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Comparative study on the immunogenicity between an HLA-A24-restricted cytotoxic T-cell epitope derived from survivin and that from its splice variant survivin-2B in oral cancer patients

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Abstract

Background: We previously reported an HLA-A24-restricted cytotoxic T-cell epitope, Survivin-2B80-88, derived from a splice variant of survivin, survivin-2B. In this report, we show a novel HLA-A24-restricted T-cell epitope, Survivin-C58, derived from a wild type survivin, and compared their immunogenicity in oral cancer patients.

Methods: By stimulating peripheral blood lymphocytes of HLA-A24-positive cancer patients with Survivin-C58 peptide *in vitro*, the peptide-specific CTLs were induced. In order to compare the immunogenic potential between C58 peptide and 2B80-88 peptide, peripheral blood T-cells from thirteen HLA-A24-positive oral cancer patients were stimulated with either or both of these two peptides.

Results: Survivin-2B80-88 peptide-specific CTLs were induced from four patients, and C58 peptide-specific CTLs were induced from three out of eight patients with over stage II progression. The CTLs exerted cytotoxicity against HLA-A24-positive tumor cells. In contrast, CTL induction failed from a healthy volunteer and all four patients with cancer stage I.

Conclusion: It was indicated that a splicing variant-derived peptide and wild type survivin-derived peptide might have a comparable potency of CTL induction, and survivin targeting immunotherapy using survivin-2B80-88 and C58 peptide cocktail should be suitable for HLA-A24+ oral cancer patients.

Background

Survivin, an inhibitor of apoptosis protein, is highly expressed in the vast majority of cancers [1,2]. Survivin has been shown to increase tumor resistance to apoptotic stimuli, such as radiation and chemotherapy [3,4]. In agreement with these findings, a number of reports demonstrate that survivin expression in cancer cells has a prognostic value and is associated with increased tumor recurrence and shorter patient survival [5-10], although the opposite correlation is reported in certain cancers [11]. So far, four different splicing variants of human survivin have been described, including survivin-2α, survivin-2B, survivin-ΔEx3, and survivin-3B [12-15]. While survivin-2α and survivin-3B are truncated forms, survivin-2B results from alternating splicing at the interface between exon 2 and exon 3, leading to insertion of an additional exon, termed exon 2B, in BIR domain. Since BIR domain is a functional domain that is important for the anti-apoptotic activity of survivin, survivin-2B is predicted to be non-anti-apoptotic [16,17].

Survivin was originally detected only in normal thymus, testis and placenta; however, low levels of wild type survivin was detected in other normal tissues, such as activated T-cells, vascular endothelial cells, and hematopoietic cells by more sensitive methods [3,18,19]. Wild type survivin is known to have an essential role in the mitosis [3,18,20]. It forms a complex with the chromosomal passenger proteins during mitosis and regulates mitotic progression. In contrast, the protein levels as well as the mRNA levels of survivin-2B and other survivin variants are far less than that of wild type survivin, and they are dispensable in such a mitotic checkpoint [17,21].

Since survivin expression is very low in normal differentiated adult tissues as compared with that in cancer tissues, survivin is considered to be an ideal molecular target for cancer immunotherapy. With this mind, we attempted to identify a HLA-A24-restricted cytotoxic T-lymphocyte (CTL) epitopes of survivin that were suitable for cancer vaccine, since HLA-A24 was the most frequent allele in Japanese. In our previous report, three peptides derived from survivin and its splicing variant survivin-2B were examined for HLA-A24-binding affinity and immunogenicity [22]. It was shown that Survivin-2B80-88 peptide (amino acid sequence AYACNTSTL), which was derived from a splicing variant survivin-2B-specific exon2B, was capable of inducing CTLs that had killing activity to HLA-A24^{+} cancer cells. Following this report, we provided further evidence that Survivin-2B80-88 was highly immunogenic in various cancer patients, including those with gastric cancer, breast cancer, and colorectal cancer [23]. Based on these results *ex vivo*, we have conducted phase I clinical trials assessing the adverse event and efficacy of Survivin-2B80-88 peptide vaccination in patients with advanced colorectal cancer, breast cancer, lung cancer, bladder cancer, and oral cancer [24-26].

Though we failed to identify an HLA-A24-restricted CTL epitope derived from wild type survivin in the initial study, a number of epitopes have been identified from wild type survivin that are restricted to other HLA class I alleles, such as A1, A2, A11, and B35 [27-29], some of which have been applied for clinical trials [30,31]. More recently, Andersen, et al. demonstrated that wild type survivin-derived Sur20-28 peptide (amino acid sequence STFKNWPFL) was capable of inducing the peptide-specific CD8-positive T-cells from PBMCs of HLA-A24^{+} cancer patients, although HLA-A24-restricted killing activity of the peptide-specific T-cells against survivin-positive cancer cells has not been assessed [32]. In this study, we present a novel CTL epitope Survivin-C58 peptide derived from wild type survivin. The peptide-specific CTLs induced from peripheral blood mononuclear cells (PBMCs) of oral cancer patient exerted HLA-A24-restricted cytotoxicity against the tumor cells. Then, we stimulated PBMCs of oral cancer patients with either or both Survivin-C58 and Survivin-2B80-88 peptides, and the consequent CTLs were examined for the peptide-specificity and cytotoxicity against HLA-A24+ tumor cells. We demonstrate here for the first time a comparative study on the potency of inducing CTLs *in vitro* between wild type survivin-derived peptide and survivin-2B-derived peptide, which indicates the comparable potency of CTL induction in oral cancer patients.

Materials and methods

Patients and samples

Surgically-resected cancer specimens and PBMCs used in this study were obtained from HLA-A*2402^{+} patients with breast cancer or oral cancer who were hospitalized at Sapporo Medical University Hospital after obtaining their informed consent. The patients' clinicopathological profiles were listed on the table.

