Declaration of Financial Disclosure

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

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ORIGINAL ARTICLE

Nuclear, but not cytoplasmic, localization of survivin as a negative prognostic factor for survival in upper urinary tract urothelial carcinoma

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Abstract Survivin, a member of the inhibitor of apoptosis protein gene family, inhibits apoptosis and promotes mitosis. We determined whether nuclear or cytoplasmic localization of survivin could predict survival of patients with upper urinary tract urothelial carcinoma (UUTUC). Immunohistochemical staining for survivin was carried out on archival specimens from 125 consecutive patients with UUTUC who underwent radical nephroureterectomy. Nuclear and cytoplasmic staining of survivin was scored and compared with clinicopathologic features and cancer-specific survival (CSS). Nuclear expression of survivin was significantly correlated with tumor grade ($p<0.001$), lymphovascular invasion ($p=0.022$) and poor survival with an estimated 5-year CSS probability of 54 % for tumors with nuclear expression of survivin vs. 73 % for those without nuclear expression of survivin (hazard ratio=2.19; 95 % confidence interval=1.02–4.70; $p=0.043$). The 5-year cancer-specific survival rates of patients with cytoplasmic survivin-negative and -positive tumors were 66 and 67 %, respectively. There was no difference in survival between patients with cytoplasmic survivin-negative tumors and those with cytoplasmic survivin-positive tumors. Using univariate analysis, nuclear survivin expression, tumor grade, pathological T stage, pathological N stage, and lymphovascular invasion were the predictive variables for CSS. In contrast, cytoplasmic survivin expression had no prognostic relevance. These data suggest that nuclear accumulation of survivin represents biologic aggressiveness and that nuclear survivin is a negative prognostic marker in patients with resected UUTUC.

Keywords Survivin · Urothelial carcinoma · Upper urinary tract · Survival

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Introduction

Upper urinary tract urothelial carcinomas (UUTUC) are uncommon and account for only 5–10 % of urothelial carcinomas [1]. The primarily recognized prognostic factors are tumor stage and grade, whereas gender, age and the initial location of the tumor within the upper urinary tract are no longer accepted as prognostic factors [1]. Lymphovascular invasion [2–4], tumor necrosis [5, 6], tumor architecture [7], and concomitant carcinoma in situ [8, 9] are associated with higher risks of recurrent disease and cancer-specific mortality. Molecular markers such as microsatellite instabilities [10], E-cadherin, hypoxia-inducible factor-1α and a telomerase RNA component [11] have been shown to be useful for prognosis, although none of the markers has been externally validated.

Survivin, an inhibitor of apoptosis protein (IAP) family member that inhibits caspases and blocks cell death, is overexpressed in various human malignancies [12]. Survivin also plays a central role in cell division, and it is expressed in the nuclear or cytosolic pool in cancer cells [13]. Many studies have reported a correlation between survivin expression and an either unfavorable or favorable prognosis [14]. Recently,

nuclear expression of survivin has been reported to be associated with unfavorable outcomes in head and neck squamous cell carcinoma (SCC) [15–18], hepatocellular carcinoma [19, 20], esophageal SCC [21, 22], melanoma [23], glioblastoma [24], Merkel cell carcinoma [25], and bladder cancer [26–30]. Conversely, survivin nuclear positivity correlated with favorable prognoses in gastric [31], colorectal [32], and ovarian cancers [33, 34]. In non-small cell lung cancer, nuclear accumulation of survivin was a positive prognostic factor for survival in advanced disease [35], but a negative prognostic factor in patients with resected Stage I and II diseases [36].

To date, two studies have examined whether survivin expression has an impact on prognosis in patients with UUTUC. One study indicated no relationship between survivin expression and survival [37], whereas the other one showed that survivin expression was a poor prognostic factor [38]. In those studies, however, cells were considered survivin-positive when a distinct granular pattern was apparent within the cytoplasm of tumor cells. Therefore, we investigated the nuclear expression of survivin in patients with UUTUC and determined its prognostic relevance.

Materials and methods

Patients

We reviewed the clinical pathology archives of 181 consecutive patients who underwent radical nephroureterectomy and were diagnosed as having UUTUCs at the Sapporo Medical University Hospital from June 1995 through May 2010. Patients with a previous history of bladder cancer and patients with concomitant bladder cancer were excluded. Finally, a total of 125 patients were enrolled in this study. Informed consent was obtained from the patients to use the surgical specimens remaining after pathological diagnosis for the investigational study, which was approved by the Institutional Review Board for Clinical Research at our university. All hematoxylin and eosin stained slides were reviewed, and all of these specimens showed urothelial carcinoma. The median age at operation of the 89 male and 36 female patients was 69 years (range 32 to 88). Median follow-up was 69 months (range 6 to 192). The clinical stage was assigned using the American Joint Committee on Cancer TNM Staging System for Renal Pelvis and Ureter Cancer (7th ed., 2010). Tumor grading was assessed according to the 1973 World Health Organization classification. The patients' characteristics are shown in Table 1.

Table 1 Characteristics of the 125 patients

Characteristic	*N*	(%)
Median age (range)	69 (32–88)	
Median follow-up (months)	69	
Sex		
Male	89	(71)
Female	36	(29)
Side		
Right	54	(43)
Left	71	(57)
Primary site (main)		
Renal pelvis	75	(60)
Ureter upper	11	(9)
middle	10	(8)
lower	29	(23)
Tumor architecture		
Papillary	57	(46)
Sessile	66	(53)
Flat	2	(1)
Tumor grade (1973 WHO)		
G1	3	(2)
G2	53	(43)
G3	69	(55)
Pathological stage		
Stage 0a	16	(13)
Stage 0is	2	(1)
Stage I	17	(14)
Stage II	21	(17)
Stage III	50	(40)
Stage IV	19	(15)
Chemotherapy		
Neoadjuvant	10	(8)
Adjuvant	6	(5)

Immunohistochemistry and scoring

Sections (4 μm) of the formalin-fixed, paraffin-embedded tumor specimens were immunostained after heat-induced epitope retrieval in citrate buffer (pH6.0) using an autoclave with a polyclonal antibody against survivin (1:200, Novus Biologicals, Littleton, CO, USA). Subsequent incubations with a secondary biotinylated antibody, avidin-conjugated peroxidase complex, and chromogen were done on Ventana NexES (Ventana Medical Systems, Tucson, AZ, USA). The slides were then counterstained with hematoxylin, rinsed, dehydrated through graded alcohols into nonaqueous solution, and coverslipped with mounting media. Sections of colorectal adenocarcinoma were used as positive controls for survivin. Negative controls had the primary antibody replaced by buffer. All specimens were reviewed and scored independently using light microscopy in at least 5 areas at ×400 magnification by investigators who were blinded to clinicopathological data (TT and YH). Cases were scored positive when >10 % of the cells reacted

with the anti-survivin antibody, as proposed previously [26, 30].

Statistical analysis

We tested the relationships between nuclear or cytoplasmic survivin expression and the other clinicopathological parameters, i.e., the pathological T stage, pathological N stage, tumor grade and lymphovascular invasion by chi-square tests. Cancer-specific survival was assessed by the Kaplan–Meier method, and differences between two groups were compared using the log-rank test. Univariate and multivariate regression analyses according to the Cox proportional hazards regression model, with cancer-specific survival as the dependent variable, were used to evaluate the survivin expression as a potential independent prognostic factor. A value of $p<0.05$ was considered to indicate statistical significance. The calculations were performed using JMP™ software.

Results

Survivin expression in UUTUC and its associations with clinicopathological variables

Nuclear and cytoplasmic expression of survivin (Fig. 1) was found in 48 (38 %) and 30 (24 %) of the 125 cases, respectively. Coexistence of nuclear and cytoplasmic staining was observed in 15 cases (12 %). No normal urothelial cells were stained with the anti-survivin antibody either in the nucleus or in cytoplasm. Expression of nuclear and cytoplasmic survivin was positive in 20 (35 %) and 9 (11 %) of the 57 papillary tumors, respectively. In the 77 sessile tumors, expression of nuclear and cytoplasmic survivin was positive in 27 (41 %) and 19 (29 %), respectively. There was no relationship between tumor architecture and nuclear or cytoplasmic survivin expression. Nuclear survivin expression was significantly associated with higher tumor grade ($p<0.001$) and lymphovascular invasion ($p=0.022$), whereas no clinicopathological variables were linked to cytoplasmic survivin expression (Table 2).

