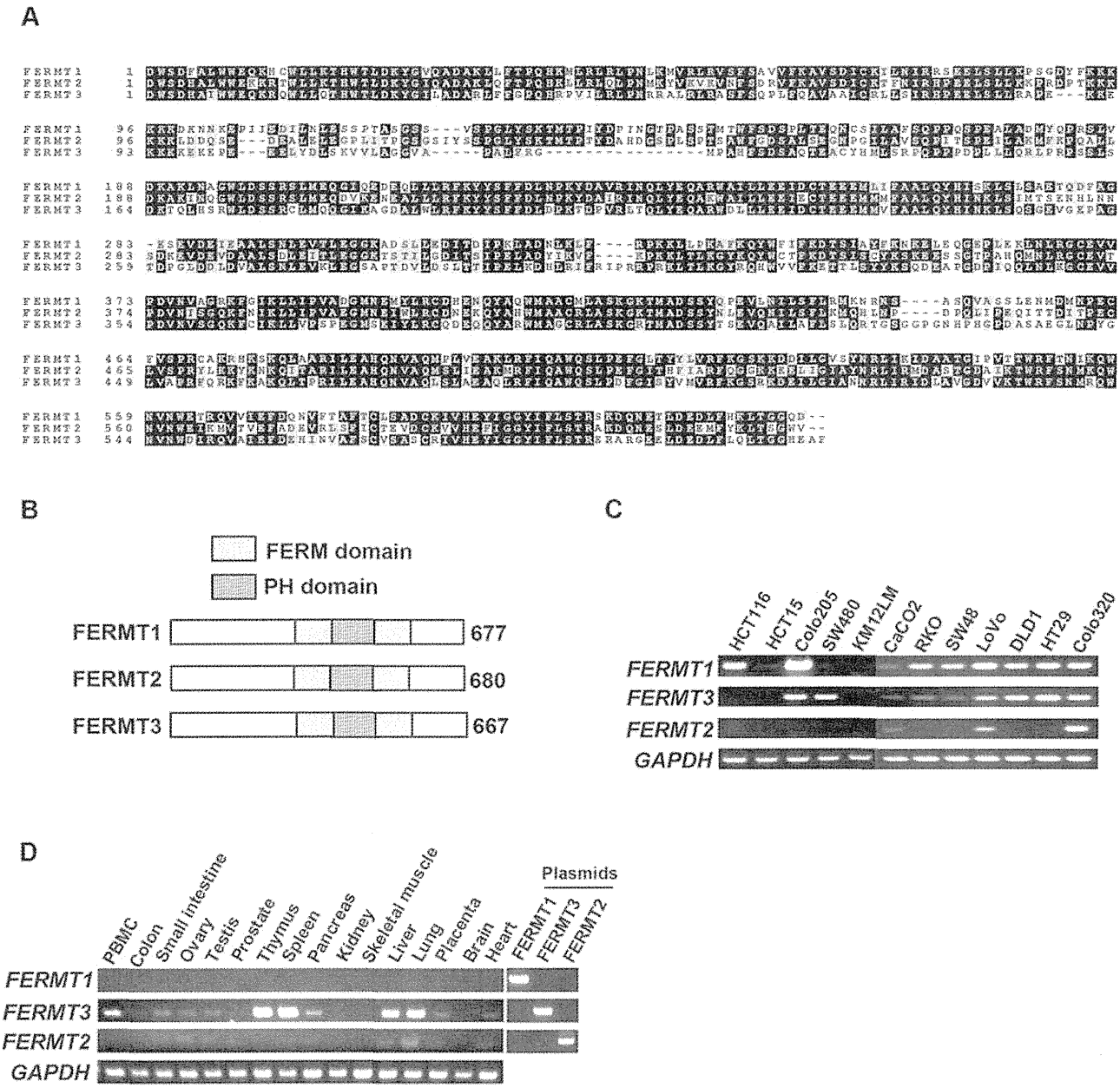

Figure 1. *Expression profiles of fermitin family member (FERMT) family genes. A: Sequence alignment of FERMT proteins. FERMT1, FERMT2 and FERMT3 amino acid sequences are shown. A black box indicates the same alignment, a gray box indicates similar alignment. B: Molecular structure of FERMT family proteins.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