Cell lines and culture media

Human breast cancer cell lines, HMC-1 and HMC-2, human oral squamous cell carcinoma (OSCC) cell lines, OSC19, OSC20, OSC30, OSC40, OSC70, and POT1 were established in our laboratory. OSCC cell lines HO-1-NH, KOSC-3, HSC-2, HSC- 3, and HSC-4 were purchased from the Human Science Research Resources Bank (HSRRB, Osaka, Japan). OSCC cell line SAS was obtained from the Institute of Development, Aging and Cancer Tohoku University (Tohoku, Japan). Human embryonic kidney cell line 293T, breast cancer cell line MCF7, lymphoma cell line Daudi and leukemia cell line K562 were purchased from American Type Culture Collection (Manassas, VA). C1R-A24 and C1R-A31, lymphoblastoid cell line C1R transfectants with HLA-A*2402 and HLA-A*31012 cDNA

respectively, were kind gifts from Dr. M. Takiguchi (Kumamoto University School of Medicine, Kumamoto, Japan). T2-A24, a stable transfectant of T2 cells with HLA-A*2402 cDNA was a kind gift from Dr. K. Kuzushima (Aichi Cancer Research Institute, Nagoya, Japan).

293T cells and breast cancer lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO) with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin G, and 100 mg/ml streptomycin at 37°C in humidified 5% CO_2 atmosphere. All the OSCC cell lines were cultured in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin at 37°C in a humidified 5% CO2 atmosphere. OSC20-A24, a stable transfectant of OSC20 with HLA-A*2402 cDNA was cultured in a medium supplemented with 800 ng/ml of puromycin (Sigma-Aldrich, St. Louis, MO). Hygromycin B (0.5 mg/ml, WAKO chemicals, Osaka, Japan) or G418 (800 μg/ml, GIBCO/Invitrogen Corp., Carlsbad, CA) was continuously added to the culture medium for C1R transfectants and T2 transfectant, respectively.

RT-PCR Analysis

A set of total RNA from normal human adult tissues was purchased from Clontech (human total RNA master panel). Total RNA was isolated from cultured cells by using ISOGEN reagent (Nippon Gene, Tokyo, Japan). The cDNA mixture was synthesized from 1 mg of total RNA by reverse transcription using Superscript II and oligo(dT) primer (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's protocol. PCR amplification was performed in 50 ml of PCR mixture containing 1 mL of the cDNA mixture, KOD Plus DNA polymerase (Toyobo, Osaka, Japan), and 50 pmol of primers. The PCR mixture was initially incubated at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 57°C for 30 s, and extension at 68°C for 1 min. Primer pairs used for RT-PCR analysis were 5'-TCAAGGACCACCGCATCTCTAC-3' and 5'-GCACTTTCT-TCGCAGTTTCCTC-3' as a forward and a reverse primer, respectively. Expected sizes of PCR products for wild type survivin, survivin-2B, and survivin-DEx3 were 355 bp, 424 bp, and 236 bp, respectively. As an internal control glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was detected by using a forward primer 5'-ACCACAGTC-CATGCCATCAC-3' and a reverse primer 5'-TCCACCAC-CCTGTTGCTGTA-3' with an expected PCR product of 452 bp. The PCR products were visualized with ethidium bromide staining under UV light after electrophoresis on 1.0% agarose gel. Nucleotide sequence of the PCR products was confirmed by direct sequencing using ABI Genetic analyzer PRIM 310 and an AmpliCycle sequencing kit (Perkin-Elmer, Foster City, CA).

Western blotting

Cultured cells were washed in ice-cold PBS, lysed by incubation on ice in a lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% NP40, protease inhibitor cocktail; Complete, Roche Diagnostics, Inc., Basel, Switzerland], and clarified by centrifugation at 15,000 rpm for 20 minutes at 4°C. The whole-cell lysates were boiled for 5 minutes in the presence of SDS sample buffer, resolved by 10% SDS-PAGE, and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Billerica, MA). The membranes were then incubated with blocking buffer (5% nonfat dry milk in PBS) for 1 hour at room temperature and then incubated for 40 minutes with mouse anti-human survivin monoclonal antibody (Santa Cruz Biotechnology) or mouse anti-β-actin monoclonal antibody AC-15 (Sigma-Aldrich). After washing three times with wash buffer (0.1% Tween-20 in PBS), the membrane was reacted with peroxidase-labeled goat anti-mouse IgG antibody (KPL, Gaithersburg, MD) for two hours. Finally, the signal was visualized by using an enhanced chemiluminescence (ECL) detection system (Amersham Life Science, Arlington Heights, IL) according to the manufacturer's protocol.

Peptides and Cytokines

Wild type survivin-derived peptides carrying HLA-A24 binding motif Survivin-C58 (amino acid sequence FFCFKELEGW), a splicing variant survivin-2B-derived peptide Survivin-2B80-88 (AYACNTSTL) [22], EBV LMP2-derived HLA-A24 binding peptide (TYGPVFMSL) [33], HIV env-derived HLA-A24 binding peptide (RYL-RDQQLLGI) [34], CMV pp65-derived HLA-A24 binding peptide (QVDPVAALF), mouse VSV-derived peptide VSV8 (RGYVYQGL), and synovial sarcoma chromosomal translocation product SYT-SSX-derived SS393 peptide and K9I peptide (GYDQIMPKK and GYDQIMPKI respectively) [35,36] were purchased from SIGMA Genosys (Ishikari, Japan). They were resolved in DMSO at the concentration of 5 mg/ml and stored at -80°C. Human recombinant interleukin (IL)-2 was a kind gift from Takeda Pharmaceutical Co. (Osaka, Japan). Human recombinant GM-CSF was a kind gift from Kirin (Tokyo, Japan) and Novartis Pharmaceutical (Basel, Switzerland). Human recombinant IL-4 and IL-7 were purchased from Invitrogen (San Diego, CA).