Association of nuclear or cytoplasmic survivin expression with survival and recurrence

The 5-year cancer-specific survival rates of patients with nuclear survivin-positive and -negative tumors were 54 and 73 %, respectively (Fig. 2a). The 5-year cancer-specific survival rates of patients with cytoplasmic survivin-positive and -negative tumors were 67 and 66 %, respectively (Fig. 2b). There were significant differences in cancer-specific survival between patients with nuclear survivin-negative tumors and those with nuclear survivin-positive tumors (hazard ratio=2.19; 95 % confidence interval= 1.02–4.70; $p=0.043$) (Fig. 2a), but no significant differences between those with cytoplasmic survivin-negative tumors

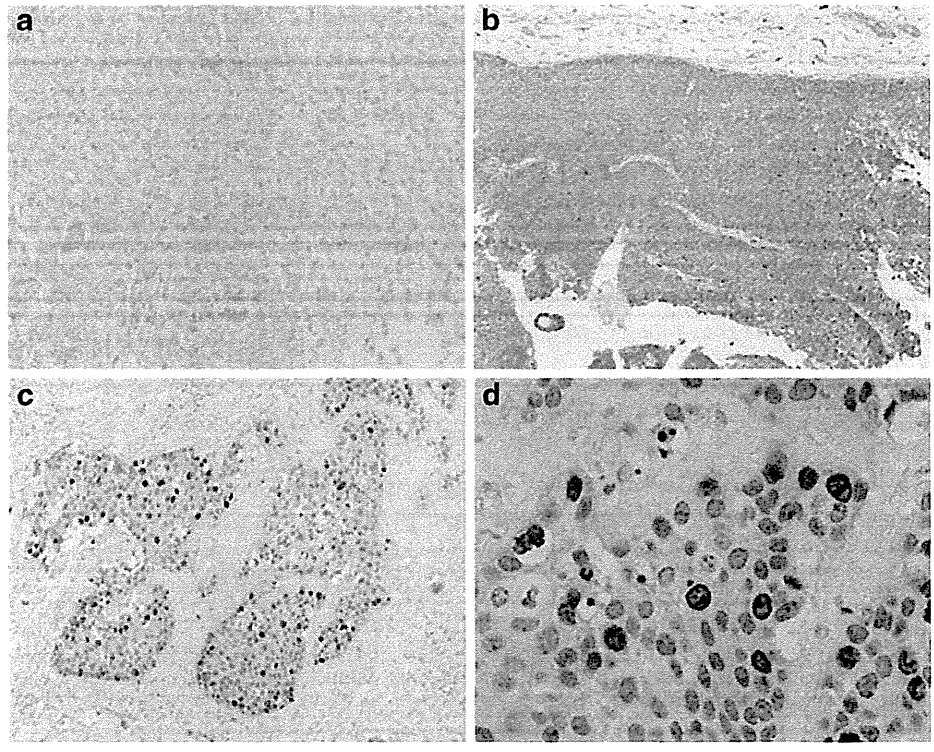

Fig. 1 Immunohistochemical staining for survivin in upper urinary tract urothelial carcinoma: **a** survivin-negative expression; **b** cytoplasmic survivin-positive expression; **c–d** nuclear survivin-positive expression

Table 2 Survivin staining patterns and pathological factors of patients with upper urinary tract urothelial carcinoma

Variable	Nuclear survivin expression			Cytoplasmic survivin expression		
	Positive (%)	Negative (%)	*p* value	Positive (%)	Negative (%)	*p* value
Pathological T stage						
pTis	1 (2)	1 (1)	0.058	2 (7)	0 (0)	0.222
pTa	5 (10)	11 (14)		4 (13)	12 (13)	
pT1	2 (4)	16 (21)		4 (13)	14 (15)	
pT2	9 (19)	14 (18)		4 (13)	19 (20)	
pT3	27 (56)	34 (44)		15 (50)	46 (48)	
pT4	4 (8)	1 (1)		1 (3)	4 (4)	
Pathological N stage						
pN0	42 (88)	70 (91)	0.107	26 (86)	87 (92)	0.656
pN1	1 (2)	5 (6)		2 (7)	3 (3)	
pN2	5 (10)	2 (3)		2 (7)	5 (5)	
Grade						
G1	0 (0)	3 (4)	<0.001	1 (3)	2 (2)	0.736
G2	11 (23)	42 (54)		11 (37)	42 (44)	
G3	37 (77)	32 (42)		18 (60)	51 (54)	
Lymphovascular invasion						
Negative	25 (52)	56 (73)	0.022	19 (63)	62 (65)	0.831
Positive	23 (48)	21 (27)		11 (37)	33 (35)	

and those with cytoplasmic survivin-positive tumors (hazard ratio=1.11; 95 % confidence interval=0.44–2.78; p=0.832) (Fig. 2b). A significant difference in CSS was observed between patients with nuclear survivin-positive tumors and those with survivin-negative tumors (hazard ratio=3.71; 95 % confidence interval=1.16–11.8; p=0.027) (Fig. 3), when the cutoff was set at 20 %.

In univariate analysis, the pathological T stage, pathological N stage, tumor grade, lymphovascular invasion, and nuclear survivin expression were associated with a poor prognosis (Table 3). In multivariate analysis, the independent factors of prognosis were the pathological T stage, pathological N stage and tumor grade (Table 3).

Discussion

Two studies have investigated the relationships between survivin expression and survival in patients with UUTUC. Nakanishi et al. [37] examined survivin expression in UUTUC using immunohistochemistry and its relationship with the prognosis, clinicopathologic parameters, bcl-2, p53 and proliferating cell nuclear antigen (PCNA) immunoreactivity. They found no correlation between survivin expression and prognosis, clinicopathologic findings, bcl-2, p53 or PCNA, and concluded the survivin cytoplasmic expression

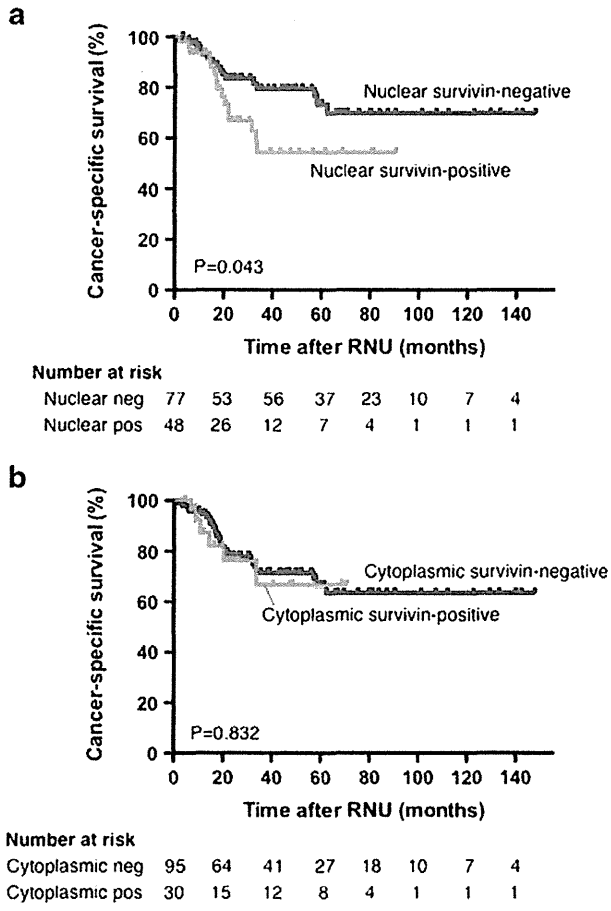

Fig. 2 Kaplan–Meier curves for cancer-specific survival rates according to **a** nuclear survivin expression status and **b** cytoplasmic survivin expression status. *RNU* radical nephroureterectomy

