showed that cytosolic glycine decarboxylase was related to the tumor-initiating ability of lung cancers [9]. However, this is the first report that mitochondria-related gene has tumorigenic potential.

The product of the *SMCP* gene localizes to the capsule associated with the mitochondrial outer membranes and is thought to function in the organization and stabilization of the crescent structure of the sperm's mitochondrial sheath [41]. Sperm mitochondria differ in morphology and subcellular localization from those of somatic cells. They are elongated, flattened, and arranged circumferentially to form a helical coiled sheath in the midpiece of the sperm flagellum. In this study, we investigated the distributions of *SMCP* protein, and we found that GFP-fused SMCP protein is expressed in the mitochondria of lung carcinoma cells. Since SMCP is expressed in the testis and cancer cells, SMCP is a novel cancer-testis (CT) gene [51]. The testis is also an immune-privileged site [52]. Therefore, CT antigens are highly immunogenic and are promising targets for cancer immunotherapy [53–55]. Some cancer–testis antigens have been isolated by analyzing a testis cDNA expression library with cancer patients' sera [54]. Although *SMCP* has not been reported by screening using cancer patients' sera, SMCP was reported to be recognized by sera from rats immunized by sperm [56]. Thus, SMCP might be immunogenic to the humoral immune system. Indeed, we could detect anti-SMCP antibody in cancer patients' sera by an ELISA assay using SMCP recombinant protein (unpublished data). Anti-SMCP antibody might therefore be a new useful biomarker for detection of CSCs/CICs that is related to prognosis of cancer patients.

RACE and RT-PCR analysis revealed that the transcript of SMCP in CSCs/CICs was a variant form (SMCP vt2). SMCP vt2 lacks exon 1 and has only one exon 2 which has 889 base pair extension to the 5′-end. Since wild-type SMCP (SMCP vt1) and SMCP vt2 share the same coding sequence, the SMCP protein structures in CSCs/CICs and the testis should be same. However, the transcriptional start point of SMCP vt2 is approximately 6000 base pairs downstream of the transcriptional start point of SMCP vt1. Thus, the transcription factor and promoter that are responsible for the transcription of SMCP vt 2 should be different from those of SMCP vt 1. Further molecular analysis is needed.

In this study, overexpression of SMCP enhanced tumorigenicity *in vivo*. Moreover, transient knockdown of *SMCP* mRNA by SMCP-specific siRNA completely abrogated the tumorigenicity of LHK2 bulk cells and even SP cells. These observations indicate that SMCP has a pivotal role in tumorigenicity of lung cancer; however, its exact molecular mechanisms are still elusive.

In summary, we identified a novel variant form of the sperm-specific antigen *SMCP* that is expressed in CSCs/CICs and showed that *SMCP* plays a role in lung CSC/CIC tumorigenicity. Since *SMCP* is expressed in cancer tissues but not in normal tissues except for the testis, *SMCP* might be a novel CSC/CIC marker and a promising and potential target of CSC/CIC-targeting therapy.

Supporting Information

Table S1 Summary of upregulated genes in LHK2 SP cells. The summary of upregulated genes in SP cells derived from LHK2 cells. Cy5/Cy3: SP cells were labeled with Cy5 and MP cells were labeled with Cy3. Cy3/Cy5: SP cells were labeled with Cy3 and MP cells were labeled with Cy5.
(XLS)

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Author Contributions

Conceived and designed the experiments: AT YH T. Torigoe NS. Performed the experiments: AT T. Kanaseki T. Kubo MN HA HS. Analyzed the data: AT YH T. Torigoe YT T. Tsukahara T. Kondo VK TH NS. Contributed reagents/materials/analysis tools: AT T. Torigoe TH NS. Wrote the paper: AT YH T. Torigoe NS.

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Clinical Study

Potential Survival Benefit of Anti-Apoptosis Protein: Survivin-Derived Peptide Vaccine with and without Interferon Alpha Therapy for Patients with Advanced or Recurrent Urothelial Cancer—Results from Phase I Clinical Trials

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We previously identified a human leukocyte antigen (HLA)-A24-restricted antigenic peptide, survivin-2B80–88, a member of the inhibitor of apoptosis protein family, recognized by CD8+cytotoxic T lymphocytes (CTL). In a phase I clinical trial of survivin-2B80-88 vaccination for metastatic urothelial cancer (MUC), we achieved clinical and immunological responses with safety. Moreover, our previous study indicated that interferon alpha (IFNα) enhanced the effects of the vaccine for colorectal cancer. Therefore, we started a new phase I clinical trial of survivin-2B80–88 vaccination with IFNα for MUC patients. Twenty-one patients were enrolled and no severe adverse event was observed. HLA-A24/survivin-2B80–88 tetramer analysis and ELISPOT assay revealed a significant increase in the frequency of the peptide-specific CTLs after vaccination in nine patients. Six patients had stable disease. The effects of IFNα on the vaccination were unclear for MUC. Throughout two trials, 30 MUO patients received survivin-2B80–88 vaccination. Patients receiving the vaccination had significantly better overall survival than a comparable control group of MUO patients without vaccination ($P = 0.0009$). Survivin-2B80–88 vaccination may be a promising therapy for selected patients with MUC refractory to standard chemotherapy. This trial was registered with UMIN00005859.

1. Introduction

Urothelial carcinoma of the bladder is the fourth most common cancer in men [1]. Systemic chemotherapy has been the mainstay of management for metastatic urothelial cancer [2, 3], and cisplatin-based combinations have evolved as the standard first-line therapy. The regimens consisting of methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC) and gemcitabine and cisplatin (GC) are currently employed and provide prolongation of survival up to 14.8 and 13.8 months, respectively [3]. However, no standard therapy has been established for patients with progressive disease after the first-line chemotherapy [2, 3], and some new regimens including other anticancerous agents such as paclitaxel, ifosphamide, nedaplatin, and vinflunine are used in this setting [4–6], although they have not been proven to have sufficient clinical efficacy.

On the other hand, during the past two decades, research on human tumor immunology and cancer immunotherapy has progressed. Immunization with peptides derived from cancer-specific antigen induces antitumor cytotoxic T lymphocytes (CTLs) [7–9]. A large number of cancer-specific antigens have been identified from melanomas and other cancers, and clinical trials of peptide-based immunotherapy have been carried out.

We previously reported that survivin and its splicing variant survivin-2B were expressed abundantly in various cancer tissues and cancer cell lines, including urothelial cancer,

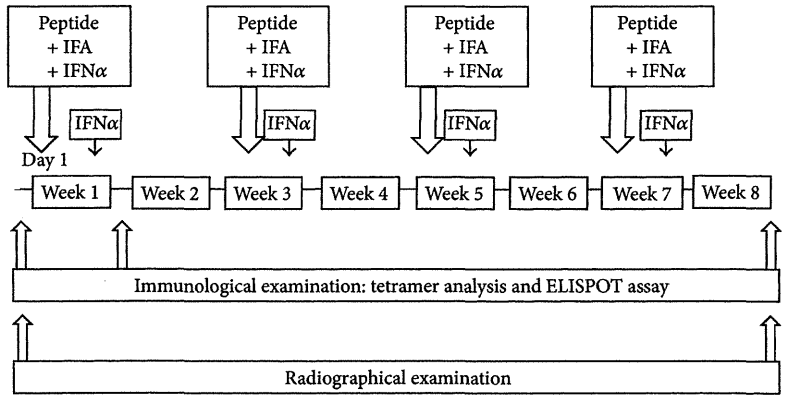


FIGURE 1: Protocol of Study 2. The protocol consisted of survivin-2B80–88 peptide, IFA, and IFNα. IFA: incomplete Freund's adjuvant; IFN: interferon.

and were suitable as target antigens for active-specific anticancer immunization [10]. Subsequently, we identified the human leukocyte antigen (HLA)-A24-restricted antigenic peptide survivin-2B80–88 (AYACNTSTL) derived from the exon 2B-encoded region and recognized by CTLs in the context of HLA-A24 molecules. In addition, we reported further evidence that the survivin-2B80–88 peptide might serve as a potent immunogenic cancer vaccine for various cancers, including bladder cancer [11]. On the basis of these studies, we started a phase I clinical study using survivin-2B80–88 peptide vaccination for urothelial cancers (Study 1) [12]. This study revealed that survivin-2B80–88 peptide vaccination was safe and well tolerated without severe side effects and could induce survivin-2B80–88 peptide-specific CTLs. Moreover, we previously reported that combination with interferon (IFN) alpha successfully enhanced the immunological responses of patients who received survivin-2B80–88 peptide vaccination for colorectal [13] and pancreatic cancers [14]. Therefore we conducted a phase I clinical study of survivin-2B80–88 peptide vaccination in combination with IFN alpha for patients with advanced or recurrent urothelial cancer expressing survivin to assess the safety and immunological efficacy (Study 2). In addition, we analyzed the effects on survival of survivin-2B80–88 peptide vaccination therapy with and without IFN alpha using the pooled data of Study 1 and Study 2.

2. Materials and Methods

2.1. Patient Selection. The study protocol was approved by the Clinical Institutional Ethical Review Board of the Medical Institute of Bioregulation, Sapporo Medical University, Japan. The HLA-A typing and immunohistochemical study were performed after obtaining informed consent from all candidate patients. Patients enrolled in this study were required to conform to the following criteria: (1) histologically proven urothelial cancer, (2) HLA-A*2402 positive, (3) survivin- and HLA class I-positive carcinomatous lesions on the primary site demonstrated by immunohistochemistry, (4) age between 20 and 85 years old, (5) surgical excision of the primary tumor, and (6) Eastern Cooperative Oncology Group (ECOG) performance status between 0 and 3. Exclusion criteria included (1) prior cancer therapy such as chemotherapy, radiation therapy, steroid therapy, or other immunotherapies within the previous 4 weeks, (2) the presence of other cancers that might influence the prognosis, (3) immunodeficiency or a history of splenectomy, (4) severe cardiac insufficiency, acute infection, or hematopoietic failure, and (5) unsuitability for the trial based on clinical judgment. This study was carried out at the Department of Urology, Sapporo Medical University Hospital from May 2009 to June 2013.