Peptide Binding Assay

Peptide binding affinity to HLA-A24 was assessed by HLA-A24 stabilization assay as described previously [22]. Briefly, after incubation of T2-A24 cells in culture medium at 26°C for 18 h, cells (2×10^5) were washed with PBS and suspended with 1 ml of Opti-MEM (Life Technologies,

Inc.) containing 3 µg/ml of β 2-microglobulin with or without 100 µg of peptide, followed by incubation at 26°C for 3 h and then at 37°C for 3 h. After washing with PBS, the cells were incubated with anti-HLA-A24 monoclonal antibody (c7709A2.6, kindly provided by Dr. P. G. Coulie, Ludwig Institute for Cancer Research, Brussels Branch) at 4°C for 30 min, followed by incubation with FITC-conjugated rabbit anti-mouse IgG at 4°C for 30 min. The cells were then suspended with 1 ml of PBS containing 1% formaldehyde and analyzed by FACScan (Becton Dickinson, Mountain View, CA). Binding affinity was evaluated by comparing mean fluorescence intensity of HLA-A24 expression in the presence of peptide pulsation to mean fluorescence intensity in the absence of the peptide.

Peptide specific CTL induction with immature dendritic cells and phytohemagglutinin blasts

CTLs were induced from PBMCs by using autologous dendritic cells (DCs) and phytohemagglutinin (PHA) blasts as antigen presenting cells (APC). Briefly, PBMCs were isolated from one healthy volunteer and 12 cancer patients (one breast cancer and eleven oral cancer) by standard density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway) and cultured in AIM-V medium (Life Technologies) at 37°C for 2 h to separate adherent cells and non-adherent cells. Autologous immature DCs were generated from adherent cells in the plastic flask by culturing in AIM-V medium supplemented with HEPES (10 mmol/L), 2-mercaptoethanol (50 µmol/L), GM-CSF (1000 units/mL) and IL-4 (1000 units/mL) for 7 days. $CD8^+$ cells were isolated from non-adherent cells in the plastic flask by the MACS separation system (Miltenyi Boitech, Bergish Blabach, Germany) using anti-CD8 monoclonal antibody coupled with magnetic microbeads according to manufacturer's instruction. PHA blasts were derived from $CD8^-$ cells by culturing in AIM-V medium containing IL-2 (100 units/mL) and PHA (1 µg/mL, Wako Chemicals, Osaka, Japan) for 3 days, followed by washing and cultivation in the presence of IL-2 (100 units/ml) for 4 days. DCs and PHA blasts were cultured in AIM-V medium supplemented with 50 µmol/L of peptide at room temperature for 2 h, washed with AIM-V, and then irradiated (100 Gy) before use.

CTL induction procedure was initiated by stimulating 2×10^6 $CD8^+$ cells with peptide-pulsed autologous DCs at a 20:1 effecter/APC ratio in AIM-V supplemented with IL-7 (10 ng/mL) for 7 days at 37°C. The following stimulation was performed with peptide-pulsed PHA blasts at a 5:1 effector/APC ratio. On the next day of the second stimulation, IL-2 was added to the culture at a concentration of 50 units/mL. The same CTL stimulation cycle with PHA blasts was then performed twice more over the period of two weeks. One week after the 4th stimulation, cytotoxic activity of the CTLs was measured by ^{51}Cr release assay.

Cytotoxicity assay

The cytotoxic activities of CTLs were measured by ^{51}Cr release assay as described previously [22]. Briefly, target cells were labeled with 100 µCi of ^{51}Cr for 1 hr at 37°C, washed thrice, and resuspended in AIM-V medium. Then, 3×10^3 ^{51}Cr-labeled target cells were incubated with effecter cells at various effector/target (E/T) ratios at 37°C for 6 h in V-bottom 96-well microtiter plates. Then supernatants were collected and the radioactivity was measured by a gamma-counter. The percentage of specific lysis was calculated as following: % specific lysis = (test sample release - spontaneous release) × 100/(maximum release - spontaneous release). For preparation of peptide-pulsed target cells, T2-A24 cells or C1R-A24 cells were incubated with 50 µg/mL of peptide at room temperature for 2 h before the assay. For preparation of tumor target cells, target cells were treated with 100 units/ml of IFN-γ for 48–72 h before the assay.

Results

Survivin expression in oral cancer cells

We previously showed that the survivin mRNA level was elevated in various cancer cell lines, including gastric cancer cells, colon cancer cells, breast cancer cells, lung cancer cells, bladder cancer cells, renal cancer cells, and melanoma cells [22]. In the present study, we focused on the survivin expression in oral cancer cell lines. In concurrence with previous reports [5], survivin was highly expressed in oral cancer tissues as well as oral cancer cell lines. In the RT-PCR analysis, three bands were detected, corresponding to survivin-2B, wild type survivin, and survivin-ΔEx3 respectively (Fig. 1A), which were confirmed by DNA sequence analysis. By the same RT-PCR method, wild type survivin expression was detected only in the placenta, thymus, and testis among normal adult tissues; however, survivin-2B and survivin-ΔEx3 were barely detected (Fig. 1B). By using more sensitive RT-PCR analysis, expression of these splicing variants was shown only in the thymus [22].

We then analyzed the survivin expression in the protein level. In all the oral cancer cell lines examined in the present study, wild type survivin was detected, but not in normal oral mucosal tissue by Western blotting (Fig. 2). A small amount of survivin-2B protein was also detected in some cell lines. These data indicate that the expression of survivin-2B was more restricted to cancer tissues, though its level was far less as compared to that of wild type survivin.