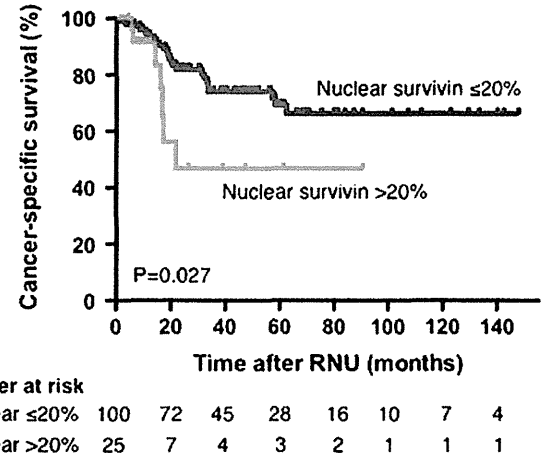

Fig. 3 Kaplan–Meier curves for recurrence-free survival rates according to nuclear survivin expression status (>20 % vs. ≤20 %). *RNU* radical nephroureterectomy

Table 3 Prognostic factors for cancer-specific survival in univariate and multivariate analyses

Factor	Univariate analysis		Multivariate analysis	
	HR (95 % CI)	*p* value	HR (95 % CI)	*p* value
Pathological T stage	2.76 (1.69–4.89)	<0.001	1.92 (1.09–3.59)	0.023
Pathological N stage	2.75 (1.75–4.09)	<0.001	2.00 (1.24–3.12)	0.006
Grade	6.02 (2.53–17.7)	<0.001	3.72 (1.42–11.7)	0.006
Lymphovascular invasion	2.18 (1.52–3.25)	<0.001	1.30 (0.85–2.04)	0.236
Nuclear survivin	1.61 (1.01–2.44)	0.049	1.32 (0.81–2.02)	0.261
Cytoplasmic survivin	1.05 (0.64–1.59)	0.834	0.90 (0.54–1.39)	0.652

did not predict prognosis in UUTUC [37]. Jeong et al. [38] investigated the expression of apoptosis-related markers, including survivin, by using immunohistochemistry and the association with the clinical outcomes of patients with UUTUC. They demonstrated that survivin expression, the apoptosis index, pathological T stage, and pathological N stage were significantly associated with disease-specific survival in multivariate analysis [38]. Thus, the prognostic value of cytoplasmic expression of survivin in tumor cells was different in these studies and is still controversial, although we found that cytoplasmic survivin expression had no impact on survival in patients with UUTUC. One of the reasons is the difficulty and ambiguity of immunohistochemical scoring, especially when cells are weakly stained in the cytoplasm.

Recent reports [26–30] concerning survivin expression in urothelial carcinoma of the bladder indicated that nuclear expression of survivin correlated with clinical outcome and prognostic factors. Two of those studies compared the predictive value of nuclear versus cytoplasmic expression of survivin in bladder cancer cells. Yin et al. [29] evaluated the expression profile of the major apoptosis regulators, including caspases, IAPs (survivin, livin, XIAP, etc.), APAF1, SMAC, and BCL2 in non-muscle-invasive bladder cancer by immunohistochemistry. They demonstrated that survivin nuclear, but not cytoplasmic, expression was the only apoptotic marker that correlated significantly with tumor grade, stage, and patient outcome [29]. Another study, by Skagias et al. [30], analyzed tissues from 80 bladder cancers, including both non-muscle- and muscle-invasive diseases, by immunohistochemistry. They found correlations between nuclear survivin expression and increased grade, stage and the probability of tumor recurrence, but no relationship between cytoplasmic survivin expression and any clinicopathological parameter [30]. Several studies [13, 39] have reported that the nuclear pool of survivin is involved in promoting cell proliferation, whereas the cytoplasmic pool of survivin may participate in controlling cell survival but not cell proliferation. Furthermore, it is known that several splice variants of survivin have differential intracellular localization, e.g., survivin-ΔEx3 in the nucleus and survivin-2B in the cytoplasm [40, 41]. Nouraee et al. [42] examined the expression pattern of survivin and its major splice variants (survivin-ΔEx3 and survivin-2B) and their prognostic values by reverse transcriptase polymerase chain reaction. They demonstrated that the expression of survivin and survivin-ΔEx3 was preferentially elevated in tumors with high grades, whereas survivin-2B expression was lower in high-grade tumors [42]. Nuclear expression of survivin, including survivin itself and survivin-ΔEx3, is positively correlated with tumor cell proliferation [41], which may explain the reason why nuclear survivin correlates with survival and prognostic factors in various cancers.

To the best of our knowledge, this is the first study in which the relationships between nuclear expression of survivin and most clinically relevant features of UUTUC were evaluated. In the present study, we demonstrated that the UUTUC patients with nuclear survivin-positive tumor cells had significantly shorter OS, than those whose tumors were nuclear survivin-negative. In contrast, cytoplasmic survivin expression had neither prognostic relevance nor any association with other clinicopathological variables. Although nuclear survivin expression was not an independent prognostic factor in multivariate analysis, it was linked to tumor grade and lymphovascular invasion. Several investigators have found the prognostic impact of tumor grade on survival [43–45], and two large, independent, and multicenter studies [2, 4] showed that lymphovascular invasion was an independent prognostic factor in UUTUC and was associated with established features of biologically aggressive disease [46]. Therefore, nuclear accumulation of survivin represents biologic aggressiveness, which may have a prognostic impact on survival in patients with UUTUC. However, we did not find an independently increased risk of cancer-specific mortality with nuclear survivin expression.

This study has several potential limitations. First are the limitations inherent in any retrospective data collection. Second, radical nephroureterectomy was performed by various surgeons over a long time period. Third, immunohistochemistry has inherent limitations such as reproducibility and reliability. The data should be validated by molecular biology methods.

In summary, the current results demonstrate a negative association between the nuclear expression of survivin and patient survival in UUTUC. We conclude that nuclear survivin expression may be a superior biologic and prognostic marker for UUTUC.

Conflict of interest The authors declare no conflict of interest.

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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).

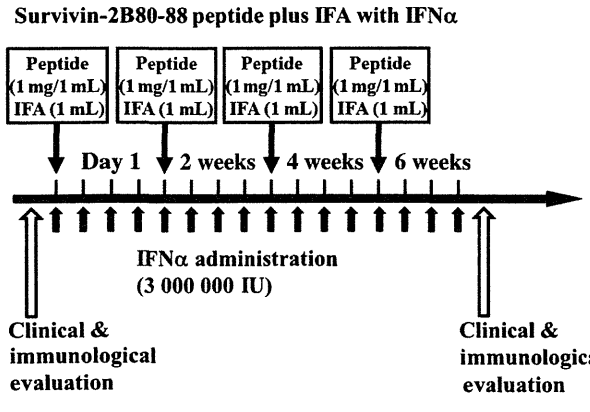

Fig. 1. Clinical protocol of study. Survivin-2B80-88 and incomplete Freund's adjuvant (IFA) were mixed immediately before vaccination. The patients were then vaccinated s.c. four times at 14-day intervals. In addition, α-interferon (IFNα) was given twice a week close to the site of vaccination. For this,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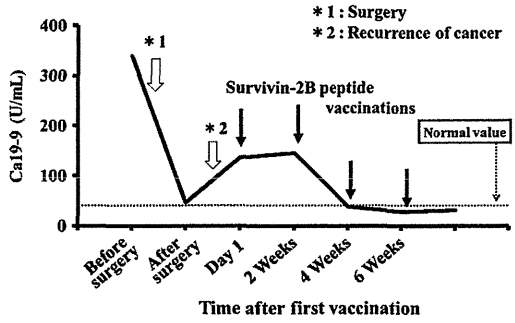

Fig. 2. Representative illustration of the clinical effect in patient No. 1 as assessed by the serum Ca19-9 level. Arrows indicate vaccinations with survivin-2B80-88 plus incomplete Freund's adjuvant with α-interferon (IFNα).