2.2. Peptide 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) [12–14]. The identity of the peptide was confirmed by mass spectrometry analysis and the purity was shown to be more than 98% as assessed by high-pressure liquid chromatography analysis. The peptide was supplied as a freeze-dried, sterile white powder. It was dissolved in 1.0 mL of physiological saline (Otsuka Pharmaceutical Co., Ltd, Tokyo, Japan) and stored at −80°C until just before use.

2.3. IFA and IFN Alpha Preparation. Montanide ISA 51 (Seppic, Paris, France) was used as IFA. Human IFN alpha was purchased from Dainippon-Sumitomo Pharmaceutical Co. (Osaka, Japan).

2.4. Patient Treatment. In Study 1 we administered the survivin-2B80–88 peptide plus IFA [12]. In Study 2, the survivin-2B80–88 peptide plus IFA and a type-I IFN, IFN alpha, were used as illustrated in Figure 1. The doses were determined according to previous studies [13, 14]. Survivin-2B80–88 at a dose of 1 mg/1 mL and IFA at a dose of 1 mL were mixed immediately before vaccination. The patients were then vaccinated subcutaneously four times at 14-day intervals. In addition, IFN alpha at a dose of 3,000,000 IU was administered subcutaneously immediately before vaccination and three days after vaccination at the site of vaccination. The primary endpoint was safety. The secondary endpoints were investigations about antitumor effects and clinical and immunological monitoring.

Table 1: Profiles of patients with advanced urothelial cancer enrolled in Study 2.

No.	Age	Sex	Primary site	Recurrence site	ECOG PS	Prior chemotherapy (number of cycles)
1	72	f	UUT	LN in neck, mediastinum, and abdomen	1	MVAC (5), TIN (2)
2	36	m	Bladder	Abdominal LN Bone	0	MVAC (2), TIN (1)
3	61	m	UUT	Pelvic soft tissue	0	GC (3), TIN (2)
4	75	m	Bladder	Abdominal LN	0	MVAC (2)
5	76	m	UUT	Renal pelvis, urethra	2	GC (3), TIN (1)
6	60	f	UUT	Abdominal LN	1	MVAC (3)
7	72	f	UUT	Mediastinal LN	1	MEC (1), GEM (1), TIN (2)
8	77	m	Bladder	Lung	0	None
9	75	f	UUT	Pelvic soft tissue	1	None
10	68	m	UUT	Abdominal LN Lung	0	GC (4)
11	72	m	Bladder	Pelvic LN, liver	0	TG (1)
12	58	m	Bladder	LN in neck, abdomen, and pelvis	0	GC (2), TIN (2)
13	64	m	Bladder	LN in abdomen and pelvis	1	GC (2)
14	73	f	Bladder	Lung, liver, and bone	0	GC (3), TIN (2)
15	62	m	Bladder	Lung	1	GC (1), GCar (1)
16	74	m	Bladder	Abdominal LN	2	GC (4), TIN (2)
17	53	m	UUT	Lung, subcutaneous	2	None
18	61	m	Bladder	Lung	1	GC (6), TIN (3)
19	56	m	Bladder	Abdominal LN, liver	1	GCar (2)
20	63	f	Bladder	Abdominal LN, lung, and liver	1	GC (4)
21	73	m	Bladder	Abdominal LN, liver	0	GC (4)

UUT: upper urinary tract; LN: lymph node; ECOG PS: Eastern Cooperative Oncology Group performance status; MVAC: methotrexate, vinblastine, adriamycin, and cisplatin; TIN: paclitaxel, ifosphamide, and nedaplatin; GC: gemcitabine and cisplatin; MEC: methotrexate, etoposide, and cisplatin; GEM: gemcitabine; TG: paclitaxel and gemcitabine; GCar: gemcitabine and carboplatin.

2.5. Toxicity Evaluation. Patients were examined closely for signs of toxicity during and after vaccination. Adverse events were recorded using the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0 (CTCAE v4.0) [15].

2.6. Clinical Response Evaluation. Physical examinations and hematological examinations were conducted before and after each vaccination [12–14]. Immunohistochemical study of the HLA class I expression in patients' primary urothelial cancer tissues was done with anti-HLA class I heavy chain monoclonal antibody EMR-8-5 (Funakoshi Co., Tokyo, Japan). We evaluated tumor size using 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 diseases. A partial response (PR) was defined as a ≥30% decrease from 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 CR, PR, or PD [12–14].

2.7. In Vitro Stimulation of PBMC. PBMCs were isolated from blood samples by Ficoll-Conray density gradient centrifugation. They were then 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 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.

2.8. Tetramer Staining. FITC-labeled HLA-A*2402-human immunodeficiency virus (HIV) peptide (RYLRDQQLL) and PE-labeled HLA-A*2402-survivin-2B80–88 peptide tetramers were purchased from MBL, Inc. (Nagoya, Japan). For flow cytometric analysis, PBMCs, which were stimulated in vitro as above, were stained with the PE-labeled tetramer at 37°C for 20 min, followed by staining with an FITC-conjugated anti-CD8 mAb (Beckton Dickinson 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 performed using FACSCalibur and CellQuest software (Beckton Dickinson Biosciences, San Jose, CA, USA). The frequency of CTL precursors was calculated as the number of tetramer-positive cells divided by the number of CD8-positive cells [12–14].

2.9. ELISPOT Assay. ELISPOT plates were coated sterilely overnight with an IFN-c capture antibody (Beckton Dickinson 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' PBMC (5×10^3 cells/well), which were stimulated in vitro as above, were then added to each

Table 2: Summary of clinical and immunological responses to vaccination with survivin-2B80-88 peptide, IFA, and IFN alpha.

No.	Adverse events (Grade)*	Tetramer staining† After/before vaccination	ELISPOT‡ After/before vaccination	Clinical response	Followup (months)	Outcome
1	Fever (1)	3100/3300	98/3	PD	6.5	DOD
2	Fever (1)	2700/600	31/20	SD	14.5	DOD
3	Fever (1)	4400/600	62/36	SD	17.0	DOD
4	Fever (1) Induration at injection site (1)	16400/1700	49/12	SD	32.5	AWD
5	Fever (1)	500/10900	29/8	PD	2.0	DOD
6	Fever (1)	2000/300	32/29	PD	4.0	DOD
7	Induration at injection site (1)	4100/0	41/15	PD	6.5	DOD
8	Fever (1)	2100/2000	49/33	PD	14.5	DOD
9	Fever (1)	2000/500	65/21	SD	7.0	DOD
10	Fever (1)	0/4500	53/6	PD	6.5	AWD
11	None	38900/0	10/0	PD	10.0	DOD
12	Fever (1)	2400/0	117/80	SD	6.0	AWD
13	Fever (1)	0/0	28/9	PD	9.5	DOD
14	None	1200/800	95/14	PD	4.0	AWD
15	Fever (1)	1600/200	616/68	SD	8.5	AWD
16	None	700/300	11/63	PD	5.5	DOD
17	Fever (1)	200/400	39/0	PD	5.5	AWD
18	Fever (1) Induration at injection site (1)	3700/4600	61/32	PD	4.0	AWD
19	None	1400/200	7/5	PD	1.0	AWD
20	None	900/800	25/0	PD	2.0	AWD
21	None	2000/300	0/0	PD	2.0	AWD

*Adverse events were recorded using the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0 (CTCAE v4.0). †Cytotoxic T-lymphocyte (CTL) frequency before and after treatment 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. ‡Interferon gamma secretion of pre- and postvaccinated patients' CD8 T cells was assessed 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. SD: stable disease; PD: progressive disease; DOD: dead of disease; AWD: alive with disease.

well along with HLA-A24-transfected CIR cells (CIR-A24) (5×10^4 cells/well), which 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 IFN-c antibody and horseradish peroxidase-conjugated avidin. Spots were visualized and analyzed using KS ELISPOT (Carl Zeiss, Jena, Germany). In the present study, the cutoff point for ELISPOT was determined according to previous studies; positive (+) ELISPOT represented a more than twofold increase of survivin-2B80–88 peptide-specific CD8 T cell IFNc-positive spots compared with HIV peptide-specific CD8 T-cell spots, whereas negative (–) represented a less than twofold increase [13, 14].

2.10. Statistical Analysis. Continuous variables were compared using the Student's t-test. Given the small size, we confirmed all results with the Mann-Whitney U test. Categorized variables were compared using Fisher's exact probability test. Overall survival rates (OS) were evaluated by the Kaplan-Meier method, and differences between two groups were compared using the log-rank test and Cox proportional hazards regression models. A value of $P < 0.05$ was considered to indicate statistical significance. The calculations were performed using Statview 5.0 (SAS Institute, Cary, NC).

3. Results

3.1. Patient Profile. Twenty-one patients were enrolled in Study 2 (Table 1). They consisted of 15 men and 6 women, whose age range was 36–77 years. Three patients did not receive chemotherapy before vaccination because they were unfit for cisplatin-based chemotherapy due to impaired renal function.