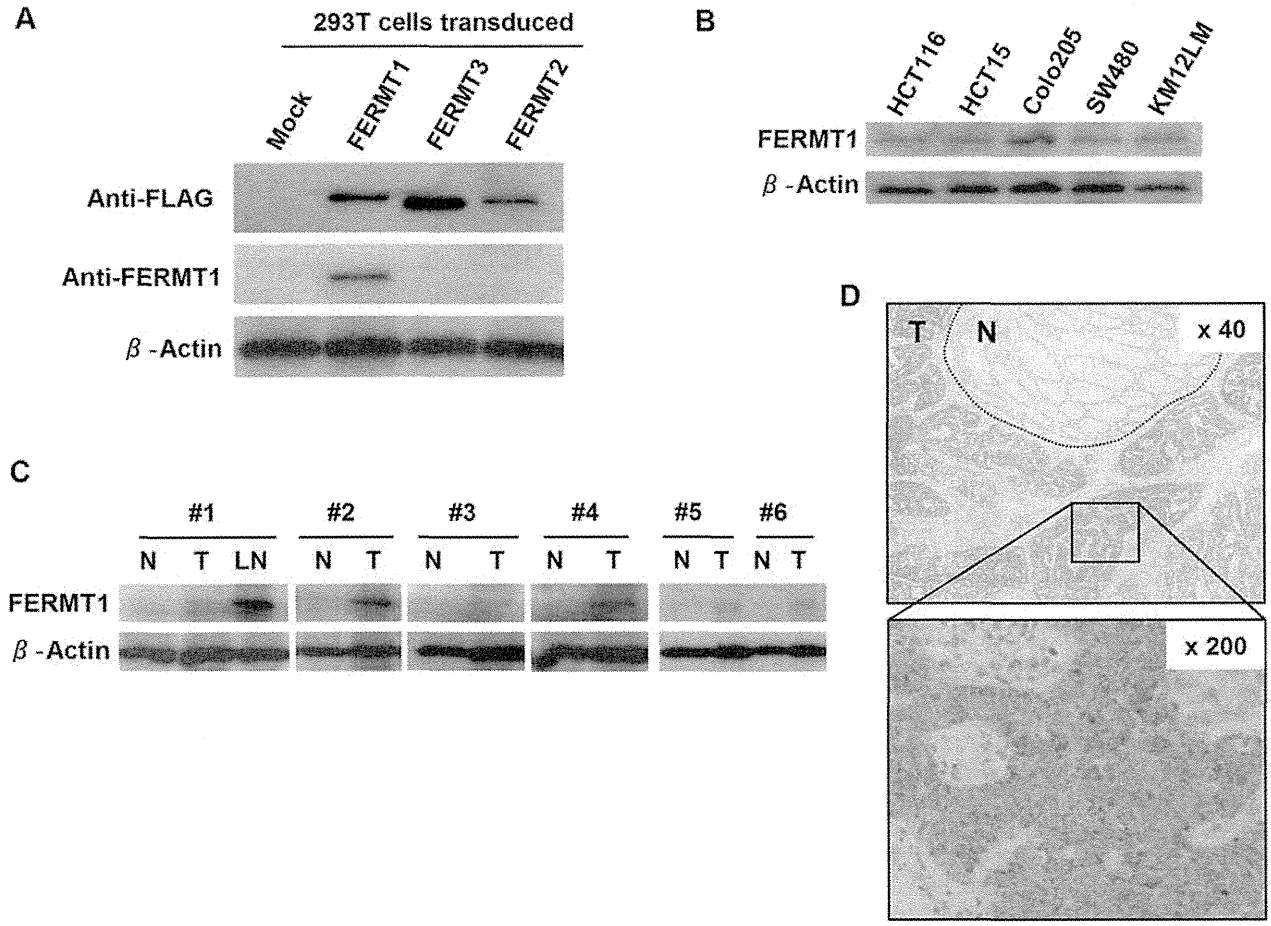

Figure 2. *Fermitin family member 1 (FERMT1) protein expression in colonic carcinomas. A: Western blotting using monoclonal antibody (mAb) against FERMT1. 293T cells were transfected with FERMT1, FERMT2 and FERMT3 plasmids. Western blotting using anti-FLAG mAb and anti-FERMT1 mAb was performed. Anti-FLAG 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.

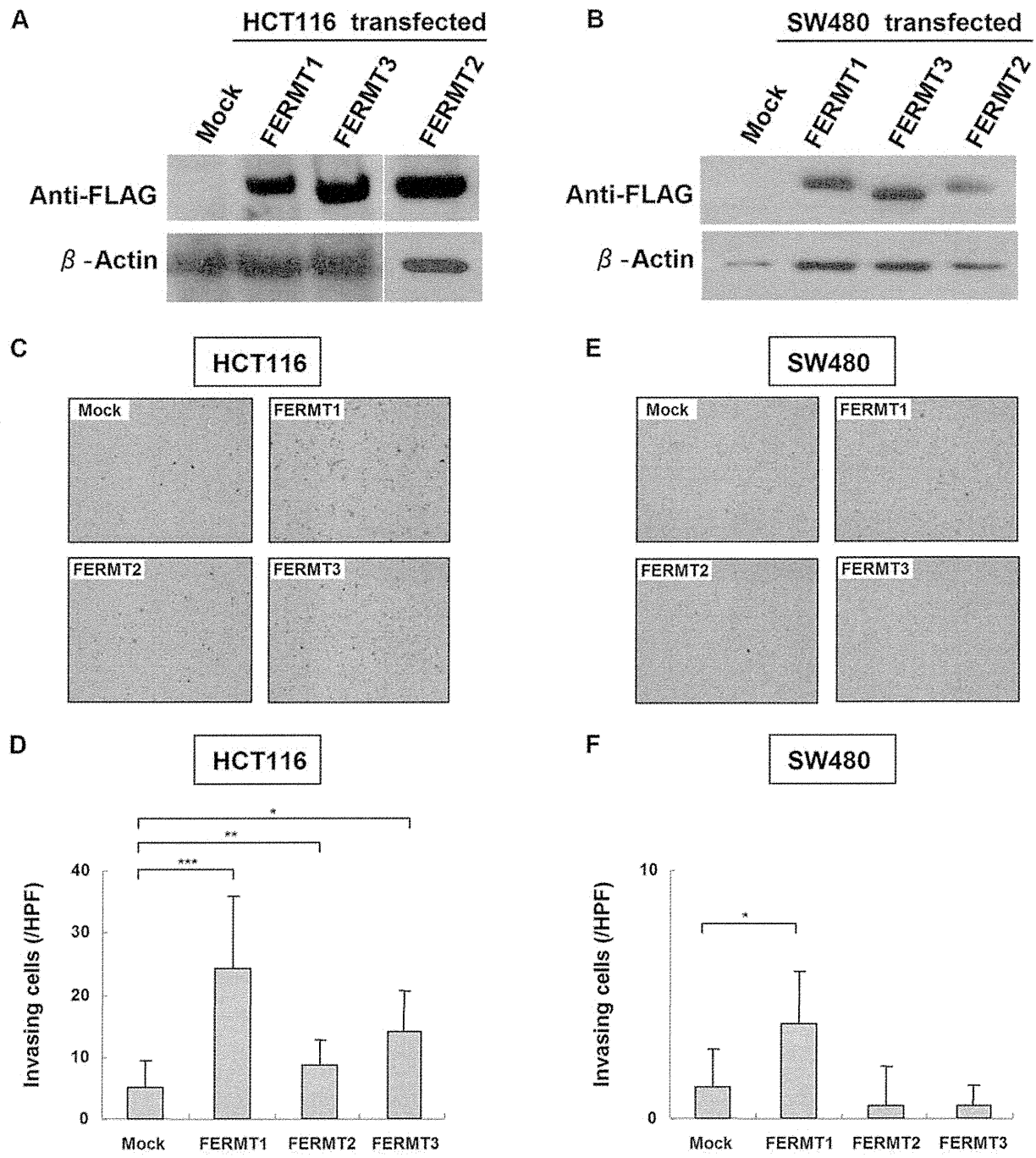

Figure 3. *Molecular function of FERMT1 in colon carcinoma cells. A: Western blotting using monoclonal antibody (mAb) to FLAG-tag. HCT116 cells were transfected with FREMT1, FERMT3, FERMT2 plasmids, and analyzed by western blot using mAb to FLAG-tag. β-Actin was used as an internal positive control.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