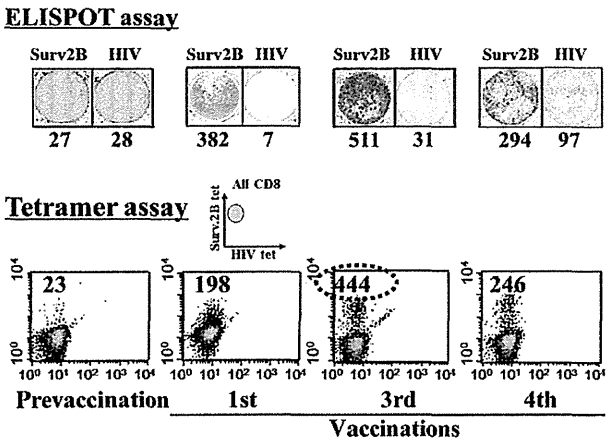

Fig. 3. Immunological analysis of CTL responses against HLA-A24 restricted survivin-2B80-88 peptide (surv2B) before and after vaccinations as assessed by enzyme-linked immunosorbent spot (ELISPOT) and tetramer (tet) analyses. Numbers in the ELISPOT assay indicate γ-interferon (IFNγ) secretion against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

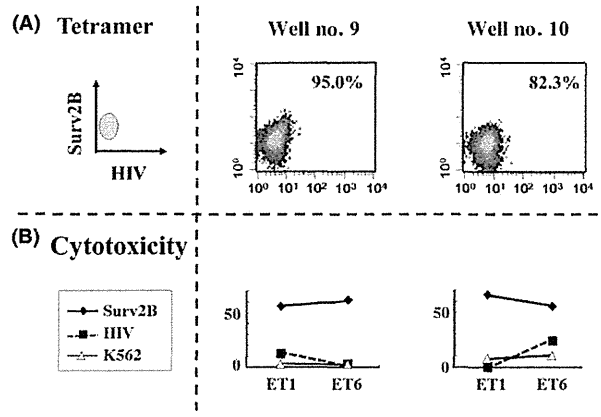

Fig. 4. Single-cell analysis of survivin-2B80-88 peptide tetramer-positive CD8 CTL cells). Survivin-2B80-88 peptide tetramer-positive CD8 T cells in Fig. 3 (circled) were sorted and cultured at 1, 3, and 10 cells /well for 7–10 days. Subsequently, clonal CTL cells were examined for their reactivity to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

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ABSTRACT

DNA methyltransferase 1 (Dnmt1) is essential for the maintenance of hematopoietic and somatic stem cells in mice; however, its roles in human cancer stem-like cells (CSCs)/cancer-initiating cells (CICs) are still elusive. In the present study, we investigated DNMT1 functions in the maintenance of human colon CSCs/CICs using the human colon cancer cell line HCT116 (HCT116 w/t) and its *DNMT1* knockout cell line (*DNMT1*$^{-/-}$). The rates of CSCs/CICs were evaluated by side population (SP) analysis, ALDEFLUOR assay and expression of CD44 and CD24. SP, ALDEFLUOR-positive (ALDEFLUOR^{+}) and CD44-positive and CD24-positive (CD44^{+}CD24^{+}) cell rates were lower in *DNMT1*$^{-/-}$ cells than in HCT116 w/t cells. Since CSCs/CICs have higher tumor-initiating ability than that of non-CSCs/CICs, the tumor-initiating abilities were addressed by injecting immune deficient (NOD/SCID) mice. *DNMT1*$^{-/-}$ cells showed less tumor-initiating ability than did HCT116 w/t cells, whereas the growing rate of *DNMT1*$^{-/-}$ cells showed no significant difference from that of HCT116 cells both *in vitro* and *in vivo*. Similar results were obtained for cells in which *DNMT1* had been transiently knocked-down using gene-specific siRNAs. Taken together, these results indicate that DNMT1 is essential for maintenance of colon CSCs/CICs and that short-term suppression of *DNMT1* might be sufficient to disrupt CSCs/CICs.

Introduction

Cancer tissues are composed from several heterogeneous cancer cells and small population of cancer cells are supposed to have higher tumor-initiating ability. These higher tumorigenic populations are named "cancer stem-like cells (CSCs)" or "cancer initiating cells (CICs)". CSCs/CICs are defined as small population of cancer cells which has (1) higher tumor-initiating ability, (2) self-renewal, (3) differentiation. (Clarke et al., 2006; Dalerba et al., 2007; Huang et al., 2009; O'Brien et, al., 2007; Ricci-Vitiani et al., 2007) CSCs/CICs have been reported to be resistant to chemotherapy, radiotherapy and certain molecular targeting therapies (Dean et al., 2005); thus, elucidation of the molecular mechanisms of the maintenance of CSCs/CICs should be useful for establishing efficient CSC/CIC targeting treatment.

Trowbridge et al. reported that hematopoietic stem cell self-renewal was abrogated by conditional gene knockout of *Dnmt1*, while the mature differentiated hematopoietic lineage was not affected. (Trowbridge et al., 2009) *Dnmt1* is essential for maintenance of the leukemia stem cells of bilinear myeloid-B lymphoid leukemia induced by transduction of *c-Myc* and *Bcl-2*. (Broske et al., 2009) *Dnmt1* was also shown to be essential for the self-renewal of skin progenitor cells. (Sen et al., 2010) Results of these studies indicate that DNMT1 is essential for self-renewal of progenitor or stem cells. However, no study has been mentioned the relation between *DNMT1* and CSCs/CICs in solid tumors including colon cancer.

In the present study, we investigated the functions of *DNMT1* in maintenance of colon CSCs/CICs. We investigated DNMT1 functions by using a *DNMT1*$^{-/-}$ cell line and also cells in which *DNMT1* was transiently knocked-down by siRNAs. Permanent DNMT1 gene knockout and transient *DNMT1* gene knock down by specific siRNAs reduced the population of CSCs/CICs. These results of this study suggest that *DNMT1* has an essential role in maintenance of CSCs/CICs, and transient inhibition of DNMT1 might be sufficient to eradicate CSCs/CICs.

Abbreviations: CSC, cancer stem-like cell; CIC, cancer-initiating cell; DNMT1, DNA methyltransferase 1; SP, side population; MP, main population.

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Materials and methods

Cell lines

The colon adenocarcinoma cell lines wild-type HCT116 and SW480 were kind gifts from Dr. K. Imai (Sapporo, Japan). A *DNMT1* knockout HCT116 cell line (*DNMT1*$^{-/-}$) that lacks exons 2–5 of *DNMT1* (Rhee et al., 2000) was a kind gift from Dr. B. Vogelstein (Baltimore, MD). HCT116 and *DNMT1*$^{-/-}$ cells were cultured in McCoy's 5A modified medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Grand Island, NY). SW480 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (SIGMA) supplemented with 10% FBS.

Xenograft transplantation

All mouse procedures were performed in accordance with institutional protocol guidelines at Sapporo Medical University School of Medicine. Serially diluted HCT116 w/t and *DNMT1*$^{-/-}$ cells were mixed with Matrigel (BD, Franklin Lakes, NJ) at a 1:1 volume and injected subcutaneously into the backs of 4–8-week-old female non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice. Tumor size was assessed weekly.

RT-PCR analysis and quantitative real-time PCR analysis

RT-PCR analysis was performed as described previously. (Inoda et al., 2009) Primer pairs used for RT-PCR analysis were 5′-CATGATG GAGACGGAGCTGA-3′ and 5′-ACCCCGCTCGCCATGCTATT-3′ for *SOX2* with an expected PCR product size of 410 base pairs (bps), 5′-CC TGGGGCTGCTGCTGTTTATTA-3′ and 5′-TACCTGGTGATTTGCCACAA-3′ for *PROM1*(*CD133*) with an expected PCR product size of 208 bps, 5′-ATCGCCTCTCTCCGTTTGGTA-3′ and 5′-TGGACTCATCCGATTTGGCT-3′ for *DNMT-1* with an expected PCR product size of 757 bps, 5′-TGGAGAAGGAGAAGCTGGAGCAAAA-3′ and 5′-GGCAGATGGTCGTTTG GCTGAATA-3′ for *POU5F1* with an expected PCR product size of 163 bps, 5′-CTCTTCCTCAAACCGTCTGC-3′ and 5′-GATCGGAGGCTAAG CAACTG-3′ for *LGR5* with an expected PCR product size of 181 bps and 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCT GTA-3′ for *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) with an expected product size of 452 bps. *GAPDH* was used as an internal control. PCR amplification was performed in 20 μl of PCR mixture containing 1 μl of cDNA mixture, 0.5 μl of Taq DNA polymerase (QIAGEN) and 4 pmol of primers. The PCR mixture was initially incubated at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 15 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s.