3.2. Safety. Six patients (cases 5, 6, 16, 17, 19, and 20) discontinued halfway through the protocol because of disease progression. The remaining 15 patients received the complete regimen including four vaccinations. None of the treatment interruptions was due to adverse effects of the vaccination. Peptide vaccination was well tolerated in all 21 patients. As shown in Table 2, no hematologic, cardiovascular, hepatic, or renal toxicity was observed. No other severe adverse events

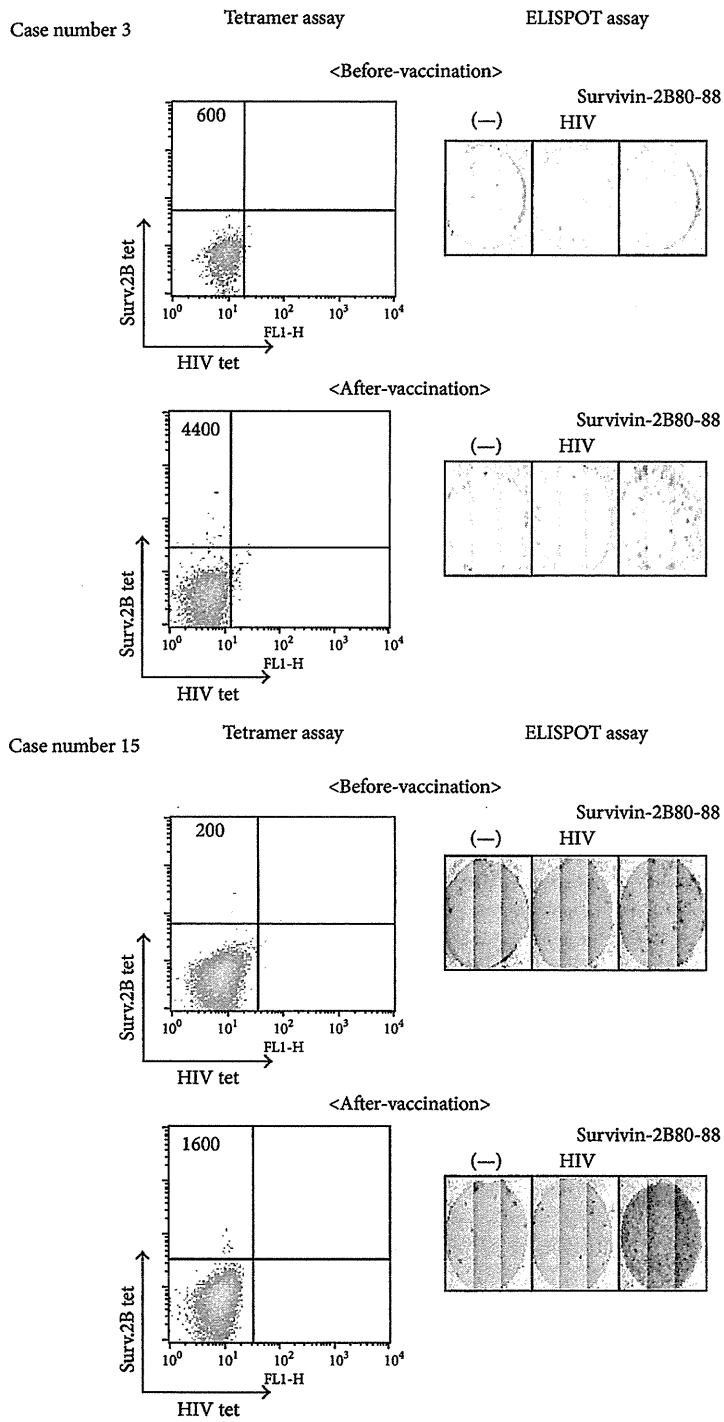


FIGURE 2: Representative illustration of immunological analysis in patients 3 and 15 who were treated with survivin-2B80–88 plus IFA with IFN alpha. Tetramer and ELISPOT analyses before and after vaccinations. The number in the tetramer analysis indicates survivin-2B80–88 peptide-specific CD8+ T cells among 10^4 CD8+ T cells. ELISPOT: enzyme-linked immunosorbent spot; HIV: human immunodeficiency virus; HLA: human leukocyte antigen; IFA: incomplete Freund's adjuvant; IFN: interferon.

Table 3: Baseline characteristics and immunological responses in Study 1 and Study 2.

	Study 1	Study 2	P value
n	9	21	
Age	58.1 ± 9.1	65.7 ± 10.3	0.0593
Sex (male/female)	4/5	15/6	0.2252
Primary site (UUT/bladder)	1/8	11/10	0.0492
Visceral metastases	4 (44.4%)	11 (52.4%)	0.9999
Prior chemotherapy	9 (100%)	18 (85.7%)	0.2320
ECOG PS (0/1/2)	4/5/0	10/9/2	0.9999
Induction of CTLs	5 (71.4%)*	9 (42.8%)	0.3845
Non-PD in clinical response	2 (33.3%)†	6 (28.6%)	0.6424

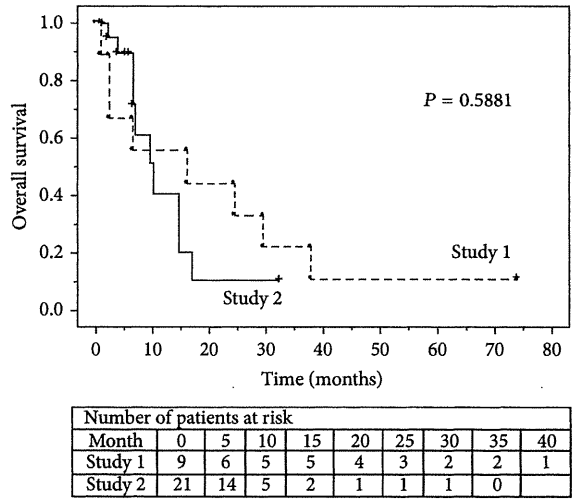
*Immunological results were obtained in 7 of 9 cases. †Clinical responses were assessed in 6 cases. UUT: upper urinary tract; ECOG PS: Eastern Cooperative Oncology Group performance status; CTL: cytotoxic T-lymphocyte; PD: progressive disease.

were observed during or after vaccination. As minor side effects, 14 patients (cases 1–6, 8–10, 12, 13, 15, 17, and 18) developed grade 1 fever, possibly due to IFN alpha, and 3 patients (cases 4, 7, and 18) developed grade 1 local skin reactions with redness and induration at the injection sites. No other severe adverse events were observed during or after vaccination.

3.3. Immunological and Clinical Responses. Representative illustrations of immunological analysis in cases 3 and 15 are shown in **Figure 2**, and **Table 2** summarizes the immunological and clinical results. HLA-A24/survivin-2B80–88 peptide tetramer analysis revealed a significant increase in the peptide-specific CTL frequency of CD8-positive T cells after vaccination in 13 patients (cases 2, 3, 4, 6, 7, 9, 11, 12, 14, 15, 16, 19, and 21), as shown in **Table 2**. Of them, however, cases 6, 16, 19, and 21 were negative in the ELISPOT study. Thus, functional peptide-specific CTLs were induced in nine patients (42.8%) by this vaccination protocol. Radiographical examination revealed SD after four vaccinations in six patients (28.6%). All of them had an increase in the peptide-specific CTLs proven in both tetramer analysis and ELISPOT assay.

3.4. Impact of IFN Alpha in Combination with the Survivin-2B80–88 Peptide on Immunological Responses and Survival. To assess the effect of additional IFN alpha, immunological and clinical outcomes were compared between Study 1 and Study 2. Baseline characteristics and immunological and clinical responses are shown in **Table 3**. There were no significant differences in either the induction of peptide-specific CTLs or radiographical responses. Furthermore, OS showed no significant difference between the two groups (**Figure 3**).

3.5. Impact of the Survivin-2B80–88 Peptide Vaccination with and without IFN Alpha on Survival. A total of 30 patients underwent the survivin-2B80–88 peptide vaccination in Study 1 and Study 2. During the course of these studies, 14 patients were excluded due to an ineligible HLA type and 4 patients eventually decided not to receive vaccination although eligible. These 18 patients were evaluated as a control group. Clinical characteristics were comparable between the vaccination group and control group, as shown in **Table 4**.



Number of patients at risk									
Month	0	5	10	15	20	25	30	35	40
Study 1	9	6	5	5	4	3	2	2	1
Study 2	21	14	5	2	1	1	1	0	

Figure 3: Kaplan-Meier estimated overall survival is shown for patients treated with survivin-2B80–88 peptide plus IFA (Study 1) versus survivin-2B80–88 peptide plus IFA in combination with IFA alpha (Study 2). IFA: incomplete Freund's adjuvant; IFN: interferon.

The vaccination group had significantly better OS than the control group (P = 0.0009), as shown in **Figure 4**. Median survival times were 10.0 months and 4.5 months in the vaccination group and control group, respectively. **Table 5** lists the results of proportional hazards regression analysis used to test the predictive value of each variable for OS. In this multivariate model adjusted for age, ECOG PS, and the presence of visceral metastases, vaccination therapy was an independent predictive factor for better OS (P = 0.0088).

4. Discussion

Survivin-2B80–88 vaccination therapy is safe and confers induction of peptide-specific CTLs in patients with metastatic urothelial cancers according to the results of Study 1 [12]. In Study 2, we used a combination protocol of survivin peptide vaccination with IFN alpha in an attempt to enhance the immunogenicity, as with colorectal [13] and pancreatic cancers [14]. The protocol was safe and well tolerated with no

Table 4: Clinical characteristics of vaccination group and control group.

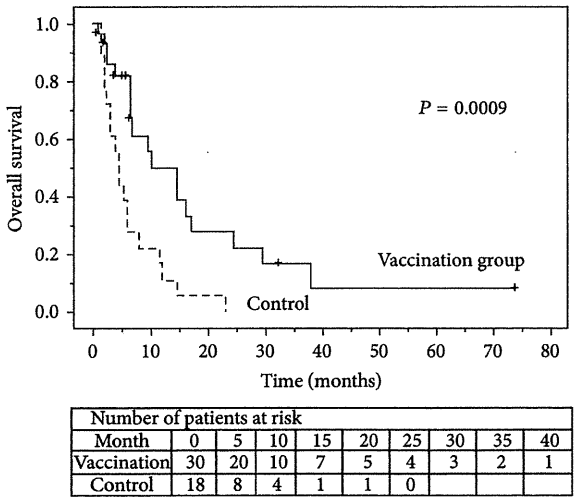
	Vaccination group	Control group	*P* value
n	30	18	
Age	63.5 ± 10.2	66.4 ± 10.3	0.3355
Sex (male/female)	19/11	15/3	0.1956
Visceral metastases	15 (50.0%)	12 (66.7%)	0.3693
Number of visceral metastatic sites	0.63 ± 0.76	1.00 ± 0.84	0.1417
Prior chemotherapy	27 (90.0%)	17 (94.4%)	0.9999
ECOG PS			
0	13 (43.3%)	8 (44.4%)	
1	14 (46.7%)	6 (33.3%)	0.4002
2	3 (10.0%)	4 (22.3%)	

ECOG PS: Eastern Cooperative Oncology Group performance status.