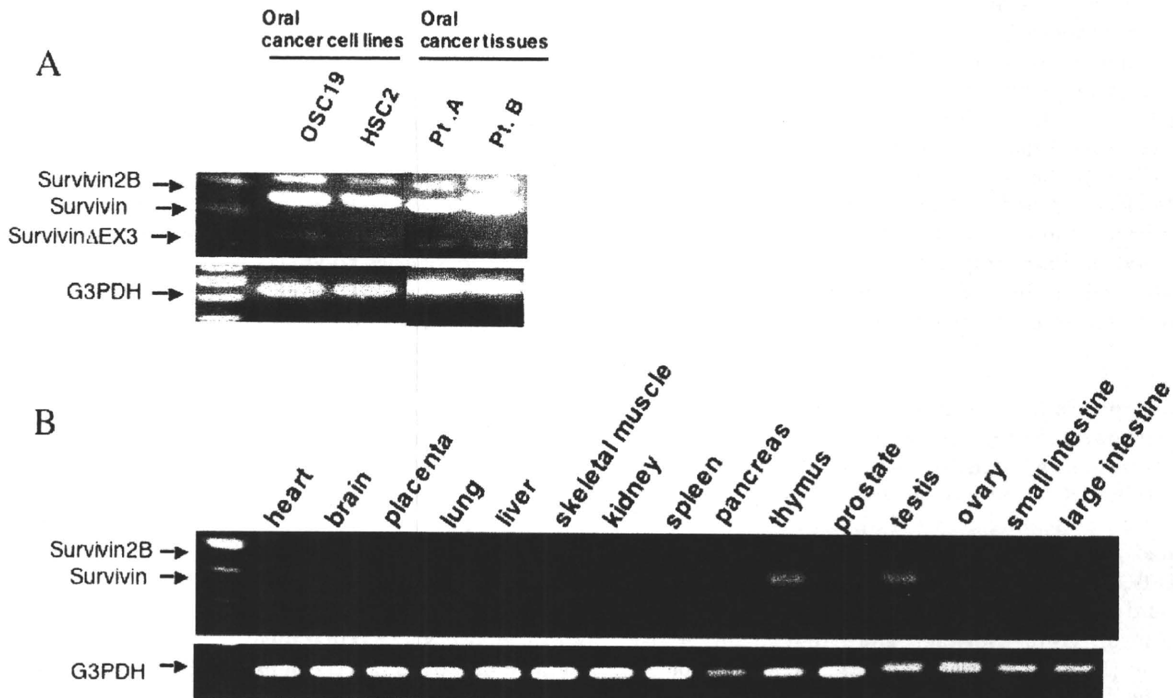

Figure 1
Expression of survivin mRNA as assessed by RT-PCR in normal tissues, and oral cancer cell lines and primary oral cancer tissues. (A) Expression of survivin mRNA in oral cancer cell lines and primary oral cancer tissues from two patients. G3PDH expression was detected as an internal control. **(B)** Expression of survivin mRNA in normal adult tissues. 293T cells transfected with myc-tagged survivin cDNA (293T-survivin) was used as a positive control for survivin expression. G3PDH expression was detected as an internal control.

HLA-A24-binding analysis of survivin-derived peptides
To evaluate if wild type survivin might become a target of immunotherapy as well as a splicing variant survivin-2B, we re-screened the total amino acid sequence of wild type survivin protein for peptides containing HLA-A24-binding motif. In our previous report, two peptides, survivin85-93 and survivin92-101, derived from exon 3-encoded region were examined; however, they did not have a significant binding affinity to HLA-A24 [22]. In the present study, we identified another peptide, designated as survivin-C58 (amino acid sequence FFCFKELEGW), which was derived from exon 2-encoded region. Survivin-C58 and survivin-2B80-88 were assessed for the binding ability to HLA-A24 molecule by HLA stabilization assay using transporters associated with antigen processing (TAP) deficient and HLA-A*2402-transfected cell line, T2-A24 cells, as described previously [35,36]. Two positive control peptides, HLA-A24-restricted CMV-pp65 epitope and HIV-env epitope, and a negative control peptide VSV8 were used in the assay. HLA-A24 level on the cell surface of T2-A24 cells is up-regulated in the presence of HLA-A24-binding peptides. Up-regulation of mean fluorescence intensity (MFI) of cell surface HLA-A24 was detected by flow cytometer (Fig. 3). Both CMV-pp65-derived peptide and HIV-env-derived peptide increased MFI of HLA-A24 clearly, while VSV8-derived peptide failed, indicating adequate qualification of this assay. Both survivin-2B80-88 and survivin-C58 peptides were capable of up-regulating the HLA-A24 levels, though survivin-C58 showed less binding capacity than survivin-2B80-88.

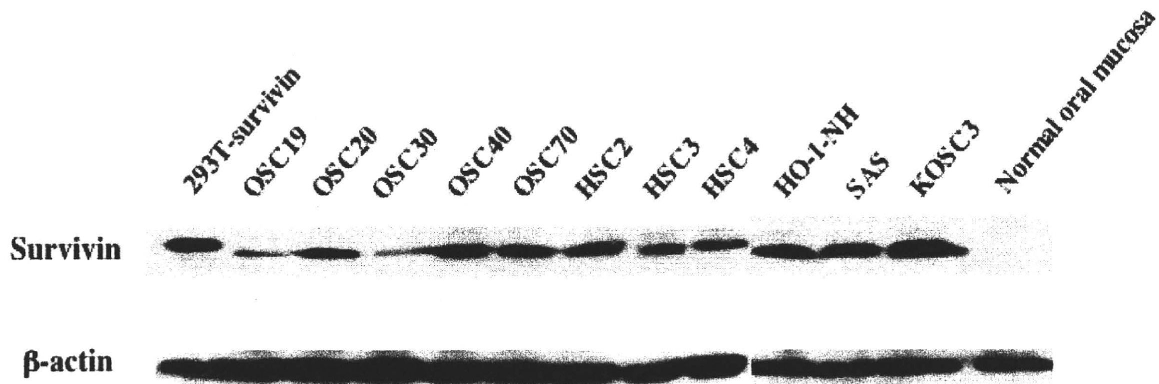

Figure 2
Western blotting analysis of survivin protein in oral cancer cell lines. Lysates from oral cancer cell lines or normal oral mucosal tissue were resolved by 10% SDS-PAGE and transferred to PVDF membranes. The membranes were then incubated with mouse anti-human survivin monoclonal antibody (upper panel) or mouse anti-β-actin monoclonal antibody AC-15 (lower panel).