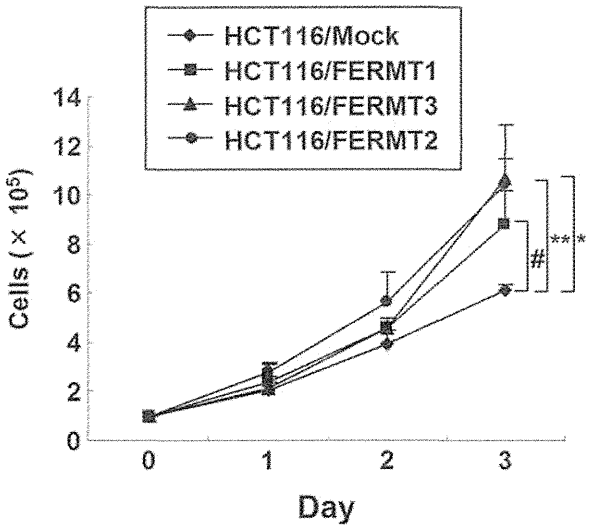

Figure 4. *Cell growth of FERMT famliy-overexpressing HCT116 cells. FERMT family cDNA-overexpressing HCT116 cells were seeded in a 6-well plate, and the cell growth rate was recorded daily. Data represent means±SD. Differences between FERMT family-overexpressing HCT116 cells and mock-transfected HCT116 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.

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

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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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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).

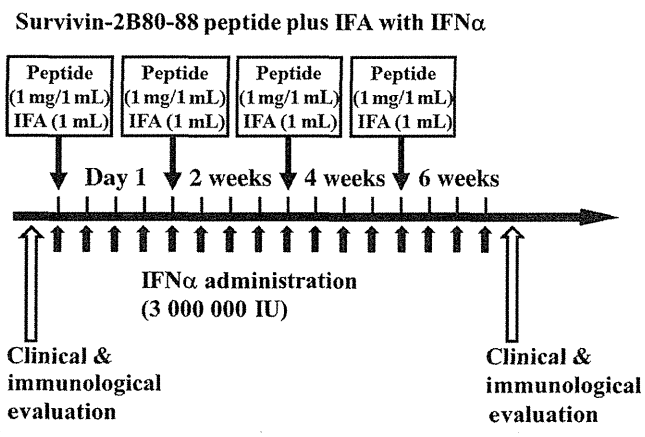

Fig. 1. Clinical protocol of study. Survivin-2B80-88 and incomplete Freund's adjuvant (IFA) were mixed immediately before vaccination. The patients were then vaccinated s.c. four times at 14-day intervals. In addition, α-interferon (IFNα) was given twice a week close to the site of vaccination. For this,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

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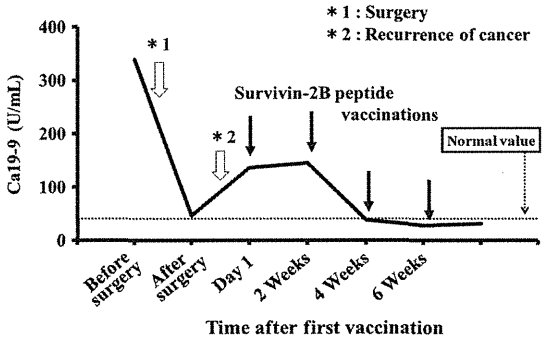

Fig. 2. Representative illustration of the clinical effect in patient No. 1 as assessed by the serum Ca19-9 level. Arrows indicate vaccinations with survivin-2B80-88 plus incomplete Freund's adjuvant with α-interferon (IFNα).

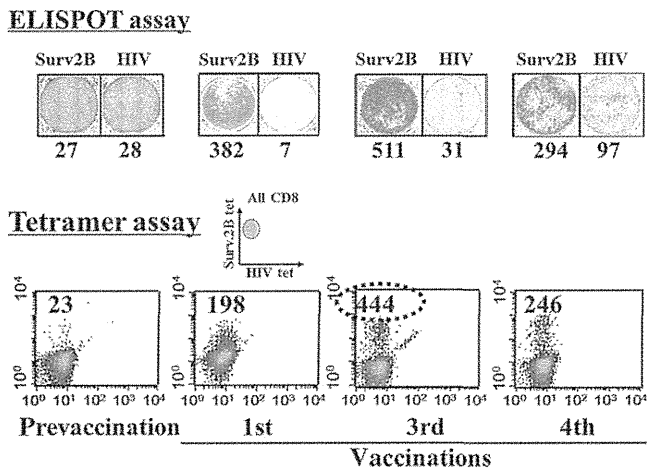

Fig. 3. Immunological analysis of CTL responses against HLA-A24 restricted survivin-2B80-88 peptide (surv2B) before and after vaccinations as assessed by enzyme-linked immunosorbent spot (ELISPOT) and tetramer (tet) analyses. Numbers in the ELISPOT assay indicate γ-interferon (IFNγ) secretion against 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

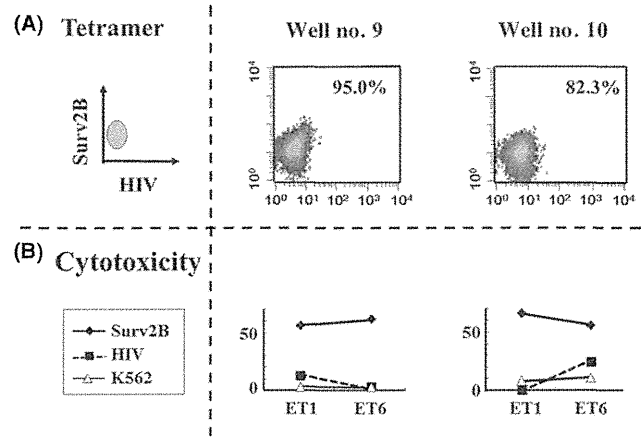

Fig. 4. Single-cell analysis of survivin-2B80-88 peptide tetramer-positive CD8 CTL cells). Survivin-2B80-88 peptide tetramer-positive CD8 T cells in Fig. 3 (circled) were sorted and cultured at 1, 3, and 10 cells /well for 7–10 days. Subsequently, clonal CTL cells were examined for their reactivity to 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.