Side population (SP) analysis, ALDEFLUOR assay and flow cytometry

Side population (SP) analysis was performed as described previously with some modifications. (Goodell et al., 1996; Inoda et al., 2011) Hoechst 33342 (Lonza, Walkersville, MD) dye was used at the concentration of 1.25 μg/ml for HCT116 and *DNMT1*$^{-/-}$. Stained cells were analyzed by a BD FACS Aria II Cell-Sorting System (BD).

Aldehyde dehydrogenase (ALDH) activity was detected using an ALDEFLUOR assay kit (StemCell Technologies) according to the manufacturer's protocol. (Ginestier et al., 2007) Cells stained by bodipy-aminoacetaldehyde (BAAA) at 1.5 mM and incubated for 30 min at 37 °C were analyzed by a BD FACS Aria II and BD FACSDiva software ver. 6.1 (BD).

CD44 and CD24 were detected using anti-CD44-APC antibody and anti-CD24-FITC antibody (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were stained with these antibodies and analyzed using a FACS Aria II.

DNMT1 mRNA knockdown

A *DNMT1* gene knockdown experiment was performed using small interfering RNA (siRNA). *DNMT1* siRNA duplexes were designed and synthesized using the BLOCK-it RNAi designer system (Life technologies). The oligonucleotide encoding DNMT1 siRNA i was 5′-AA AGATGGACAGCTTCTCATTTGTC-3′ and that encoding siRNA ii was 5′-AAATATGGGCGCATACTCGGGACTG-3′. Negative control siRNA was obtained from Invitrogen. Cells were seeded at 50% confluence, and transfections were carried out using Lipofectamine RNAi max (Life technologies) in Opti-MEM according to the manufacturer's instructions.

Bisulfite pyrosequencing

Genomic DNAs of SP and MP cells were modified with sodium bisulfite using an EpiTect Bisulfite kit (Qiagen), and bisulfite pyrosequencing analysis was done as described previously (Yamamoto et al., 2008). Primer sequences for LINE-1 methylation were the same as those described previously. (Yamamoto et al., 2008) Primer sequences for Alu Yb8 and centromeric satellite-α of chromosome 1 (Sat-α) were the same as those described previously (Igarashi et al., 2010).

Statistical analysis

In the xenograft model, cell growth *in vitro* and MIB-1 labeling index, samples were analyzed using Student's *t*-test, with $P<0.05$ conferring statistical significance.

Results

Lower rates of CSCs/CICs in DNMT1$^{-/-}$ cells

DNMT1 has been described to be essential for self-renewal of progenitor or stem cells. (Trowbridge et al., 2009; Broske et al., 2009; Sen et al., 2010). We therefore hypothesized that DNMT1 has also role in maintenance of human colon CSCs/CICs. CSC/CIC population in HCT116 *DNMT1*$^{-/-}$ cells and HCT116 w/t cells were analyzed by SP analysis, ALDEFLUOR assays and expression of cell surface CSC/CIC makers. Putative CSC/CIC including *SOX2*, *POU5F1*, *LGR5* and *PROM1* were analyzed by RT-PCR. (O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Ben-Porath et al., 2008; Vermeulen et al., 2008) HCT116 w/t cells expressed *SOX2* and *PROM1* (*CD133*), representative markers for colon CSCs/CICs; however, SOX2 and PROM1 were undetectable in *DNMT1*$^{-/-}$ cells (Fig. 1A). The other CSCs/CICs markers including *POU5F1* and *LGR5* were not detectable in both HCT116 w/t and *DNMT1*$^{-/-}$ cells by RT-PCR (data not shown).

Since CSCs/CICs were enriched in SP cells in several malignancies (Inoda et al., 2011; Kondo et al., 2004), we evaluated HCT116 w/t cells and *DNMT1*$^{-/-}$ cells by SP analysis. The SP cell rates in HCT116 w/t cells was 1.2%, whereas the SP cell rates in *DNMT1*$^{-/-}$ cells were 0.1% (Fig. 1B). The SP population was completely inhibited by Verapamil an ABCG2 transporter inhibitor, indicating that the SP population was specific for ABCG2 transporter (Fig. 1B). Since HCT116 SP cells have not been described to be enriched with CSCs/CICs, we investigated the presence of CSCs/CICs in HCT116 SP cells by RT-PCR and tumorigenicity in NOD/SCID mice (Figs. 1C, D). HCT116 SP cells expressed higher levels of *SOX2* and *PROM1* than those in HCT116 MP cells (Fig. 1C) and HCT116 SP cells showed higher tumor-initiating ability than that of HCT116 MP cells (Fig. 1D).

We also performed other methods of isolating CSCs/CICs. It has been reported that colon CSCs/CICs could be detected using ALDEFLUOR assay and surface marker analysis such as CD24 and CD44. (Huang et al., 2009; Kemper et al., 2010; Vermeulen et al., 2008; Yeung et al., 2010) ALDEFLUOR-positive (ALDEFLUOR^{+}) cell rate was 2.6% in HCT116 w/t cells, and that population was inhibited by an ALDH inhibitor (Fig. 1E). On the other hand, ALDEFLUOR^{+} cell rate in *DNMT1*$^{-/-}$