Table 5: Multivariate proportional hazards regression model for overall survival.

Variable	HR	95% CI	*P* value
Age: <65 versus ≥65 years	0.782	0.343–1.784	0.5591
ECOG PS: 0 versus ≥1	0.335	0.160–0.703	0.0038
Visceral metastases: no versus yes	0.599	0.284–1.263	0.1782
Vaccination: yes versus no	0.308	0.127–0.743	0.0088

ECOG PS: Eastern Cooperative Oncology Group performance status; HR: hazard ratio; CI: confidence interval.



Number of patients at risk									
Month	0	5	10	15	20	25	30	35	40
Vaccination	30	20	10	7	5	4	3	2	1
Control	18	8	4	1	1	0			

Figure 4: Kaplan-Meier estimated overall survival (OS) is shown for patients who received survivin-2B80–88 peptide vaccination with and without IFN alpha and did not receive survivin-2B80–88 peptide vaccination. A statistically significant difference in OS was identified between the two groups.

severe adverse effects, as in the case of colorectal and pancreatic cancers [13, 14]. Fever was the most frequent adverse effect and may have resulted from the use of interferon alpha. However, we could not find any benefit of additional interferon alpha in urothelial cancer patients in terms of enhancing the immunogenicity of the survivin-2B80–88 peptide. In a previous study, peptide-specific CTLs were induced in 50% of patients with colorectal cancer by survivin-2B80–88 peptide vaccination in combination with interferon alpha, but in 0% by that without interferon alpha [13]. On the other hand, survivin peptide vaccination without interferon alpha induced peptide-specific CTLs in 67% of urothelial cancer patients [12]. IFN alpha may be an insufficient adjuvant for further enhancement of the immunogenicity of survivin-2B80–88, and improvement of the protocol is required. Besides IFN alpha, other cytokines such as interleukin (IL)-2, IL-4, IL-15, and granulocyte macrophage colony-stimulating factor are expected to have effects leading to stronger immune responses in both the induction and effector phases [16]. Furthermore, blockade of negative regulation of the immune response is considered to be an important strategy to enhance the immunological and clinical responses of peptide vaccination therapy [16, 17]. Low-dose cyclophosphamide [18, 19] and an anti-CD25 [20] antibody are employed to suppress regulatory T cells, and low-dose gemcitabine is promising to suppress myeloid-derived suppressor cells [21]. These agents might be effective to enhance the immunological response and clinical efficacy in survivin-2B-80-88 peptide vaccination therapy for urothelial cancer.

In cancer vaccination therapy, tumor shrinkage is not expected and may not be an appropriate endpoint for evaluation of the efficacy of cancer immunotherapy [22]. Although neither CR nor PR after the vaccination was observed in our series, all six patients with SD also had increases in CTLs. SD can be considered to be a result of immunological responses to the survivin-2B80–88 peptide vaccine. Therefore, the results of the current study suggest that survivin-2B80–88 peptide vaccination therapy potentially provides survival benefit for patients with metastatic urothelial cancer. However, this study had only a small number of subjects. Although the control group was comparable, patients were not randomized. To confirm the efficacy for survival, a larger randomized clinical trial is necessary.

There is no standard therapy for metastatic urothelial cancers refractory to standard chemotherapy [2, 3]. In addition, most second-line chemotherapy regimens under investigation have severe adverse events, which can impair patients' quality of life and are often associated with life-threatening adverse effects [4–6]. On the other hand, the results of the current study suggest that survivin-2B80–88 peptide vaccination therapy is safe and well tolerated and may potentially have clinical benefits in selected patients. Thus, survivin-2B80–88 peptide vaccination appears to be a promising treatment strategy for metastatic urothelial cancers refractory to standard chemotherapy.

5. Conclusions

Although survivin-2B80–88 peptide vaccination in combination with IFN alpha is safe and well tolerated, the effects of additional IFN alpha are unclear. According to the results of pooled data analysis of Study 1 and Study 2, survivin-2B80–88 peptide vaccination therapy potentially has clinical effects; thus, it may be a promising therapy for selected patients with metastatic urothelial cancers refractory to standard chemotherapy.

Conflict of Interests

All authors of this paper reported no financial interests or potential conflict of interests.

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High Expression of CD109 Antigen Regulates the Phenotype of Cancer Stem-Like Cells/Cancer-Initiating Cells in the Novel Epithelioid Sarcoma Cell Line ESX and Is Related to Poor Prognosis of Soft Tissue Sarcoma

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Abstract

Epithelioid sarcoma (ES) is a relatively rare, highly malignant soft tissue sarcoma. The mainstay of treatment is resection or amputation. Currently other therapeutic options available for this disease are limited. Therefore, a novel therapeutic option needs to be developed. In the present study, we established a new human ES cell line (ESX) and analyzed the characteristics of its cancer stem-like cells/cancer-initiating cells (CSCs/CICs) based on ALDH1 activity. We demonstrated that a subpopulation of ESX cells with high ALDH1 activity ($ALDH^{high}$ cells) correlated with enhanced clonogenic ability, sphere-formation ability, and invasiveness *in vitro* and showed higher tumorigenicity *in vivo*. Next, using gene expression profiling, we identified CD109, a GPI-anchored protein upregulated in the $ALDH^{high}$ cells. CD109 mRNA was highly expressed in various sarcoma cell lines, but weakly expressed in normal adult tissues. CD109-positive cells in ESX predominantly formed spheres in culture, whereas siCD109 reduced ALDH1 expression and inhibited the cell proliferation *in vitro*. Subsequently, we evaluated the expression of CD109 protein in 80 clinical specimens of soft tissue sarcoma. We found a strong correlation between CD109 protein expression and the prognosis (P = 0.009). In conclusion, CD109 might be a CSC/CIC marker in epithelioid sarcoma. Moreover, CD109 is a promising prognostic biomarker and a molecular target of cancer therapy for sarcomas including ES.

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Introduction

Epithelioid sarcoma (ES) is a relatively rare and highly malignant soft tissue sarcoma (STS) accounting for <1% of all STSs [1]. The mainstay of treatment is aggressive, radical local resection or amputation. Currently other therapeutic options available for ES are limited. Therefore, a novel therapeutic option needs to be developed.

Recent studies have revealed that several human cancers contain a small subpopulation of cells called cancer stem-like cells (CSCs)/cancer initiating cells (CICs), which are defined by the ability of self-renewal, multi-differentiation potential, and tumorigenesis. Therefore, CSCs/CICs are believed to be responsible for the progression and relapse of cancer [2]. In the current study, we isolated CSCs/CICs based on aldehyde dehydrogenase 1 (ALDH1) activity. Human ALDHs are a family of NAD (P)+-dependent enzymes involved in detoxifying a wide

variety of aldehydes to their corresponding weak carboxylic acids [3]. They serve to detoxify both xenobiotic aldehydes (eg. cyclophosphamide) and many other intracellular aldehydes, including ethanol and vitamin A [4]. Therefore, ALDH activity is important for drug resistance and the response to oxidative stress [5]. Recently ALDH1 activity was used, either alone or in combination with cell surface markers, to identify CSCs/CICs in hematologic malignancies and carcinomas derived from the lung and prostate [6-8].

We established a new ES cell line (designated ESX) from a 73-year-old woman. Next, we investigated CICs/CSCs in ES cell lines and isolated CSCs/CICs based on ALDH activity. Finally, we demonstrate that CD109 is a potential CSC/CIC marker that may be useful as a prognostic biomarker and a molecular target of cancer therapy for sarcomas, including ES.

Materials and Methods

Ethics Statement

Mice were maintained and experimented on in accordance with the guidelines of and after approval by the Ethics Committee of Sapporo Medical University School of Medicine, Animal Experimentation Center under permit number 08-006. Any animal found unhealthy or sick was promptly euthanized. All studies were approved by the Institutional Review Board of Sapporo Medical University Hospital. Written informed consent was obtained from all patients according to the guidelines of the Declaration of Helsinki.

Primary tumor

A 73-year-old Japanese woman was admitted to our hospital with a 9-month history of swelling of the left thigh. The swelling had gradually enlarged and become painful. A well-demarcated elastic soft mass was palpable in the medial aspect of the left thigh. Magnetic resonance imaging revealed a subcutaneous tumor and lymph node metastases in the inguinal region (Figure S1A). The tumor (3×3 cm) was homogeneously isointense relative to skeletal muscle in T1-weighted images, whereas it was heterogeneously iso- and hyperintense relative to skeletal muscle in T2-weighted images. Computed tomography revealed no pulmonary metastasis. The serum CA125 level was 6.6 U/ml (normal: <40 U/ml). Open biopsy showed that the tumor was composed of sheets of large cells with vesicular chromatin, prominent nucleoli, and amphophilic cytoplasm, with peripheral palisading of epithelioid cells around necrotic areas (Figure S1B). Immunohistochemical analysis revealed that the tumor was positive for AE1/AE3 and vimentin, but negative for CD34, CA125, and S-100. (Figure S1C). Although the tumor was weakly positive for INI1 analyzed by immunohistochemistry, fluorescence in situ hybridization (FISH) analysis revealed the heterozygous deletion of INI1 in 17 of 50 tumor cells (34%) (Figure S1D). Upon these findings, the tumor was diagnosed as proximal-type epithelioid sarcoma. Wide resection of the tumor and lymph node dissection were performed, but systemic chemotherapy was not. Unfortunately, pulmonary metastases developed 12 weeks after surgery and the patient died 16 weeks after the definitive surgery.