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Disclosure Statement

The authors have no conflict of interest.

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Preoperative CT evaluation of intraductal spread of breast cancer and surgical treatment

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Abstract It is always a challenge to accurately determine the appropriate extent of resection in breast-conserving surgery (BCS), in order to reduce the need for re-excision, prevent local recurrence, and optimize cosmetic results. Detecting intraductal spread alone with high sensitivity may not be enough to realize safe BCS. Computed tomography carried out with the patient in the supine position accompanied by adequate marking is effective for preoperative determination of the optimum extent of BCS.

Keywords Breast cancer · CT · Breast-conserving surgery · Extent of surgery · Extensive intraductal component

Abbreviations

BCS	Breast-conserving surgery
CT	Computed tomography
EIC	Extensive intraductal component
HU	Hounsfield units
MD-CT	Multidetector-row computed tomography
MIP	Maximum intensity projection
MMG	Mammography
US	Ultrasonography

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Breast cancer diagnosis

Although computed tomography (CT) is not a primary modality for screening the breast or differentiating between malignant and benign breast lesions, some studies have reported that CT was able to reveal the primary tumor with high sensitivity [1]. Diagnostic criteria for breast cancer using CT include an irregular margin, irregular shape, and rim enhancement [2]. Spiculation was strongly suggestive of malignancy when detected incidentally by use of CT [3 5]. Irregular shape and axillary lymphadenopathy are also morphological predictors. The CT values of malignant lesions were higher than those of benign lesions. The cut-off value ranged from 60 Hounsfield units (HU) at 30 s [6, 7] to 90 HU on the 1-min images [8]. Optimum timing of the early phase scan was 80 s after injection of contrast media [9].

Multidetector-row computed tomography (MD-CT) detected contralateral breast cancer in 2.6% of newly diagnosed breast cancer cases [10].

Preoperative MD-CT evaluation of the extent of cancer in the breast

Extensive intraductal spread is often accompanied by invasive ductal carcinoma and becomes a major cause of positive margins after breast-conserving surgery (BCS). It is always a challenge to accurately determine the appropriate extent of resection in order to prevent local recurrence, reduce the need for re-excision, and optimize cosmetic results. Diagnostic criteria for intraductal spread using CT (axial image) are non-mass-like enhancement which is contiguous with and enhanced to the same extent as the index tumor, and the presence of linear or segmental enhancement around the main tumor [11]. The maximum

intensity projection (MIP) image is also useful in diagnosing the extent of breast cancer [12]. The morphological type of intraductal spread using the MIP image is continuous extension from the index tumor (Fig. 1) [13]. Linear enhancement at the edge of the mammary gland, detected using either axial or coronal sections, or diffuse punctate enhancement with smooth margin, are associated with fibrocystic change [13]. They are sometimes seen bilaterally.

Sensitivity and specificity in the detection of the intraductal spread have varied from 71.8 to 88.0% and from 67.8 to 81.9%, respectively (Table 1) [11 15]. CT evaluation of the maximum diameter of the extent of breast cancer has been shown to be substantially better correlated with histopathological diameter than that determined by mammography (MMG) [13, 16]. The median deviation of the tumor extension revealed by 3D CT from pathological size was reported to be 7.7 mm [17]. CT is more accurate than MMG or ultrasonography (US) in determining the extent of invasive lobular carcinoma, with or without neoadjuvant chemotherapy [18].

CT has been shown to detect multiple lesions that are undetectable by conventional methods in 6 18.6% of breast cancer cases [13, 19]. The sensitivity, specificity, and accuracy of the CT diagnosis of otherwise occult sites of cancer have been shown to be 93.3, 98.3, and 97.3%, respectively [13].

High sensitivity may not be enough

It was believed that the incidence of positive margins was certain to decrease if they could be depicted accurately. MRI is the most sensitive modality available to date for identifying the extent of cancer within the breast. However, findings reported in 2008 that were related to the retrospective analysis of preoperative MRI as compared with no MRI were received with great disappointment, because use of MRI failed to reduce the incidence of positive margins [20]. Subsequently, two randomized control studies that assessed the effectiveness of preoperative MRI in terms of the need for re-excision were reported [21, 22]. The COMICE trial included 1623 women with biopsy-proven primary breast cancer who were randomly assigned to MRI and non-MRI groups before surgery [21]. Addition of MRI to conventional triple assessment was not significantly associated with a reduction in the need for reoperation, with 19% of patients in the MRI group requiring reoperation compared with 19% in the non-MRI group [21]. The primary endpoint of another clinical study, the MONET trial, also involved assessment of the need for additional surgical procedures (re-excision and conversion to mastectomy) for non-palpable breast tumors. The need for additional surgical intervention after initial BCS was 45% in the MRI group versus 28% in the conventional non-MRI group. Thus, addition of MRI to routine clinical care in patients with non-palpable breast cancer was paradoxically associated with an increase in the need for re-excision.

Positive results had been expected from these two randomized controlled trials. Why did MRI fail to reduce the incidence of positive margins and re-excision in BCS despite excellent sensitivity? One reason is speculated to be the change of the shape of the breast because of the different positions used during MRI examination and subsequent surgery. Thus, there is a possibility that even if the lesion can be revealed by MRI, the extent of excision cannot be accurately determined. We should therefore be very careful in not only depicting the tumor margins but also in preventing errors in determining the excision margins that are associated with changes in position of the breast.