*CTL induction from PBMCs of HLA-A*2402⁺ cancer patients*

In order to know if HLA-A24-restricted peptide-specific CTLs are induced from PBMCs of cancer patients, PBMCs were collected from HLA-A*2402-positive cancer patients (one breast cancer patient and one oral cancer patient), and stimulated *in vitro* with survivin-C58 peptide in the presence of autologous monocyte-derived DC or autologous PHA blasts. After 4 times stimulation, cytotoxic activity against peptide-pulsed target cells was examined by ^{51}Cr release assay. As shown in Fig. 4A, CTLs induced from PBMCs of a breast cancer patient were capable of killing survivin-C58-pulsed T2-A24 target cells, but they failed in killing SYT-SSX-derived peptide-pulsed T2-A24 cells or survivin-C58-pulsed HLA-A24-negative target cells. The same CTLs showed a significant cytotoxicity to HLA-A*2402-positive breast cancer cells, HMC2 and HMC1, but not to HLA-A*2402-negative breast cancer cells MCF7. The similar result was shown in Fig. 4B, when CTLs were induced from an oral cancer patient (Case #13 in Table 1). Survivin-C58 peptide-specific CTLs showed cytotoxicity against HLA-A*2402-transfected oral cancer cell line OSC20. Therefore, it was indicated that wild type survivin-derived survivin-C58 peptide could be presented on tumor cells in the context of HLA-A24 and recognized by CTLs.

CTL induction efficiency with survivin-2B80-88 or survivin-C58 from PBMCs of HLA-A24+ oral cancer patients

Previously we showed that survivin-2B80-88-specific CTLs were induced efficiently from PBMCs of HLA-A24+ patients with survivin-positive breast cancer, colorectal cancer, and gastric cancer [23]. In the present study, we examined if survivin-2B80-88-specific CTLs and survivin-C58-specific CTLs could be induced from PBMCs of HLA-A24+ oral cancer patients. PBMCs were collected from thirteen patients with survivin-positive oral cancer and one healthy volunteer with HLA-A*2402 genotype (Table 1), and stimulated with either or both of these two peptides *in vitro* in the presence of autologous DC or PHA blasts as APCs. After 4 times stimulation over a period of four weeks, CTLs were examined for their peptide-specific killing activity by 51-Cr release assay using peptide-pulsed T2-A24 target cells. Survivin-2B-specific CTLs were induced from four patients out of twelve patients examined, and survivin-C58-specific CTLs were induced from three patients out of twelve patients examined. Though the number of patients in this study was too few to discuss the exact correlation, it is possible that the CTL induction efficiency might be related to the disease progression stage of the patients, since CTLs could not be induced from any of four patients with stage I (cases #2, #9, #11, and #12), nor from a healthy volunteer.

PBMCs from eleven patients were stimulated with survivin-2B80-88 and survivin-C58 peptides in separate

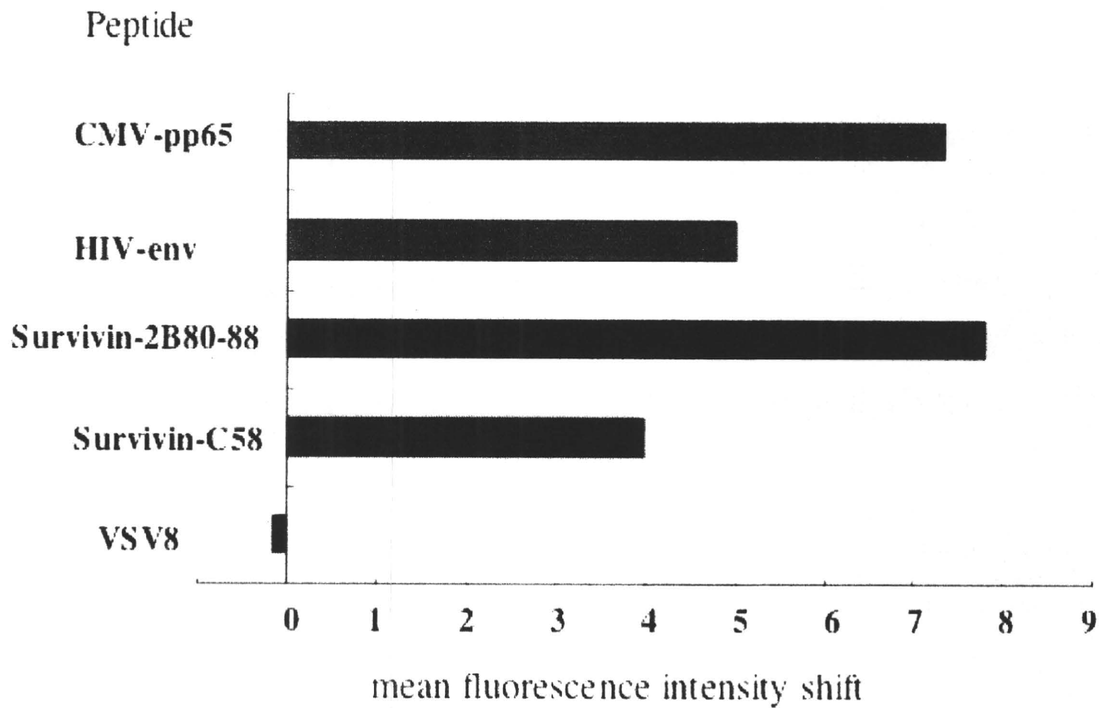

Figure 3
HLA-A24-binding assay of peptides. Binding affinity of peptide to HLA-A24 molecule was evaluated by mean fluorescent intensity (MFI) shift of cell surface HLA-A24 level on T2-A24 cells that were pulsed with each peptide. CMV pp65-derived HLA-A24-binding peptide (QVDPVAALF) and HIV env-derived HLA-A24-binding peptide (RYLRDQQLLGI) were used as positive controls. VSV-derived peptide VSV8 (RGYVYQGL) was used as a negative control. Histograms of MFI shift were displayed for each peptide. MFI shift was calculated as; MFI shift = (MFI of T2-A24 cells pulsed with the peptide) - (MFI of T2-A24 cells without peptide pulsation).