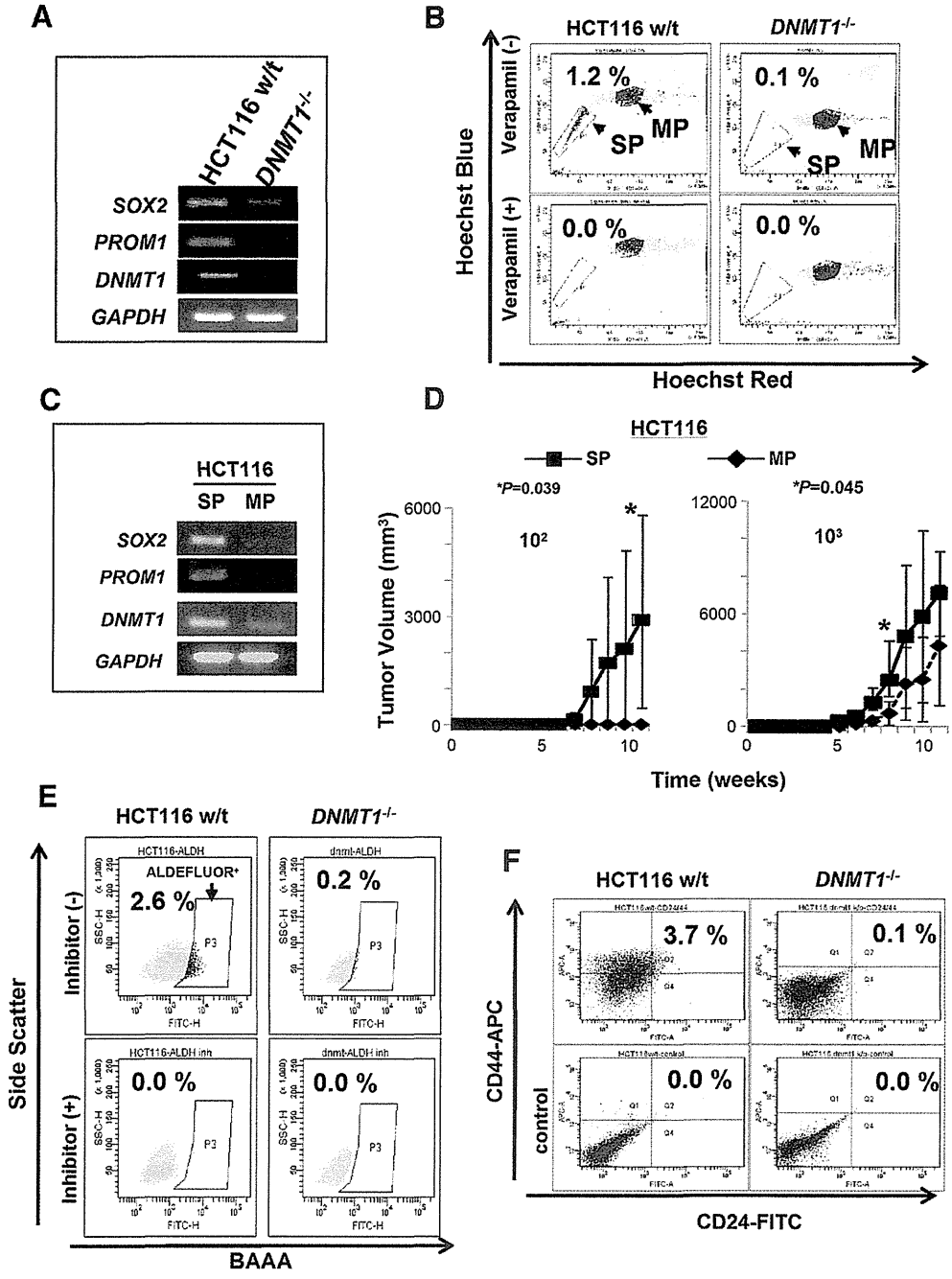

Fig. 1. *DNMT1*$^{-/-}$ cells contain lower CSCs/CICs than HCT116 w/t cells. A. RT-PCR of CSCs/CICs markers in HCT116 w/t and *DNMT1*$^{-/-}$ cells. B. Isolation of SP cells from colon cancer cell lines. HCT116 w/t and *DNMT1*$^{-/-}$ cells were stained with Hoechst 33342 dye and analyzed using a FACS Aria II cell sorter. SP: side population, MP: main population. C. RT-PCR of CSC/CIC markers in HCT116 SP and MP cells. D. Tumor growth of HCT116 SP and MP cells. 10^2 and 10^3 HCT116 SP and MP cells were inoculated subcutaneously into the backs of NOD/SCID mice, and tumor growth was measured weekly. Data represent means ± SD. Differences between HCT116 SP and MP cells were examined for statistical significance using Student's *t*-test. *P values. E. ALDEFLUOR Assay. ALDH activity was detected using the ALDEFLUOR assay kit as described in Materials and methods. A specific inhibitor of ALDH is diethylaminobenzaldehyde (DEAB). Stained cells were analyzed using a FACS Aria II cell sorter. F. Expression of CD44 and CD24. The numerical value in the dots plot graph is $CD24^{+}44^{+}$ cells rate. Stained cells were analyzed using a FACS Aria II cell sorter.

cells was 0.2%. Cell surface CD24 expression was decreased and CD44 expression greatly decreased in *DNMT1*$^{-/-}$ cells compared with that in HCT116 w/t cells (Fig. 1F). CD24/44-double positive population was 3.7% in HCT116 w/t cells and 0.1% in *DNMT1*$^{-/-}$ cells, respectively.

Lower tumor-initiating ability of DNMT1$^{-/-}$ *cells*

CSCs/CICs are known to have greater tumor-initiating ability than that of non-CSCs/CICs (Al-Hajj et al., 2003), we therefore evaluated the tumorigenicities of HCT116 w/t and *DNMT1*$^{-/-}$ cells. HCT116 w/t cells could initiate tumors with a minimum of 10^2 cells; however, 10^5 cells were needed to initiate tumors with *DNMT1*$^{-/-}$ cells (Table 1). Volumes of tumors derived from HCT116 w/t cells were also significantly higher than those of tumors derived from *DNMT1*$^{-/-}$ cells (Fig. 2A). The histology of tumors derived from each HCT116 w/t and *DNMT1*$^{-/-}$ cells showed high-grade poorly differentiated adenocarcinomas, and no significant histological difference was observed (Fig. 2B).

We investigated the growth rates of HCT116 w/t cells and *DNMT1*$^{-/-}$ cells. There was no significant difference between growth rates *in vitro* of HCT116 w/t cells and *DNMT1*$^{-/-}$ cells (Fig. 2C). No significant difference in MIB-1 labeling index, which represents cells undergoing the cell cycle, was observed in tumors derived from

Table 1
Tumor initiating ability in HCT116 and DNMT1 knockdown cells.

Cell lines	Percentage of SP cells	10^5	10^4	10^3	10^2	10^1
HCT116 w/t	1.2%	5/5	5/5*	3/5	2/5	n.d.
HCT116 *DNMT1*$^{-/-}$	0.1%	5/5	0/5	0/5	0/5	n.d.
HCT116 control siRNA	0.8%	n.d.	5/5*	5/5*	5/5*	0/5
DNMT1 siRNA	0.0%	n.d.	1/5	0/5	0/5	0/5
SW480 control siRNA	2.2%	n.d.	5/5	5/5*	1/5	0/5
DNMT1 siRNA	0.2%	n.d.	5/5	1/5	0/5	0/5

The analysis was completed 10 weeks following injection. Data are expressed as number of tumors formed/number of injections. Differences between HCT116 w/t cells and DNMT1$^{-/-}$ cells were examined for statistical significance using Student's *t*-test. *P<0.05, w/t: wild type, *DNMT1*$^{-/-}$: *DNMT1* knock out cell, n.d.: not done.

HCT116 w/t cells and *DNMT1*$^{-/-}$ cells (Fig. 2D). These observations indicate that *DNMT1*$^{-/-}$ cells sustain growth ability both *in vitro* and *in vivo*; however, they have very little tumor-initiating ability compared with that of HCT116 w/t cells.

DNMT1 gene knockdown experiments

DNMT1$^{-/-}$ cell phenotypes were confirmed by transient gene knockdown using gene-specific siRNAs. We designed two different siRNAs and confirmed suppression of *DNMT1* mRNA by RT-PCR (Fig. 3A). Transfection of siRNAs decreased the expression levels of *SOX2* and *PROM1* (Fig. 3A). Transfection of siRNAs drastically decreased SP cell ratios of HCT116 w/t cells (Fig. 3B). Furthermore, siRNA transfection decreased the ratio of ALDEFLUOR$^+$ cells in HCT116 w/t cells (Fig. 3C). CD24/44-double positive population was also decreased in HCT116 *DNMT1*-knockdown cells (Fig. 3D). We