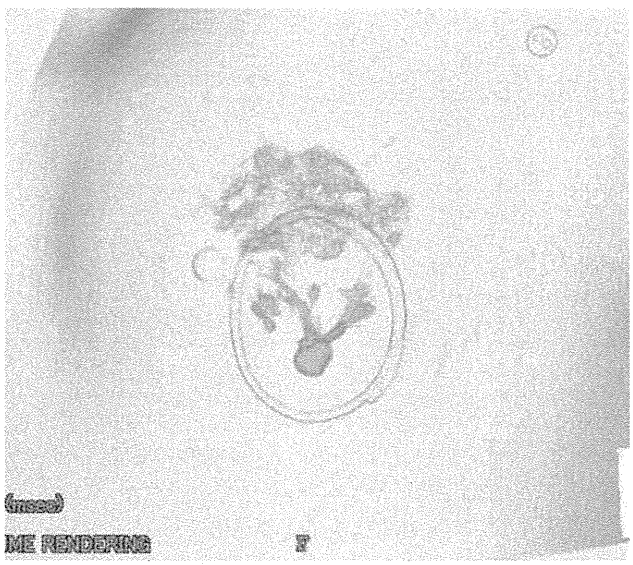

Fig. 1 Reconstructed 3D CT image. Intraductal extension continuous from the index cancer

Important factors in determining the extent of surgery

The accuracy with which the surgery is aligned with the image-detected lesion is an important concern. Accurate

Table 1 Sensitivity, specificity, and accuracy of detection of intra ductal spread by CT

	Published in	No. of patients	Sensitivity	Specificity	Accuracy
Akashi Tanaka	1998	122	91	79	
Uematsu	2001	135	77	87	
Fujita	2005	81	81	68	73
Doihara	2006	72	72	86	

and facile skin markings are one solution to this problem. This author conducted a multicenter prospective study on the effectiveness of pre-operative breast CT imaging in surgical planning for patients undergoing BCS [23]. The surgeons marked the line of planned excision on the skin based on information from palpation, MMG, and US before CT, which was also recorded on the CT image. Contrast-enhanced breast CT was performed in the supine surgical position. The CT results were used to help determine the extent of resection. The surgeons widened the extent of resection in 42 (14.1%; 95% confidence interval 10.1 18.1%) out of a total of 297 patients based on the CT findings. Breast CT correctly modified the extent of surgery in 13.1% and overexcision in 1%. An example of a correctly modified case using CT is shown in Fig. 2. CT was especially effective in cases of invasive lobular carcinoma and apocrine carcinoma. The efforts taken to simulate the patient's positioning that was subsequently used in the

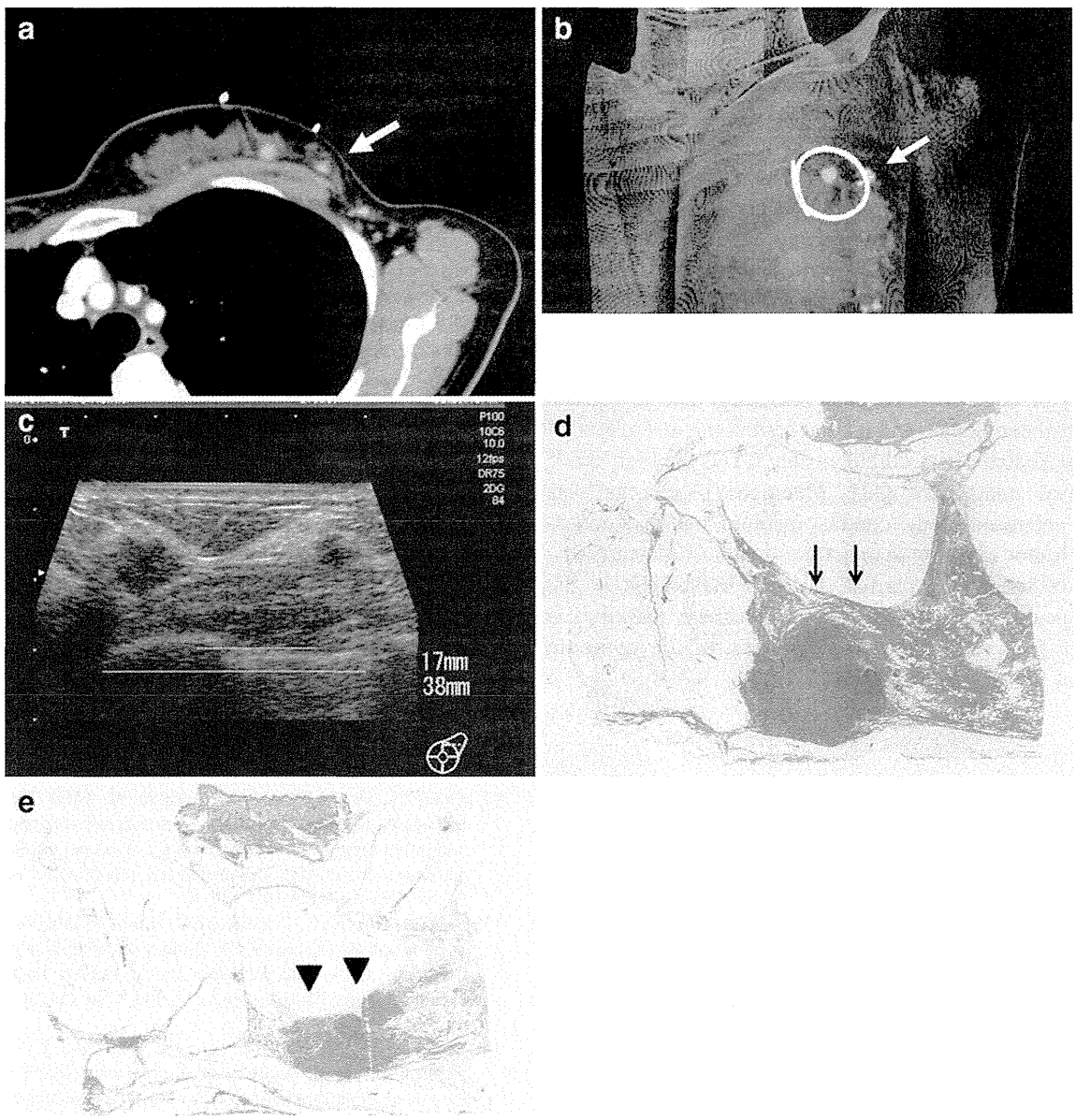

Fig. 2 An instance in which CT successfully affected surgical management. **a** CT image showing an enhancing lesion (*arrow*) lateral to the main tumor which suggested that it was located outside the planned resection line. The angiocatheter can be seen on the skin demarcating the pre CT planned resection line. **b** Maximum intensity projection image of the right breast. The *white line* indicates the surgical line that was originally planned. **c** Second look ultrasonography revealed a second tumor with an 8 mm diameter located 17 mm lateral to the main tumor. We modified the resection line to widen the lateral side. **d** Surgical specimen (H&E). The *arrows* indicate the main tumor which was an invasive ductal carcinoma. **e** The *triangles* indicate the second tumor which was an invasive ductal carcinoma located in the modified excised specimen

operating room, and surgical marking, brought about this excellent result.