Establishment of a new ES cell line, ESX

The resected specimen of the primary tumor was rinsed with phosphate-buffered saline, cut into small pieces with a scalpel and cultured in Iscove's modified Dulbecco's Eagle's medium (IMDM; GIBCO BRL, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Inc., South Logan, UT). The tumors were incubated at 37°C in 5% CO_2. The cell line (ESX) was maintained for more than 24 months.

Cell lines

Human osteosarcoma cell lines (NY, U2OS and HOS), human Ewing sarcoma cell lines (SKES, WES, and RDES), the human synovial sarcoma cell line FUJI, and the human ES cell line VA-ES-BJ were purchased from the Japanese Collection of Research Bioresources Cell Bank (Tokyo, Japan) and American Type Culture Collection (Manassas, VA, USA). The human synovial sarcoma cell line YaFuSS and FU-EPS-1 were gifts from Dr J. Toguchida (Kyoto University) [9] and Dr H. Iwasaki (Fukuoka University) [10]. The human osteosarcoma cell line OS2000 and KIKU, and the human malignant fibrous histiocytoma cell lines MFH2003 and MFH2004 were established in our laboratory [11-14].

ALDEFLUOR assay

The ALDEFLUOR kit (StemCell Technologies, Vancouver, Canada) was used to separate the population with high ALDH1 activity. Cells (1×10^6) were suspended in ALDEFLUOR assay buffer containing an ALDH1 substrate, bodipy-aminoacetaldehyde, at the concentration of 1μmol/L and incubated for 50 min at 37°C according to the manufacturer's protocol. A specific inhibitor of ALDH1, diethylaminobenzaldehyde (DEAB), was used at 50mmol/L as a negative control.

CD109-positive cell sorting

The cells were washed once with PBS and then centrifuged at 440g at 4°C for 5 min using an LX120 (Tomy, Tokyo, Japan). The cell pellets were resuspended and incubated for 60 min at 4°C with a 100-fold dilution of a mouse anti-CD109 antibody (R&D Systems). Then samples were washed with PBS 3 times and stained and incubated for 60 min at 4°C with a 500-fold dilution of an FITC-labeled anti-mouse secondary antibody (KPL, Gaithersburg, MD). Cell sorting was performed using a FACSAria II (BD Bioscience, San Jose, CA). Collected data were analyzed using BD FACSDiva V6.1.3 (BD Bioscience). Propidium isodide (PI; Life Technologies Corp.) was used to stain live cells.

RNA preparation and PCR analysis

Total RNAs were extracted from cells using the RNeasyMini Kit (Qiagen, Hilden, Germany). cDNA was synthesized using Superscript III and an oligo(dT) primer (Life Technologies Corp.). Human Multiple Tissue cDNA Panels I and II, and the Human Fetal Multiple Tissue cDNA Panel (Clontech; Mountainview, CA) were used as normal tissue cDNAs. PCR was performed using KOD Dash (TOYOBO, Osaka, Japan) to

detect CD109. The primer sequences used were 5'-TTGAATTCCCAATCCTGGAG-3' and 5'-TTGTTGCCACTAACCACCAA-3'. The PCR mixture was denatured at 98°C for 2 min, followed by 30 cycles at 98°C for 15s, at 55°C for 2s, and at 74°C for 30s. GAPDH and beta-actin were used as internal controls. Real-time PCR was performed using the StepOne system (Life Technologies Corp.). Primers and probes were designed using the TapMan Gene expression assay (Life Technologies Corp.). Thermal cycling was performed with 40 cycles of 95°C for 1s, followed by 60°C for 20 min. Each experiment was done in triplicate and normalized to the GAPDH gene as an internal control.

siRNA

CD109 siRNA (siCD109) (5'-AAAGUUUGGACUCUGAUGACACCCA-3') was designed using BLOCK-it RNAi (Life Technologies Corp.). Control siRNA was obtained from Life Technologies Corp. The siRNAs were transfected using Lipofectamine RNAiMAX transfection reagent (Life Technologies Corp.)

Spherical colony formation assay

Cells were plated at 1000 cells per well in six-well ultra-low attachment plates (Corning Inc., Corning, NY) and cultured in DMEM/F12 medium with 10ng/ml hEGF, 10ng/ml hbFGF, and 2% B-27 (Life Technologies Corp.) at 37°C in 5% CO_2. On day 7, the number of colonies was counted under an inverted contrast microscope.

Cell proliferation assay

Cells were seeded in duplicate at a density of 2.5×10^4 cells/well in 24-well plates. On the following day, siRNAs were transfected. At 48 hr, 72 hr and 120 hr cells were trypsinized and counted with a Coulter Counter (Beckman Coulter, Inc. Brea, CA).

Basement membrane matrix invasion assay

Invasiveness was analyzed using a Matrigel™ Invasion Chamber (BD Biosciences). Briefly, 2.5×10^4 cells were placed on inserts in the wells in IMDM with 10% FBS. After 52 hours, the cells were stained using a Hemacolor staining kit (Merck Millipore, Billerica, MA) and the degree of migration was determined.

Gene expression profiling

RNA from $ALDH^{high}$ cells was labeled with Cy5 dye and RNA from $ALDH^{low}$ cells were labeled with Cy3 dye. The probe mixture was hybridized for 40 hours at 65°C using a Human Whole Genome Microarray (G4112F) (Agilent Technologies, Santa Clara, CA). The array was scanned after washing with a G2565BA Microarray Scanner and fluorescent signals were acquired using Feature Extraction software (Agilent Technologies). The average expression ratio of Cy5 to Cy3 was determined per gene. A dye swap experiment was also done to label $ALDH^{high}$ and $ALDH^{low}$ cells with Cy3 and Cy5, respectively. An average ratio of more than 2.0, reproducible in 2 experiments, was determined to indicate differential up-regulation in $ALDH^{high}$ cells. The accession number of ArrayExpress is E-MEXP-3826. We focused on membrane protein-related genes for cell sorting and as therapeutic targets using antibodies. Therefore, we selected membrane protein-related genes based on information obtained from GeneCards (http://www.genecards.org) (Table S2), followed by screening of mRNA expression in $ALDH^{high}$ and $ALDH^{low}$ cells by RT-PCR (data not shown).

Xenografting

$ALDH^{high}$ and $ALDH^{low}$ cells freshly sorted from cell lines were washed and suspended in PBS. Then the cells (1×10^2, 1×10^3 and 1×10^4 cells in 50μl of PBS) were mixed with an equal volume of Matrigel (BD Science) and subcutaneously injected into the bilateral sides of the lower back in female non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (NOD.CB17-Prdkc^{scid}/J, Charles River Laboratory, Yokohama, Japan). Tumor growth was monitored weekly for 10 weeks. Then the xenografted tumors were resected and analyzed.

Immunohistochemical staining

Formalin-fixed paraffin-embedded sections from 80 STS patients who underwent resection for stage I-III tumors and chemotherapy or radiotherapy for stage IV tumors between 2004 and 2009 in the Sapporo Medical University Hospital were used for CD109 staining as previously described [15]. The reactivity of the anti-CD109 antibody was determined by the staining pattern of the tumor cell membrane and graded as follows: 0 (no staining), 1 (partial staining of the membrane), 2 (mild to moderate circumferential staining of the membrane) and 3 (strong circumferential staining of the membrane). If the score was 2 or 3 in more than 10% of the tumor cells, it was considered to be positive. The clones used, antigen retrieval methods, and commercial sources of the antibodies used in the study are listed in Table S1.

Statistical methods

The Mann-Whitney test was used to compare in vitro data and the differences in tumor volume using IBM SPSS Statistics (IBM Corp., Armonk, NY). The Fisher exact test was used to compare the associations between the CD109 expression level and clinicopathological factors using SAS software 9.3 (SAS Institute, Cary, NC). Postoperative disease-free survival (DFS) and overall survival (OS) were estimated using Kaplan-Meier plots. Prognostic significance was evaluated by the log-rank test. Univariate and multivariate analyses for hazards ratios (HR) in OS were performed by Cox's proportional-hazards regression with backward selection using IBM SPSS Statistics. A probability of less than 0.05 was considered statistically significant.

Results

Establishment of the ES cell line ESX

A tumor cell culture obtained from a patient with ES of the left thigh (Figure S1) was maintained for over 1 year and designated ESX (Figure 1A). The ESX cells were spindle-

shaped with large nuclei and grew as adherent, tightly packed monolayers, but had no epithelial cell morphology. The morphology was maintained across all cell passages. Karyotype analysis revealed massive rearrangement of chromosomes (Figure 1B). Immunohistochemical examination revealed that the atypical cells were positive for vimentin and AE1/AE3, but negative for CD34 (Figure 1C). Subcutaneous inoculations of ESX cells into NOD/SCID mice produced growing tumors. Histologically the xenografted tumors consisted of a distinct nodular arrangement of the tumor cells, a tendency to undergo central degeneration and necrosis, and an epithelioid appearance with cytoplasmic eosinophilia. Immunostaining analysis of the xenografted tumors also revealed a staining pattern similar to that of the ESX cells (Figure 1C) and the original tumor (Figure S1C). These findings indicated that the established cell line was consistent with the profile of the original tumor. The characteristics of ESX were consistent with the profile of the highly malignant original tumor.