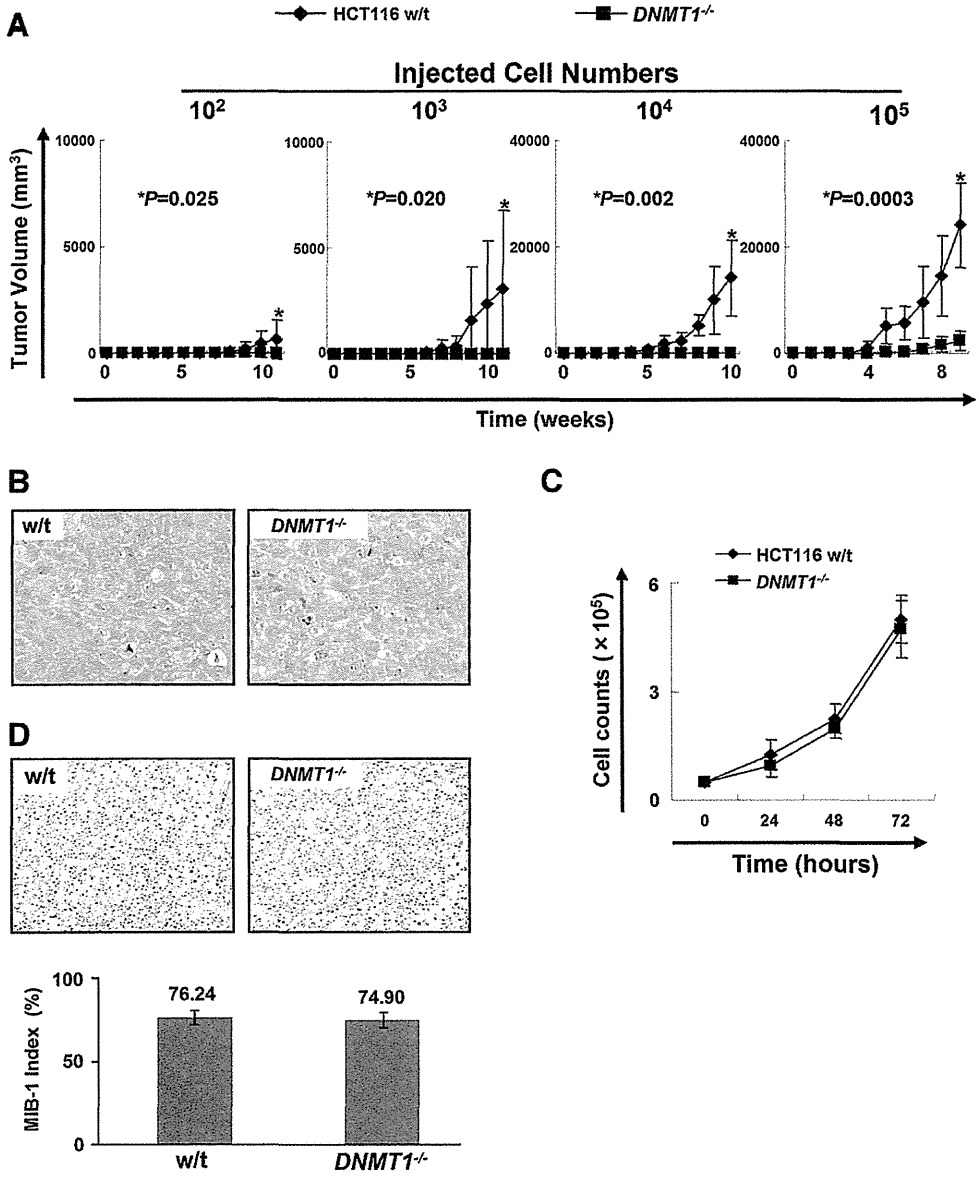

Fig. 2. Lower tumor-initiating ability of *DNMT1*$^{-/-}$ cells. A. Tumor growth of HCT116 w/t and *DNMT1*$^{-/-}$ cells. 10^2, 10^3, 10^4 or 10^5 HCT116 w/t cells and *DNMT1*$^{-/-}$ cells were inoculated subcutaneously into the backs of NOD/SCID mice, and tumor growth was measured weekly. Data represent means ± SD. Differences between HCT116 w/t and *DNMT1*$^{-/-}$ cells were examined for statistical significance using Student's *t*-test. *P values. B. Representative histology of tumors derived from HCT116 w/t and *DNMT1*$^{-/-}$ cells. Tumors derived from HCT116 w/t and DNMT1 −/− cells were stained with hematoxylin and eosin. Magnification, ×400. C. *In vitro* cell growth of HCT116 w/t and *DNMT1*$^{-/-}$ cells. 10^5 of HCT116 w/t and DNMT1 −/− cells were seeded into 6-well plates, and the cells were counted every 24 h. Data represent means ± SD. D. MIB-1 labeling index of HCT116 w/t and *DNMT1*$^{-/-}$ cells. Tumors derived from HCT116 w/t and DNMT1 −/− cells were stained with MIB-1. Magnification, ×100. The MIB-1 labeling indexes were calculated by the averages of MIB-1 positivities of 10 high power fields (H.P.F.). Data represent means ± SD.

operated xenograft model experiments using HCT116 *DNMT1*-knockdown cells and control cells. The engraftment of tumor derived from *DNMT1*-knockdown cells needed 10^4 cells (Table 1) and tumor grew slowly (Fig. 3E).

We performed similar gene knockdown experiments using another human colon cancer cell line, SW480, to generalize the effects of gene knockdown of *DNMT1*. Expression levels of *SOX2* mRNA were reduced by DNMT1 mRNA knockdown in SW480 cells (Fig. 4A). The ratios of SP cells and ALDEFLUOR$^+$ cells and the CD24$^+$44$^+$ cells were reduced by *DNMT1* mRNA knockdown in SW480 cells (Figs. 4B, C and D). Both tumor-initiating ability and tumor-growing speed were similarly reduced (Fig. 4E).

Expression of DNMT3A and DNMT3B, and methylation status of LINE-1, Alu and Sat-alfa sequences in SP and MP cells

To address the genome methylation status are related to the maintenance of CSC/CIC, we investigated the expression of DNMT3A and DNMT3B, *de novo* DNA methyltransferases, and the methylation status of repetitive sequence of genome such as LINE-1, Alu and Sat-α genes as surrogate genome methylation markers by bisulfite pyrosequencing. The expression levels of *DNMT3A* and *DNMT3B* did not show any difference in SP and MP cells derived from HCT116 cells (Fig. 5A). The methylation status of LINE-1, Alu Sat-α did not show any significant difference in HCT116 SW480 SP cells and MP cells (Figs. 5B and C).