Harada-Shoji et al. [24] reported excellent incidence of negative margins after BCS using a dedicated skin marker. Seven lines marked on the patient's skin using an oil-based paint enabled accurate resection with incidence of positive margins of 2.2%. These markings were effective when they were scanned with the patient in the supine position, which is the position used during surgery. Second-look US with the patient in the supine position in order to utilize the information obtained when the patient was in the prone position during MRI is widespread. Real time virtual sonography in the supine position has been reported to be useful for identifying enhancing breast lesions originally detected by MRI [25].

Limitations

The disadvantage of CT is radiation exposure. Some studies have compared the accuracy of MD-CT and MRI in evaluation of the intraductal spread of breast cancer. CT has been shown to be inferior in sensitivity to MRI and superior [26, 27] or equivalent [28] in specificity. The low-grade intraductal component and lobular carcinoma in situ tended not to be depicted as accurately using CT as the high-grade intraductal component [11]. Mucinous carcinoma was weakly enhanced by the contrast medium, and as a consequence tumor extent was sometimes underestimated [26].

In conclusion, CT carried out with the patient in the supine position, accompanied with adequate marking, is effective for preoperative determination of the optimum extent of breast cancer surgery.

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Axillary lymph node dissection in sentinel node positive breast cancer: is it necessary?

Seigo Nakamura

Purpose of review
Sentinel lymph node biopsy (SLNB) has become a gold standard procedure for axillary lymph node evaluation in clinically node-negative patients. In those patients with positive SLNB, completion axillary lymph node dissection (ALND) has been routely performed. Recent clinical trials suggest that ALND is not necessary in some cases, even when the sentinel lymph node (SLN) is positive. The appropriate conditions under which ALND may be eliminated are defined in this review.

Recent findings
The American College of Surgeons Oncology Group (ACOSOG) Z0011 trial studied the impact of SLNB alone versus completion axillary node dissection (AND) on survival in clinically node-negative breast cancer patients undergoing partial mastectomy and whole breast irradiation who were found to have a positive SLN on pathological evaluation. Results of this study showed no survival advantage for complete AND in patients with one or two positive SLNs. In other words, those patients appeared to be treated safely without completion AND.

Summary
Despite the small sample size and limited stastical power and the relatively short median follow up for ACOSOG Z0011, many breast cancer teams no longer believe it mandatory to perform axillary dissection for patients with one or two positive SLNs. The results of other prospective randomized trials called After Mapping of the Axilla: Radiotherapy Or Surgery study and International Breast Cancer Study Group trial 23–01 study will be available soon, and may further change the confidence with which ALND is performed or eliminated.

Keywords
After Mapping of the Axilla: Radiotherapy Or Surgery study, American College of Surgeons Oncology Group Z0011, axillary dissection, sentinel lymph node biopsy

INTRODUCTION

Sentinel lymph node biopsy has become a gold standard procedure for women with breast cancer who present with clinically negative axillary lymph nodes [1–4]. Lymphedema and paresthesias occur in approximately 5–8% of patients after sentinel node biopsy (SNB) and 10–20% of patients after axillary lymph node dissection (ALND) [5▪▪,6–9]. SNB is, thus, the optimum approach in terms of morbidity for the assessment of axillary metastasis in clinically node-negative breast cancer.

The results of American College of Surgeons Oncology Group (ACOSOG) Z0010 and National Surgical Adjuvant Breast and Bowel Project (NSABP) B32 trials help estimate the prevalence and prognostic significance of positive sentinel lymph nodes (SLNs) found only by immunohistochemistry [10–12]. Among patients with negative intraoperative frozen section who are found to be SLN positive on final pathologic examination, the risk of non-SLN metastases is low [13–15]. A growing number of patients are electing not to undergo completion ALND; a decision that may in part be due to the adoption of a predictive nomogram based on pathologic variables for the risk of non-SLN metastasis [16,17].

Retrospective studies have indicated that in up to 40–60% of cases with a positive sentinel node the sentinel node is the only positive node [13–15,18].

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KEY POINTS

- From the result of ACOSOG Z0011, AND may safely be omitted in breast conservation patients whose tumor size is 5 cm or less with clinically node negative and who will have whole breast radiation and appropriate systemic adjuvant therapy.
- Because there are several critiques to ACOSOG Z0011, we should carefully follow up such patients who have not received axillary dissection and pay attention to the result of other similar studies (AMAROS study and International Breast Cancer Study Group 23–01.)

A positive SLN will prompt a recommendation for systemic therapy in the vast majority of women. Whether surgical excision of any positive nonsentinel nodes would improve long-term outcome has been an issue of uncertainty.

ACOSOG Z0011 is a prospective randomized trial to determine the effects of complete axillary node dissection (AND) on survival of patients with SLN metastasis of breast cancer [19,20▪▪]. Women who were eligible for the trial had tumors less than 5 cm, clinically negative axillary lymph nodes, lumpectomy to negative margins, no neoadjuvant chemotherapy, planned whole breast irradiation, and 1 or 2 positive SLNs. Almost all received systemic adjuvant chemotherapy and/or endocrine therapy. The results show that ALND is not associated with 5-year overall survival and 5-year disease-free survival. Cases of lymphedema were significantly higher in the ALND group. Therefore, this study does not support the routine use of ALND in breast cancer with 1–2 involved SLNs and undergoing breast conserving therapy including whole breast irradiation. This requires that the role of ALND be reconsidered [21▪].