Identification of an ALDH^{high} population in ES cell lines

We performed ALDEFLUOR assay to detect ALDH^{high} populations containing CSCs/CICs in the epithelioid sarcoma cell lines. As shown in Figure 2A, all 3 ES cell lines (ESX, VA-ES-BJ, and FU-EPS-1) contained ALDH^{high} populations, although the proportion of ALDH^{high} cells varied. The mean proportions of ALDH^{high} cells were 36.6%, 14.2 % and 13.8% in ESX, VA-ES-BJ and FU-EPS-1, respectively. The proportions of ALDH^{high} cells in ES cell lines were higher than in the other sarcoma cell lines (Figure S2). The proportion of ALDH^{high} cells in ESX was significantly higher than in the others (p<0.001) (Figure 2B). We then analyzed the differentiation abilities of ALDH^{high} and ALDH^{low} ESX cells *in vitro* 9 days and 12 days after sorting (Figure 2C). ALDH^{high} and ALDH^{low} cells showed a tendency to differentiate into ALDH^{low} and ALDH^{high}, respectively. These results could indicate the flexible plasticity of CSCs/CICs of ES cells. The frequency of ALDH^{high} cells converted from ALDH^{low} cells after in vitro culture was 16.3% on Day 12. On the other hand, when sorted ALDH^{high} cells were cultured in vitro the frequency of remaining ALDH^{high} cells was 36.2% on Day 12, indicating that ALDH^{high} cells could maintain higher enzyme activity than ALDH^{low} cells.

Cancer-initiating ability of ALDH^{high} cells of ES cell lines

In a previous study, we showed that CSCs/CICs could generate floating spheroid-like bodies in a serum-free medium [16]. We therefore determined whether ALDH^{high} and ALDH^{low} cells of ESX could generate spherical colonies. As shown in Figure 3A, most ALDH^{low} cells died and the others formed a few small colonies. In contrast, the number of colonies derived from ALDH^{high} cells was significantly higher than that from ALDH^{low} cells (p<0.001) (Figure 3B). Next, we examined the expression of stem/progenitor cell-related genes *Sox2*, *Oct3/4, and Nanog* [17,18]. Interestingly, in the ALDH^{high} population the mRNA expression of *Sox2*, *Oct3/4*, and *Nanog* was lower than in ALDH^{low} (Figure 3C). These findings were in marked contrast to the CSCs/CICs of carcinomas, which suggested that gene expression of stem cell-related genes was not involved in the cancer-initiating ability, at least in epithelioid sarcoma [19]. In the ALDH^{high} cells of VA-ES-BJ and FU-EPS-1, the mRNA expression of stem/progenitor cell-related genes was not lower than in ALDH^{low} cells (Figure S3).

To determine whether CSCs/CICs were abundant in the ALDH^{high} population, we performed xenograft transplantation of ALDH^{high} and ALDH^{low} cells of ESX, VA-ES-BJ, and FU-EPS-1 into NOD/SCID mice. The sorted ALDH^{high} and ALDH^{low} cells were injected subcutaneously into mice, and tumor growth was monitored weekly for 10 weeks. In ESX, 1×10^3 ALDH^{high} cells showed tumorigenicity in one of four mice. In contrast, 1×10^3 of ALDH^{low} cells failed to form tumors. On the other hand, 1×10^4 ALDH^{high} and ALDH^{low} cells both showed tumorigenicity (Figure 3, D and E). However, the frequency of tumor formation was lower for ALDH^{low} cells than for ALDH^{high} cells (Figure 3F). The histology of the tumors derived from ALDH^{high} and ALDH^{low} cells of ESX showed no major differences (Figure 3G). In the case of FU-EPS-1, neither ALDH^{high} nor ALDH^{low} cells could form tumors. In VA-ES-BJ, ALDH^{low} cells formed a tumor but ALDH^{high} cells did not (Figure 3F). These findings suggested that ALDH^{high} cells of ESX contained a higher number of tumorigenic cells compatible with CSCs/CICs than ALDH^{low} cells. In contrast, we considered that VA-ES-BJ and FU-EPS-1 did not contain a CSCs/CICs population in ALDH^{high} cells that was similar to that of ESX. Therefore, ESX was used for the further characterization of the ALDH^{high} population containing CSCs/CICs.

To examine the invasive potential of ALDH^{high} and ALDH^{low} cells in ESX, we performed in vitro basement membrane matrix invasion assay. The microscopic features of the invading cells of ALDH^{high} and ALDH^{low} are shown in Figure 4A. The number of invading cells for ALDH^{high} was significantly higher than that for ALDH^{low} cells (P<0.001) (Figure 4B). Furthermore, the mRNA expression of epithelial-mesenchymal transition (EMT)-related genes *Snail1 and Twist1* was upregulated in ALDH^{high} cells, supporting the invasive ability of the ALDH^{high} population (Figure 4C), which was compatible with the characteristics of CSCs/CICs.

Identification of the novel marker CD109 for the CSCs/CICs of ES

We screened the upregulated genes in ALDH^{high} cells using a cDNA microarray to identify membrane protein-related genes for cell sorting and therapeutic targets using antibodies. The upregulated membrane protein-related genes in ALDH^{high} cells of ESX are summarized in Table S2. Among them, we selected CD109, which was upregulated in ALDH^{high} cells, as a representative marker (Figure 5A). The other molecules listed in Table S2 were not upregulated in ALDH^{high} cells assessed by RT-PCR (data not shown). CD109, a GPI-anchored glycoprotein, was originally identified as a leukemia antigen. It has been reported that CD109 is expressed on activated T lymphocytes and platelets, endothelial cells and a subpopulation of CD34+ hematopoietic stem and progenitor cells [20,21]. CD109 has also been reported as the cell surface antigen on acute myeloid leukemia [20]. On the other hand, in the other ES cell lines there was no difference of CD109 mRNA expression between ALDH^{high} and ALDH^{low} cells. These results

Figure 1

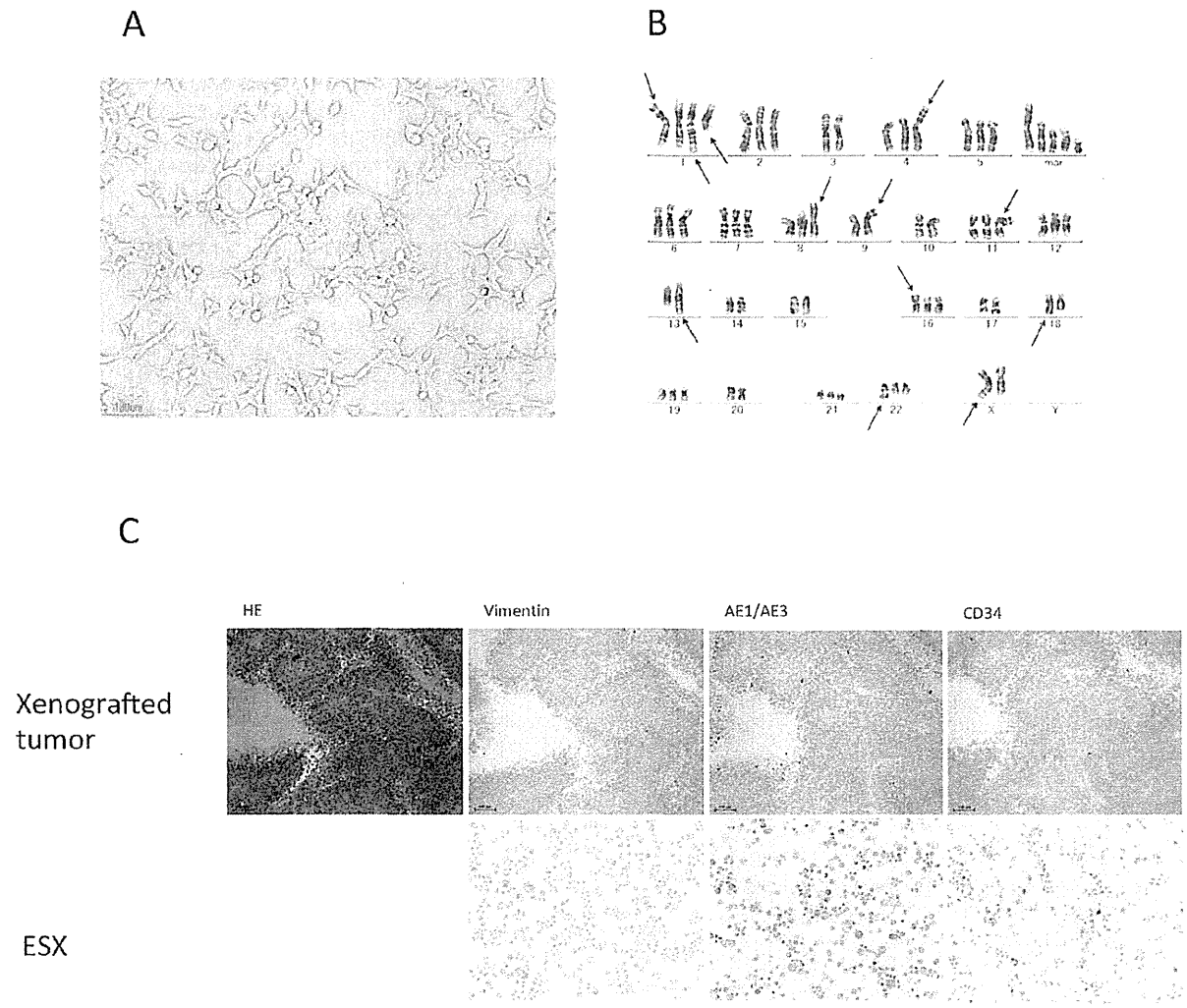


Figure 1. Establishment of the new epithelioid sarcoma cell line ESX. A. Phase-contact microscopy findings for ESX.
B. Representative G-band karyotyping of ESX. The karyotype revealed 65~68, X, -X or –Y, add (X)(q22), +1, add(1) (p32), add(1) (q21), add(1)(q42), add(1)(q42), der(4;10)(q10;q10), add(8)(p11.2), -9, add(9)(p22), der(11)t(11;14)(p13;q13),-13, add(13)(q22), -14,-15,add(16)(p13.1), -17,-18, add(18)(q21),+21,add(22)(q13), +4~6mar[cp9]. Arrows indicate deletions and derivative chromosomes.
C. Immunostaining analysis of the xenografted tumors (scale bar, 100μm) and ESX cell line for vimentin and AE1/AE3, and CD34 (original magnification ×100). Subcutaneous inoculations of ESX cells into NOD/SCID mice produced growing tumors. Histologically, the xenografted tumors consisted of a distinct nodular arrangement of the tumor cells, a tendency to undergo central degeneration and necrosis, and an epithelioid appearance with cytoplasmic eosinophilia. The atypical cells were positive for vimentin and AE1/AE3, but negative for CD34.
doi: 10.1371/journal.pone.0084187.g001

supported the hypothesis that CD109 could be essential to maintain the cancer-initiating ability of CSCs/CICs in $ALDH^{high}$ cells of ESX.