Table 1: CTL induction from PBMCs of oral cancer patients

						CTL induction		Survivin
Case no.	age	sex	stage	Origin, histology	Prior treatment	2B80-88 specific CTL	Survivin-C58-specific CTL	expression
#1	63	M	Stage II	buccal mucosa, SCC, well	Chem, Surg	-	+	+
#2	69	M	Stage I	tongue, SCC, basaloid	Surg	-	-	+
#3	60	F	Stage II	mandibular, SCC, m	Chem, Surg	-	-	+
#4	60	M	Stage III	oropharynx, SCC, mod	Chem, Surg	+	-	+
#5	50	F	Stage II	tongue, SCC, well	Chem, Surg	+	+	+
#6	83	F	Stage IVA	maxillary gingiva, SCC, well	Chem, Surg	-	-	+
#7	64	M	Stage II	tongue, SCC, well	Chem, Surg	+	n.d.	+
#8	50	F	Stage II	submaxillary gland, Adenoid cystic	Chem, Surg	-	-	+
#9	65	F	Stage I	tongue, SCC, well	Surg	-	-	+
#10	66	F	Stage II	tongue, SCC, mod	Chem, Surg	+	-	+
#11	73	F	Stage I	tongue, SCC, well	Surg	-	-	+
#12	50	F	Stage I	tongue, SCC, well	Surg	-	-	+
#13	67	M	Stage IVA	oral floor, SCC, poorly	Chem, Surg	n.d.	+	+
H#1	35	M	-	healthy volunteer	-	-	-	

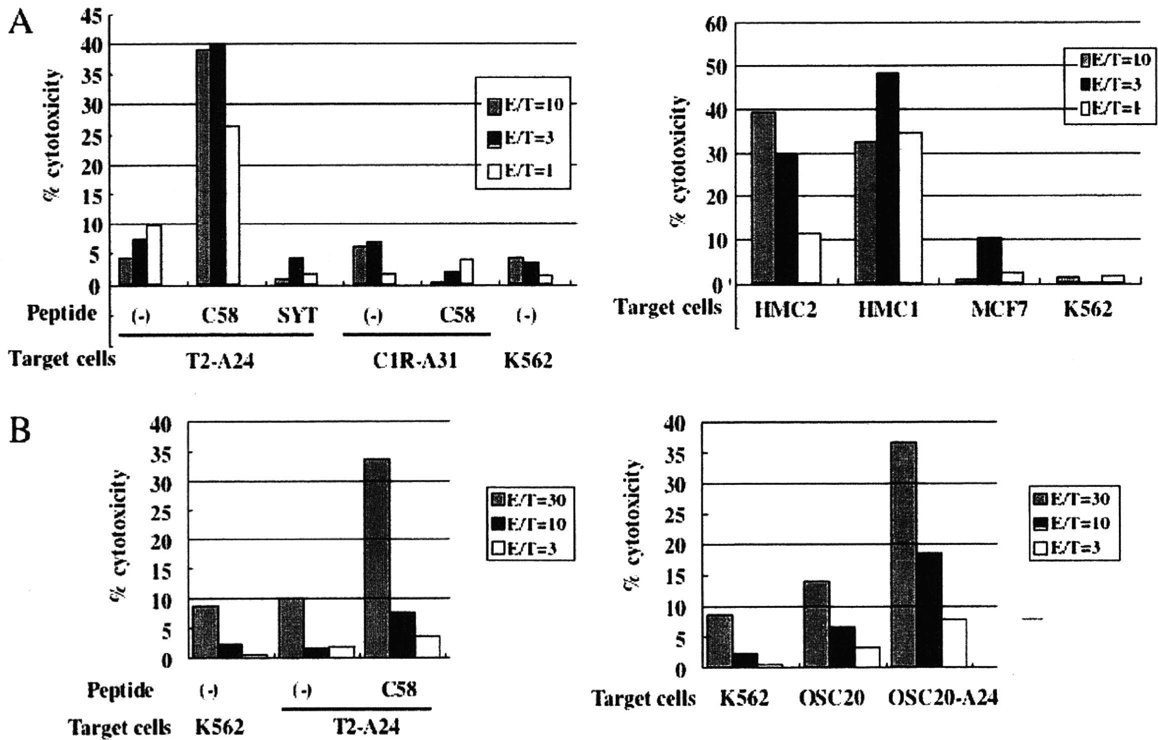

Figure 4
Induction of survivin-C58 peptide-specific CTLs and their cytotoxicity against survivin-positive cancer cell lines. CTLs were induced from PBMCs of an HLA-A*2402+ breast cancer patient by stimulating with survivin-C58 peptide-pulsed APCs. After four times stimulation, CTLs were subjected to standard ^{51}Cr release assay at the indicated effector/target (E/T) ratio. In the left panel, T2-A24 cells and C1R-A31 cells were pulsed with or without survivin-C58 peptide (C58) or SYT-SSX-derived SS393 peptide (SYT), serving as target cells. In the right panel, survivin-positive breast cancer cell lines with HLA-A*2402 (HMC1 and HMC2) or without HLA-A*2402 (MCF7 and K562) were used as target cells. **(A)** CTLs were induced from PBMCs of an HLA-A*2402+ oral cancer patient (case #13 in Table 1) by stimulating with survivin-C58 peptide-pulsed APCs. After four times stimulation, CTLs were subjected to standard ^{51}Cr release assay at the indicated effector/target (E/T) ratio. In the left panel, T2-A24 cells were pulsed with or without survivin-C58 peptide (C58), serving as target cells. In the right panel, survivin-positive HLA-A*2402-negative oral cancer cells (OSC20) and OSC20 transfectants with HLA-A*2402 cDNA (OSC20-A24) were used as target cells.

wells. CTLs with specificity to either of the two peptides were induced from three cases (case #1 specific to survivin-C58, and cases #4 and #10 specific to survivin-2B80-88), and both survivin-2B80-88-specific CTLs and survivin-C58-specific CTLs were successfully induced from one case (case #5) (Fig. 5). These data indicate that survivin-2B80-88 and survivin-C58 peptides have a comparable potency of CTL induction in oral cancer patients.