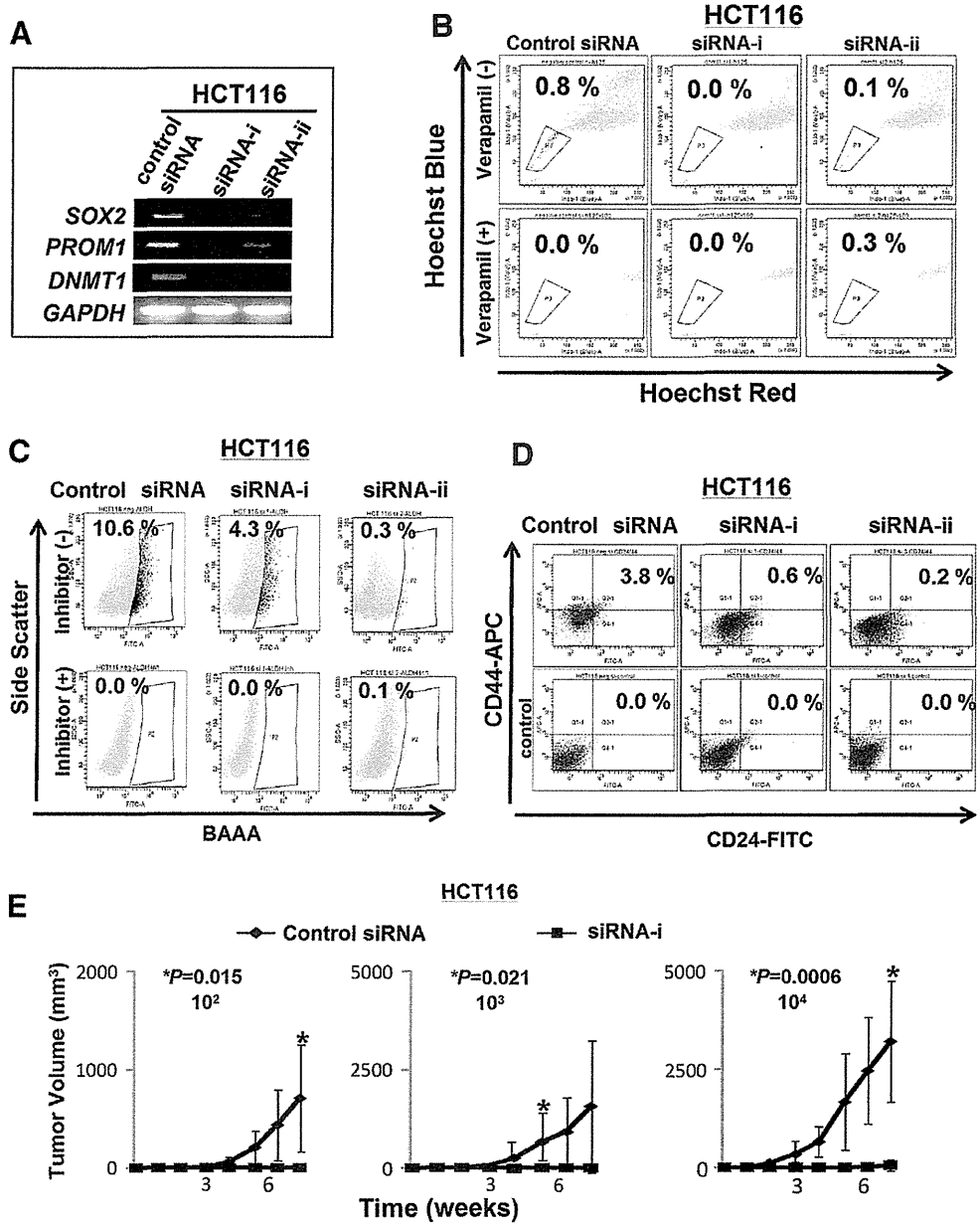

Fig. 3. *DNMT1* gene knockdown experiments in HCT116 cells. A. RT-PCR of *DNMT1* and CSCs/CICs markers. The mRNA expression levels of *DNMT1* and CSCs/CICs markers were evaluated by RT-PCR. Total RNAs were isolated 48 h after transfection of a negative control or *DNTM1*-specific siRNA i and siRNA ii. B. Isolation of SP cells from colon cancer cell lines. HCT116 *DNMT1*-knockdown cells and control cells were stained with Hoechst 33342 dye and analyzed. C. ALDEFLUOR Assay. ALDH activity was detected using the ALDEFLUOR assay kit 48 h after transfection of negative control or *DNMT1* specific siRNAs. D. Expression of CD44 and CD24. The numerical value in the dots plot graph is CD24$^+$44$^+$ cells rate. Stained cells were analyzed using a FACS Aria II cell sorter. E. Tumor growth of HCT116 *DNMT1*-knockdown cells and control cells. 10^2, 10^3 or 10^4 HCT116 *DNMT1*-knockdown cells and control cells were inoculated subcutaneously into the backs of NOD/SCID mice, and tumor growth was measured weekly. Data represent means ± SD. Differences between HCT116 *DNMT1*-knockdown cells and control cells were examined for statistical significance using Student's *t*-test. *P values.

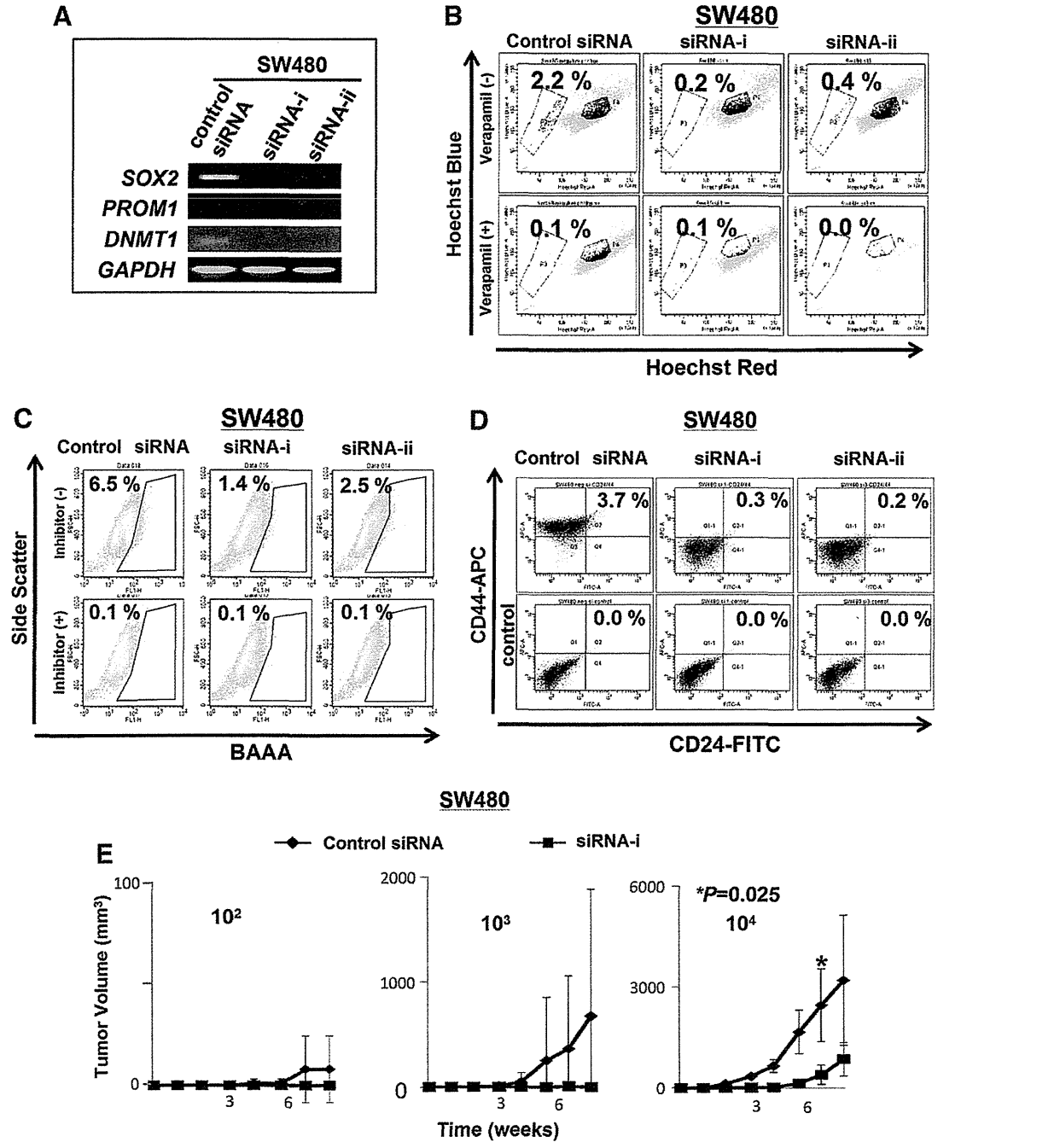

Fig. 4. *DNMT1* gene knockdown experiments in SW480 cells. A. RT-PCR of *DNMT1* and CSCs/CICs markers. The mRNA expression levels of *DNMT1* and CSCs/CICs markers were evaluated by RT-PCR. Total RNAs were isolated 48 h after transfection of a negative control or *DNTM1*-specific siRNA i and siRNA ii. B. Isolation of SP cells from colon cancer cell lines. SW480 *DNMT1*-knockdown cells and control cells were stained with Hoechst 33342 dye and analyzed. C. ALDEFLUOR Assay. ALDH activity was detected using the ALDEFLUOR assay kit 48 h after transfection of negative control or *DNMT1* specific siRNAs. D. Expression of CD44 and CD24. The numerical value in the dots plot graph is $CD24^{+}44^{+}$ cells rate. Stained cells were analyzed using a FACS Aria II cell sorter. E. Tumor growth of HCT116 *DNMT1*-knockdown cells and control cells. 10^2, 10^3 or 10^4 SW480 *DNMT1*-knockdown cells and control cells were inoculated subcutaneously into the backs of NOD/SCID mice, and tumor growth was measured weekly. Data represent means ± SD. Differences between SW480 *DNMT1*-knockdown cells and control cells were examined for statistical significance using Student's *t*-test. *P values.

Discussion

In the present study, we found that *DNMT1*$^{-/-}$ cells show much lower tumor-initiating ability than that of HCT116 w/t cells *in vivo*, whereas the growth rate of *DNMT1*$^{-/-}$ cells *in vitro* was not affected compared to that of HCT116 w/t cells, being consistent with a previous report. (Rhee et al., 2002) To address this controversial phenomenon of tumor-initiating ability and cell growth potential, we hypothesized that the CSC/CIC population is reduced in *DNMT1*$^{-/-}$ cells. We evaluated *DNMT1*$^{-/-}$ cells by SP analysis and clarified that *DNMT1*$^{-/-}$ cells contain a very small CSCs/CICs population, compared with HCT116 w/t cells. *DNMT1*$^{-/-}$ cells still have a very low tumor-initiating ability.

The *DNMT1*$^{-/-}$ cells we used in this study lack exons 2–5 of the *DNMT1* gene (Rhee et al., 2000), and *DNMT1*$^{-/-}$ cell has been proved to be a hypomorph that express a truncated form of the DNMT1 C-terminal DNA methyltransferase catalytic domain at a low level. (Egger et al., 2006). Therefore, a low level DNMT1 expression might be sufficient for cell viability. On the other hand, a low expression level might not be sufficient for maintenance of CSCs/CICs. In previous studies, human cancers have been found to overexpress DNMT1. (De