THE MANAGEMENT OF ISOLATED TUMOR CELLS OR MICROMETASTASIS IN SENTINEL NODES

It has been a standard practice to perform ALND in breast cancer patients with positive SLN, and this is done in the majority of patients. However, controversy exists over the management of patients found to have positive SLN by immunohistochemical (IHC) staining alone. Tan *et al.* [22] reported worse survival for patients with occult metastasis detected by serial sectioning and immunohistochemistry. The results of the ACOSOG Z0010 and NSABP B32 trials will help estimate the prevalence and prognostic significance of positive SLN found only by immunohistochemistry [23,24]. A systematic review by Bear *et al.* also concluded occult axillary node metastases detected by serial sections and/or IHC staining of SLN are prognostically significant [24]. However, NSABP B-32 showed the magnitude of the difference in outcome at 5 years was quite small (1.2 percentage points) [25]. Therefore, there appears to be little clinical benefit of including IHC analysis of hematoxylin and eosin stained negative sentinel nodes in patients with breast cancer [26].

THE MANAGEMENT OF AXILLARY MACROMETASTASIS: RETROSPECTIVE STUDY

Veronesi *et al.* [26] from the European Institute of Oncology presented 10-year follow up of their single-institution trial designed to compare outcomes in patients who received no axillary dissection if the sentinel node was negative, with patients who received complete axillary dissection. From March 1998 to December 1999, 516 patients with primary breast cancer under 2 cm were randomized either to SNB and complete axillary dissection (axillary dissection arm) or to SNB with axillary dissection only if the sentinel node contained metastases (sentinel node arm). Eight patients in the axillary dissection arm had false-negative sentinel nodes on histologic analysis: a similar number [8, 95% confidence interval (CI) 3–15] of patients with axillary involvement was expected in sentinel node arm patients who did not receive axillary dissection; but only two cases of overt axillary metastasis occurred. There were 23 breast cancer-related events in the sentinel node arm and 26 in the axillary dissection arm (log-rank, $P = 0.52$), whereas overall survival was greater in the sentinel node arm (log-rank, $P = 0.15$). They concluded that preservation of healthy lymph nodes may have beneficial consequences. Even though there might be around 5% false-negative rate in the sentinel node arm, axillary dissection should not be performed in clinically node-negative patients without performing SNB.

Spiguel *et al.* [27▪] retrospectively reviewed their institution's 12-year experience with SNB alone for a tumor-positive sentinel node. Among 3 806 patients who underwent SNB, 2 139 underwent SNB alone, of which 1 997 were tumor negative and 123 were tumor positive. Sentinel nodes were staged node-positive (N1mic or N1) according to American Joint Committee on Cancer criteria.

Mean age was 57 years (range 32–92 years) and mean tumor size was 1.9 cm (range 0.1–9 cm). Eighty-nine (72%) underwent lumpectomy and 34 (28%) underwent mastectomy. Ninety-three percent of patients underwent some form of adjuvant

therapy. Forty-two patients (34%) did not undergo radiation and there were no axillary recurrences in this group. At median follow-up of 95 months, there has been only one axillary recurrence (0.8%) and 13 deaths, four of which were attributed to metastatic breast cancer and the rest to nonbreast-related causes.

They also concluded that axillary recurrence is rare after SNB alone especially in case of favorable patient or tumor characteristics (older age, ER positive and Her2 negative etc.) and standard use of adjuvant therapy.

The German Clinical Interdisciplinary Sentinel study was a large prospective randomized phase III trial performed in 33 German centers [28▪]. One thousand one hundred and eighty two patients with operable, clinically node negative and invasive breast cancer were equally randomized to either a strategy of standard axillary dissection (SAD) independent of the SNB finding (SAD arm, $n = 594$), or to a strategy of performing SAD only in case of a positive SNB finding or failure of sentinel node detection (control arm, $n = 588$), but observation only in patients with negative SNB. The trial was designed to exclude an absolute difference in relapse-free survival (RFS) of 5% after 5 years with sufficient confidence. After a maximum follow-up time of 115 months, a total of 93 RFS events (40/53) and a total of 53 death events (23/30) were observed. Comparisons of RFS yielded a hormone receptor of 1.44 (95% CI 0.95–2.18; $P = 0.084$), and of overall survival yielded a hormone receptor of 1.53 (0.88–2.66; $P = 0.13$). Paresthesia, lymphedema and pain were significantly less common in the SNB-negative group. It means that this trial also showed that the false-negative rate of SNB was negligible in terms of RFS and overall survival.

THE MANAGEMENT OF AXILLARY MACROMETASTASIS: PROSPECTIVE STUDY AND ANOTHER APPROACH

ACOSOG Z0011 is a prospective randomized trial to determine the effects of complete AND on survival of patients with SLN metastasis of breast cancer. Eight hundred and ninety one clinically node-negative patients, T1N0 and T2N0, with one or two H&E positive SLNs (Fig. 1) were randomized to no further axillary surgery or to axillary dissection.

The trial was conducted among 115 centers in the United States between 1999 and 2004. The sample size was not reached to the targeted enrollment (1900 women with final analysis after 500 deaths), but the trial was closed early because mortality rate was lower than expected, and final follow up for data analysis was completed in 2010. [The result was presented at ASCO2010 (Fig. 2) and published in JAMA 2011 [19,20▪▪]].

Type of operation was not associated with outcome in 5-year overall survival (92.5% in the sentinel group, versus 91.8% in the axillary group, Fig. 3) and 5-year disease-free survival (83.9% of the sentinel node group, versus 82.2% of the ALND group (Fig. 4). About 70% of participants in the axillary lymph node group had side effects such as shoulder pain, weakness, infection and tingling, versus 25%

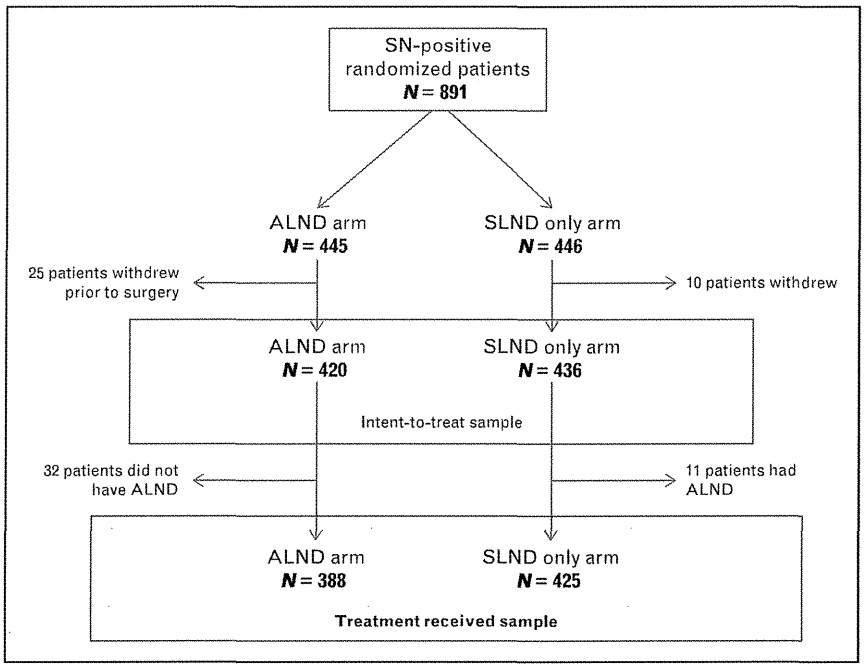

FIGURE 1. ACOSOSG Z0011 patient accrual. Adapted from [20▪▪].