We assessed the CD109 mRNA expression profiles in sarcomas and human fetal and normal adult tissues by RT-PCR. In addition to ES, CD109 mRNA expression was observed in 9 of 14 (64%) sarcoma cell lines (Figure 5B). As

Figure 2

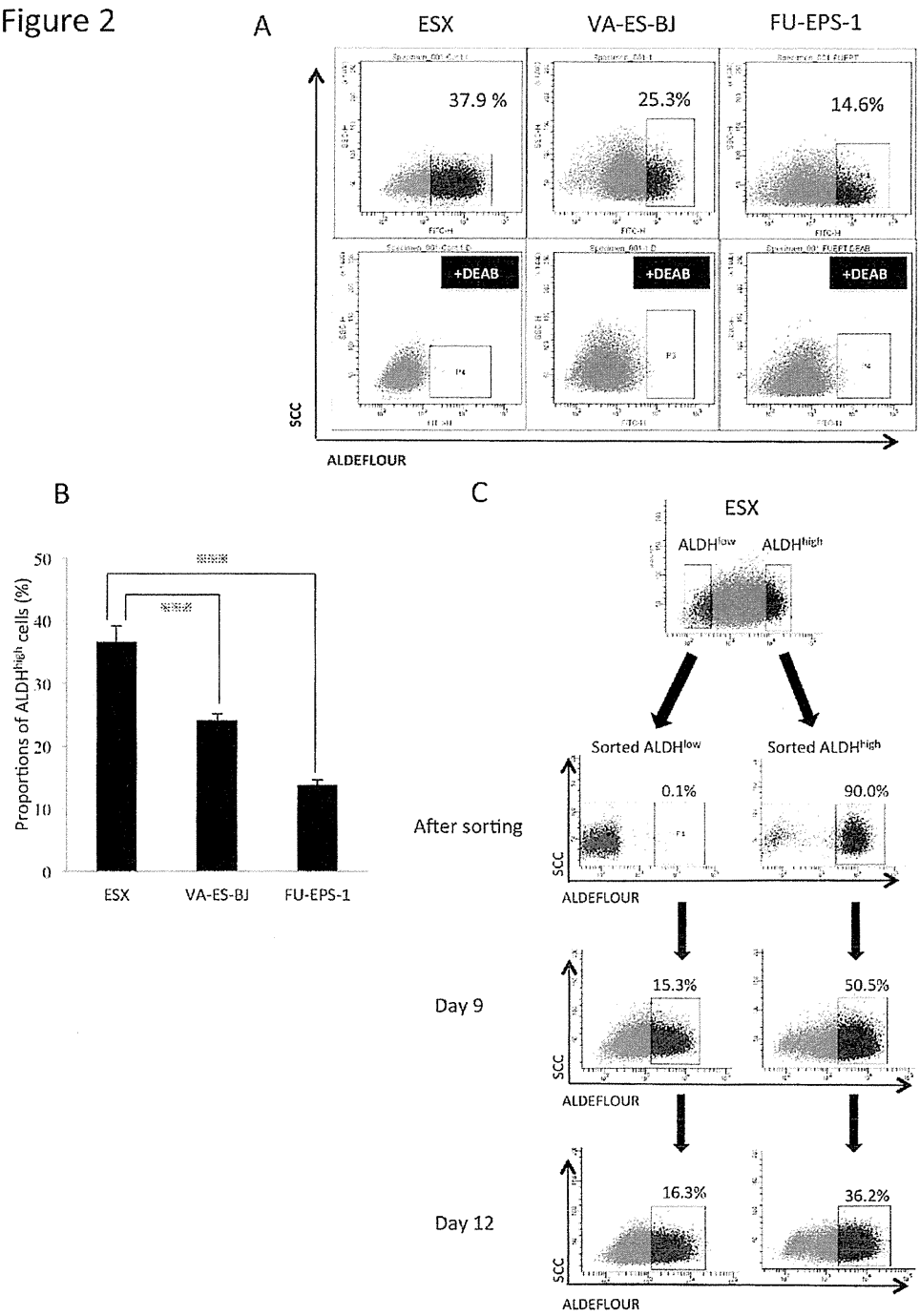
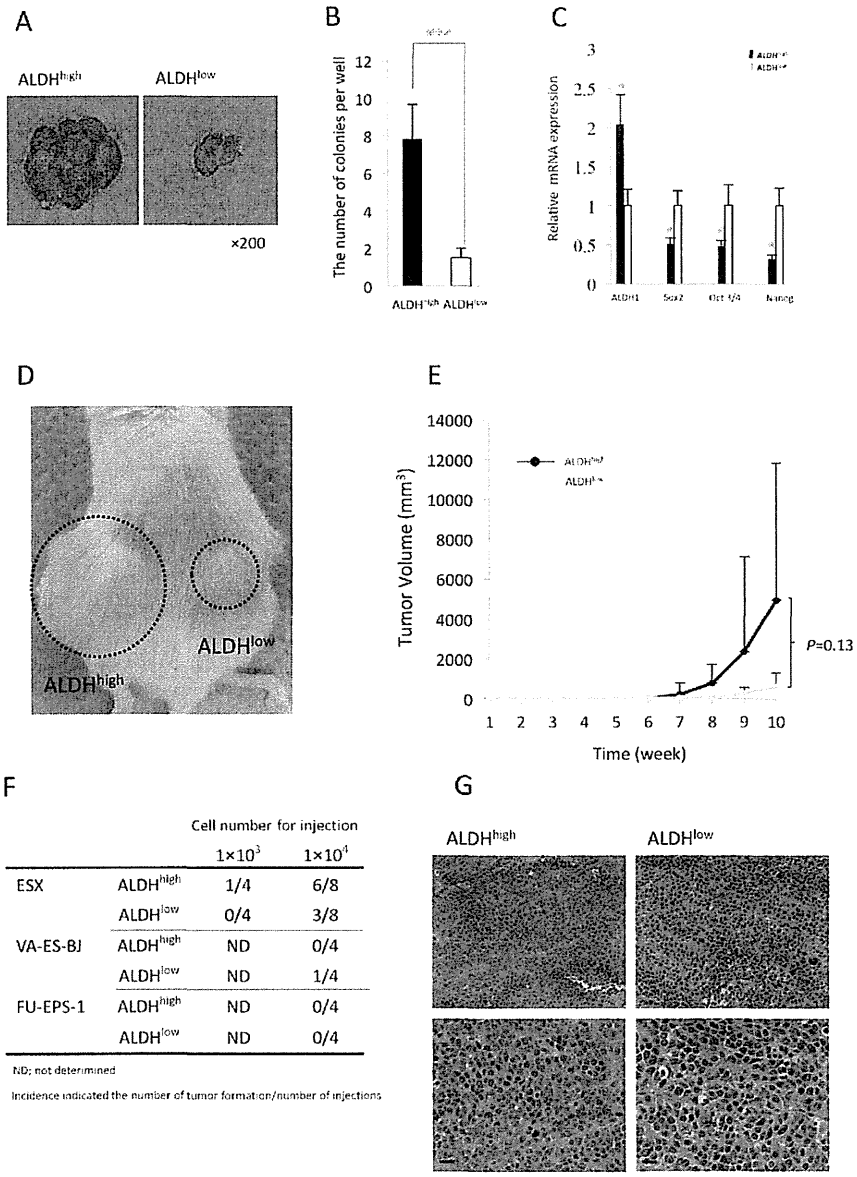


Figure 2. Identification of an ALDH^{high} population in ES cell lines. A. All 3 ES cell lines, ESX, VA-ES-BJ and FU-EPS-1, demonstrated ALDH activity. FACS analysis of ALDH1 expression in cells and the DEAB control.
B. The proportions of ALDH^{high} cells in ESX, VA-ES-BJ and FU-EPS-1. Bars represent mean±SEM (n=4) of multiple experiments. ***p<0.001, determined by the Mann-Whitney test.
C. Differentiation of ALDH^{high} and ALDH^{low} cells in vitro. Sorted ALDH^{high} and ALDH^{low} cells were analyzed on days 0 (immediately after sorting), 9 and 12. Representative fluorescence-activated cell sorting analysis is shown.
doi: 10.1371/journal.pone.0084187.g002

Figure 3



		Cell number for injection	
		1×10^3	1×10^4
ESX	ALDH^{high}	1/4	6/8
	ALDH^{low}	0/4	3/8
VA-ES-BJ	ALDH^{high}	ND	0/4
	ALDH^{low}	ND	1/4
FU-EPS-1	ALDH^{high}	ND	0/4
	ALDH^{low}	ND	0/4

ND; not determined

Incidence indicated the number of tumor formation/number of injections

Figure 3. Cancer-initiating ability of ALDH^{high} cells *in vitro* and *in vivo*. A. The features of spherical colonies derived from resultant ALDH^{high} cells and ALDH^{low} cells of ESX.

B. The number of spherical colonies from ALDH^{high} and ALDH^{low} cells of ESX. Bars represent mean±SEM (n=6). $^{***}p<0.001$, determined by the Mann-Whitney test.

C. The mRNA expression of stem/progenitor cell-related genes. RNA was isolated from freshly sorted spheroid cells (1×10^5) on day 7. Bars represent mean±SEM. $^{*}p<0.05$, determined by the Mann-Whitney test.