Discussion

Survivin is overexpressed in a variety of cancer tissues, and at least four different splicing variants have been identified so far. Wild type survivin is known to have an important role in the mitotic checkpoint in normal cells and an anti-apoptotic function in cancer cells [3,18]. In contrast, the splicing variants are dispensable in the mitotic checkpoint [21], and anti-apoptotic function is lost in some splicing variants such as survivin-2B, in which BIR domain is disrupted by the insertion of exon 2B [17]. Survivin-2B and other splicing variant proteins are unstable

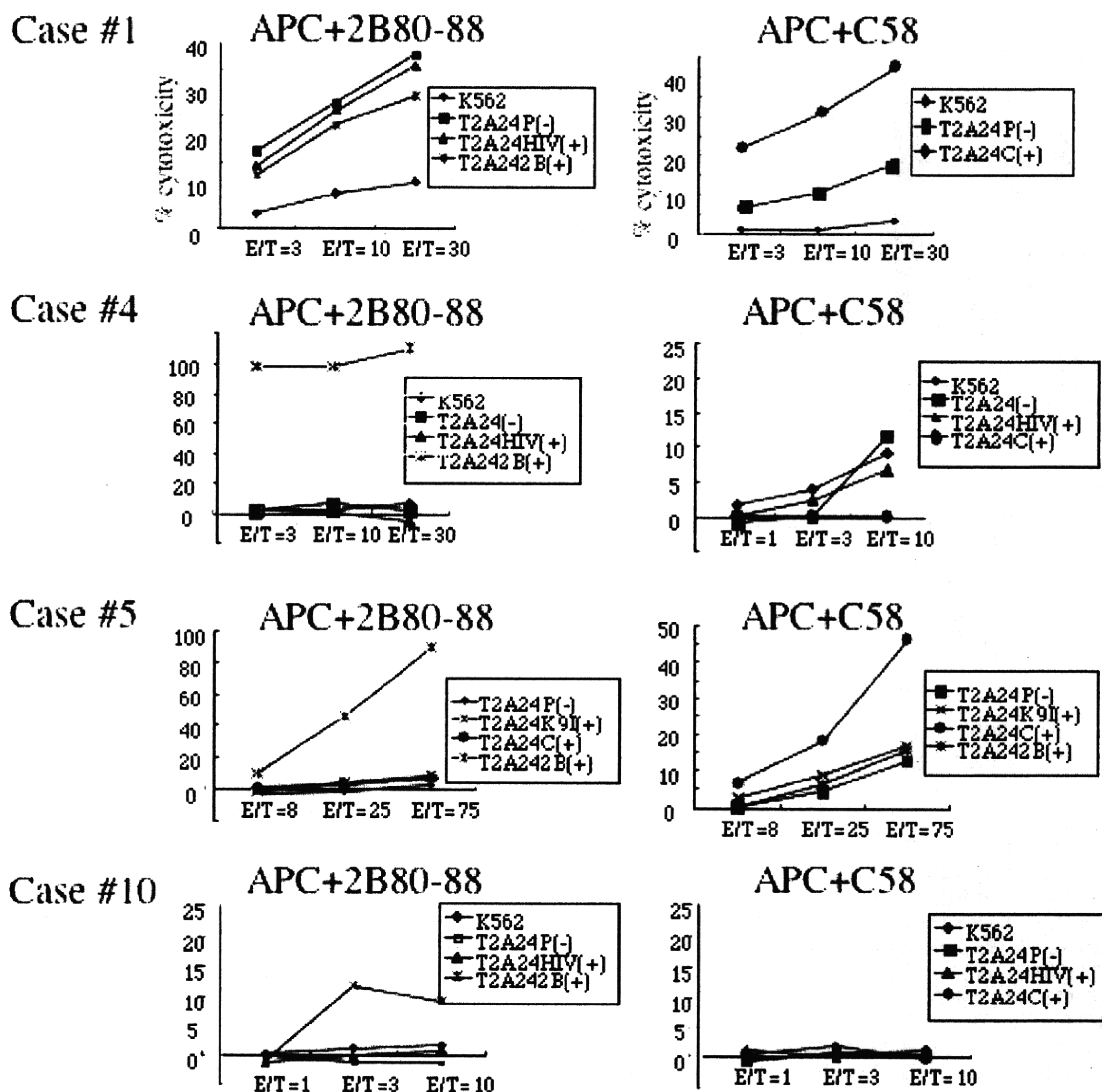

Figure 5

Peptide-specific CTL induction using survivin-2B80-88 peptide and survivin-C58 peptide from PBMCs of HLA-A*2402+ oral cancer patients. PBMCs of HLA-A*2402+ oral cancer patients were stimulated *in vitro* with survivin-2B80-88 peptide-pulsed APCs (APC+2B80-88) and survivin-C58 peptide-pulsed APCs (APC+C58) separately, followed by assessment of the peptide-specific cytotoxic activity by ^{51}Cr release assay at the indicated effector/target (E/T) ratio. T2-A24 cells were pulsed with HIV-env peptide (HIV+), SYT-SSX-derived peptide (K9I+), survivin-2B80-88 peptide (2B+), or survivin-C58 peptide (C+), serving as target cells. P(-) indicates T2-A24 target cells without peptide pulsation. K562 target cells were used for monitoring natural killer activity and lymphokine-activated non-specific cytotoxicity.