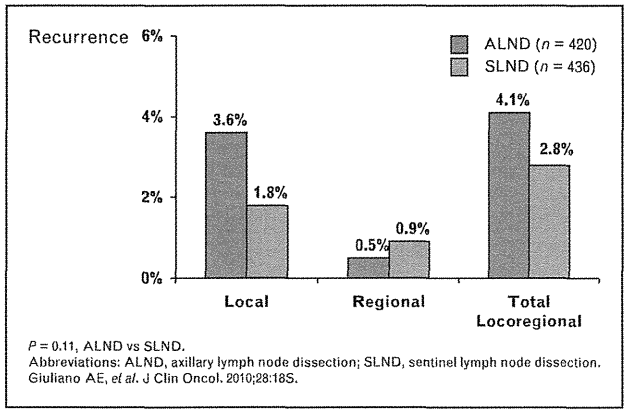

FIGURE 2. ACOSOG Z0011: 5-year recurrence rates. Adapted from [32].

in the sentinel group. Cases of lymphedema were significantly higher in the axillary group. Therefore, this study does not support the routine use of ALND in early nodal metastatic breast cancer in women undergoing breast conservation including whole breast irradiation.

Although additional axillary involvement was observed 27% in the ALND group, axillary recurrence rate was extremely low at 0.5% in the SLND group. There are several speculations. One is that systemic adjuvant treatment with hormonal therapy and/or chemotherapy has some effect in preventing locoregional recurrence. And the other is that tangent radiation fields used to the breast also covered the low axillary area and brought a therapeutic effect to the low axillary lymph nodes. Supporting this are the results of NSABP B-04, a trial comparing radical mastectomy (including ALND), total mastectomy without ALND, and total mastectomy with radiation therapy to the regional lymph nodes [27▪]. An update of this study with a median follow-up of 180 months (range 12–221 months) showed that long-term survival did not differ after axillary radiotherapy and axillary dissection. The only difference was better axillary disease control in the group with axillary dissection. In the Z0011 study, all the cases had the radiation to the residual breast, however, the radiation fields were not fully prescribed by the protocol, and the radiotherapy delivered is not fully specified.

There are several critiques for this study. First, the sample size is small because axillary recurrence was observed in two cases in the ALND group and four in the SLND group. Second, median follow up is 6.3 years and too short because most women (83%) had ER-positive cancers and would, thus, be expected to recur late.

From this study, AND may safely be omitted in breast conservation patients whose tumor size is 5 cm or less clinically node negative and who will have whole breast radiation and appropriate systemic adjuvant therapy.

There is another approach for sentinel node positive cases. Kim *et al.* [29] reported the significance of FDG-PET/CT to determine the indication of AND or SNB in breast cancer patients. They performed FDG-PET/CT scans in 137 biopsy-proven breast cancer patients planning to have an SNB to select patients for either AND (PET/CT N+) or SNB (PET/CT N0). In performing SNB, they also

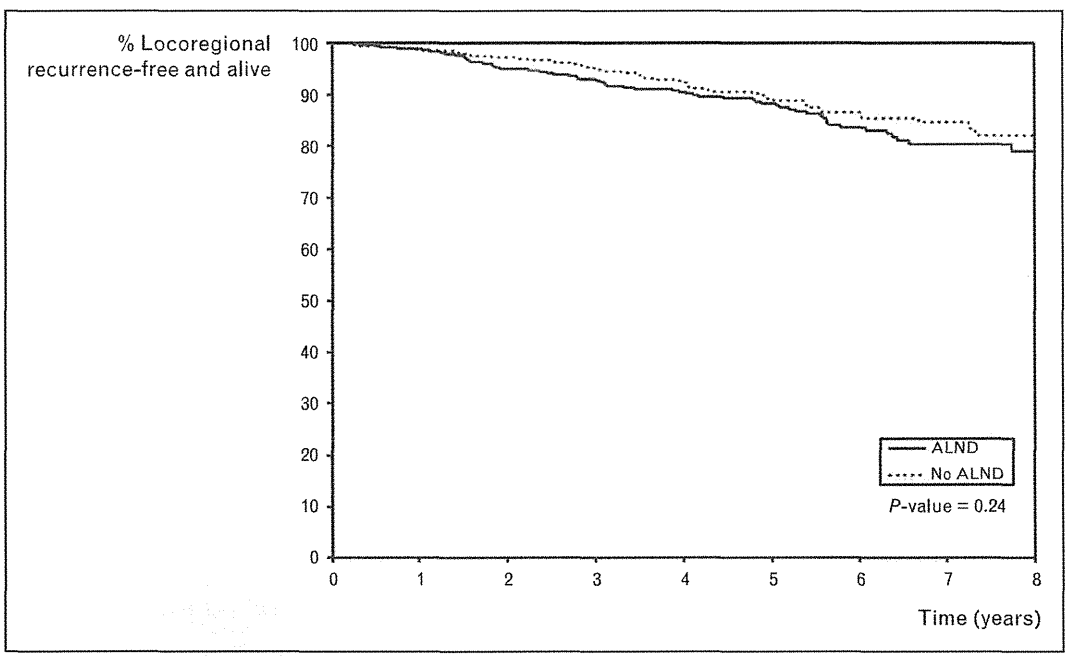

FIGURE 3. ACOSOG Z0011: recurrence-free survival. Adapted from [20▪▪].