D. The features of xenotransplanted cells *in vivo*. Macroscopic features of 1×10^4 ALDH^{high} and ALDH^{low} cells of ESX in an NOD/SCID mouse at 10 weeks after xenotransplantation.

E. Tumor growth curve of ALDH^{high} and ALDH^{low} cells of ESX. Bars represent the mean±SD (ALDH^{high} n= 4, ALDH^{low} n=2).

F. Tumorigenesis of ALDH^{high} and ALDH^{low} cells of ES cell lines in NOD/SCID mice.

G. H&E of the xenotransplanted tumors derived from ALDH^{high} and ALDH^{low}cells of ESX (1×10^4) (above; scale bar 50μm, below; 20μm).

doi: 10.1371/journal.pone.0084187.g003

Figure 4

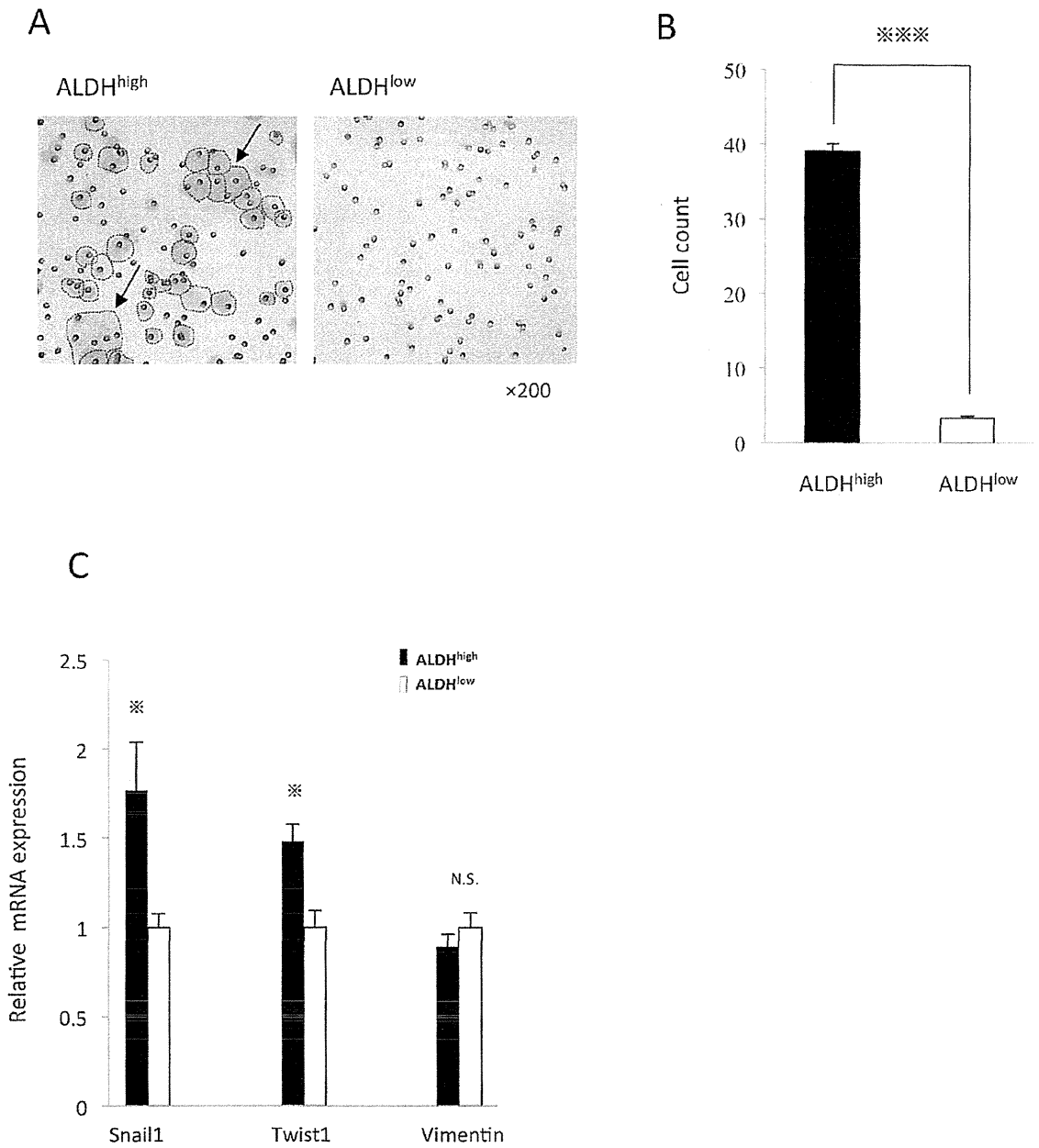


Figure 4. Invasive ability of ALDH^{high} cells of ESX. A. The features of invasion derived from resultant ALDH^{high} and ALDH^{low} cells. Invading cells are indicated by circular dots and arrows.
B. The number of invasive cells. Bars represent mean±SEM (n=4). $^{***}p<0.001$, determined by the Mann-Whitney test.
C. The mRNA expression of EMT-related genes. Bars represent mean±SEM. $^{*}p<0.05$, determined by the Mann-Whitney test.
doi: 10.1371/journal.pone.0084187.g004

Figure 5

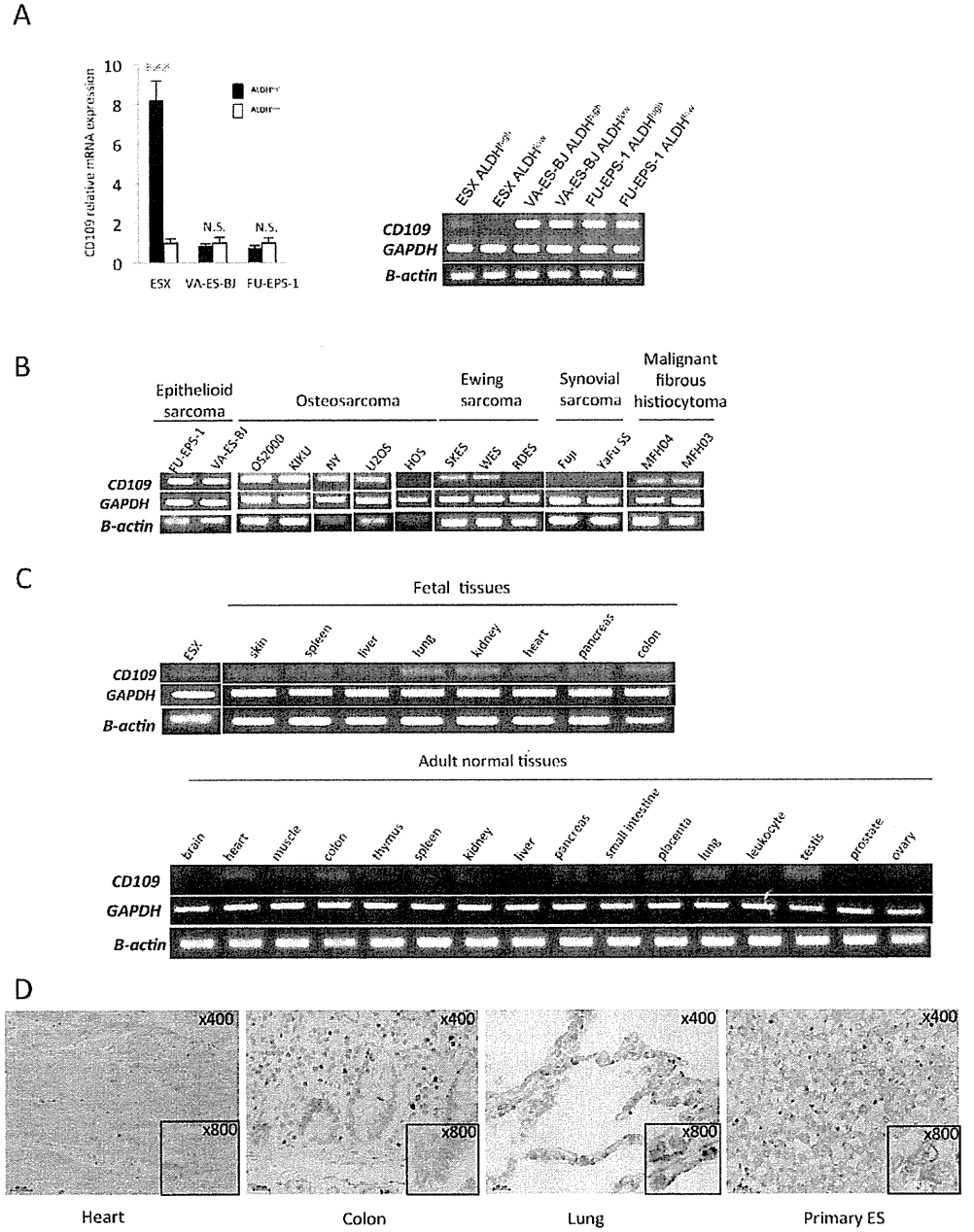


Figure 5. CD109 expression in sarcoma cells and normal adult and fetal tissues. A. Expression of CD109 mRNA in ES cell lines. Bars represent mean±SEM. ***$p<0.001$, determined by the Mann-Whitney test. N.S.: not significant.

B. Expression of CD109 mRNA in human sarcoma cell lines. Cell lines of epithelioid sarcoma (FU-EPS-1 and VA-ES-BJ), osteosarcoma (OS2000, KIKU, NY, U2OS, Saos-2, HuO9 and HOS), Ewing sarcoma (SKES, WES and RDES), synovial sarcoma (Fuji and YaFuSS) and malignant fibrous histiocytoma (MFH2003 and MFH2004) were used.

C. Expression of CD109 mRNA in human fetal tissues (upper panel) and human adult tissues (lower panel). ESX was used as a positive control.

D. Immunohistochemistry of CD109 in normal adult tissues.

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