in cells, thereby degraded rapidly. Therefore, survivin splicing variants do not appear to be suitable for the target molecules in targeting cancer therapy. However, survivin-2B is an attractive target antigen for cancer immunotherapy, since it contains a unique amino acid sequence and is barely expressed in normal adult tissue including thymus, where T-cell tolerance is induced. We have identified HLA-A24-restricted CTL epitope survivin-2B80-88 derived from survivin-2B previously and reported that it had a high potency of CTL induction in various cancer patients including breast cancer, colorectal cancer, and gastric cancer patients [23]. On the basis of these findings *in vitro*, clinical trials of survivin-2B80-88 peptide immunotherapy have been conducted for advanced cancers such as colorectal cancer, breast cancer, lung cancer, and oral cancer [24,26], in which tumor regression (partial response) was observed in certain cases. Other groups have identified the other HLA-restricted CTL epitopes from wild type survivin and applied for clinical trials [30,31]. More recently, a novel HLA-A24-restricted CTL epitope Sur20-28 was identified from wild type survivin by the screening of a peptide library of overlapping nonamers spanning the full length of survivin protein [32]. Though the peptide was shown to induce peptide-specific perforin-positive CD8+ T-cells from PBMCs of cancer patients, it remains to be determined whether the peptide-specific T-cells have a capability of killing cancer cells in an HLA-A24-restricted manner. However, it may be true that wild type survivin is also immunogenic to cancer host as well as its splicing variant survivin-2B. Therefore, we re-screened to find a novel CTL epitope derived from wild type survivin in the present study. Survivin-C58 peptide-specific CTLs were successfully induced from PBMCs of advanced oral cancer patients and exerted HLA-A24-restricted cytotoxicity against oral cancer cells. The CTL induction efficiency of survivin-C58 peptide was almost comparable to that of survivin-2B80-88 peptide, and it was noted that CTL could not be induced from PBMCs of oral cancer patients with stage I. These findings contrast with our previous report that survivin-specific CTLs were induced successfully from PBMCs of breast cancer patients and colorectal cancer patients with stage I [23]. It is speculated that immunogenicity of tumor-expressed survivin may be lower in the early oral cancer than that in other cancers. It is possible that the peptide-specific CTL efficiency might be related to the expression levels of survivin or survivin-2B proteins in the tumor tissues. As shown in Table 1, survivin expression was detected in all the cases by immunostaining. Though there were some differences in the staining intensity among the cases, we couldn't find any correlation between the staining intensity and the CTL induction efficiency.

Why does survivin have so immunogenic feature despite the abundant expression in thymus? The exact answer remains unknown. Interestingly, we observed that survivin-positive cells in thymus are mainly cortical thymocytes, but not medullary epithelial cells or dendritic cells that mediate negative selection and T-cell tolerance. It may explain at least in part the incomplete peripheral tolerance and immunogenic feature of survivin.

Conclusion

In conclusion, we provided evidence that wild type survivin is an attractive target for the immunotherapy against oral cancer as well as survivin-2B, and survivin targeting immunotherapy using survivin-2B80-88 and C58 peptide cocktail should be suitable for HLA-A24+ cancer patients.

Abbreviations

CTL: cytotoxic T-lymphocyte; PBMC: peripheral blood mononuclear cells; OSCC: oral squamous cell carcinoma; DC: dendritic cell; PHA: phytohemagglutinin; APC: antigen presenting cell.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JK carried out the CTL induction, killing assays and drafted the manuscript. TT and YH participated in the design of the study and performed the evaluation of the data. TT helped to draft the manuscript. SI contributed to the HLA-A24-binding assay and CTL induction from PBMCs. AM, AY and HH contributed to collecting patients' samples with the informed consent. HH and NS contributed to the design and coordination of this study as well as reviewing the manuscript. All authors have read and approved the final manuscript.

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Diagnosis of alveolar echinococcosis using immunoblotting with plural low molecular weight antigens

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Abstract

Alveolar echinococcosis (AE) is endemic to Hokkaido, Japan. For the past 20 years, detection of AE among inhabitants has involved serological screening using an enzyme-linked immunosorbent assay (ELISA) followed by Western blotting (WB). Between the years 1987 and 2000, antigens targeted on 66, 55 and 30–35 kDa bands were routinely used in the WB step of AE diagnosis. However, since 2001 diagnosis has been dependent on three smaller molecular weight antigens (26–28, 18 and 7–8 kDa). Due to its higher sensitivity, this improved WB approach has been used as a confirmation step in the screening process and also for the testing of suspected AE cases in hospital outpatients. Using the improved WB technique, a total of 1745 serum samples were examined in 2001–2006 with 81 patients detected and registered with AE. Interestingly, sera from 76 of the 81 diagnosed AE patients (93.8%) demonstrated reactivity with all three antigens. However, sera from the remaining five patients (6.2%) demonstrated no reactivity with the 18 kDa antigen, even though they exhibited clearly detectable levels of reactivity with the 26–28 and 7–8 kDa bands. These results suggest that medical practitioners need to pay particular attention to the specific reactions to some different diagnostic antigens to minimize the risk of misdiagnosing AE patients. In turn, these results may also provide important diagnostic information for cystic echinococcosis (CE).

Introduction

Human alveolar echinococcosis (AE) is a severe parasitic zoonosis caused by infection with the metacestode *Echinococcus multilocularis*. In Japan, *E. multilocularis* infection is prevalent in Hokkaido where local red foxes serve as the definitive host and small rodents serve as the intermediate host. In order to detect AE in Hokkaido, mass screening of residents for this disease has been undertaken over the past 20 years as a part of administrative countermeasures (Kimura *et al.*, 1999).

The initial screening process utilized to detect AE is divided into two steps. The first step involves the use of an enzyme-linked immunosorbent assay (ELISA) (Sato *et al.*, 1983a, b), and the second step consists of both a Western blotting (WB) (Furuya *et al.*, 1987, 1989, 1990; Nagano *et al.*, 1995) and ultrasound-based tests. These tests are often used in provisional confirmation prior to surgical treatment. The final diagnosis for most AE patients is based on histopathological findings following surgery (Condon *et al.*, 1988; Furuya *et al.*, 2001).

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The Hokkaido Institute of Public Health (HIPH) has played an important role in the serological aspect of the mass screening process. Furthermore, the procedures utilized in the detection of AE have also brought about numerous improvements in the testing of serological samples at a more general level.

In the original WB method undertaken between 1987 and 2000, diagnosis of AE was based on the presence of 66, 55 and 30-35 kDa bands (Furuya *et al.*, 1989). Subsequent to this time and due to a higher sensitivity and specificity, the presence of 26–28, 18 and 7–8 kDa bands is now used for the provisional confirmation of the disease (Furuya *et al.*, 2004; Yamano *et al.*, 2005).

On the basis of diagnosis using these low molecular weight antigens, numerous serum samples from people previously screened and from patients suspected to have AE in hospitals were examined between 2001 and 2006. As a result, we were able to identify 81 new AE cases in this 6-year period. However, 5 of the 81 cases demonstrated no reactivity with the 18 kDa antigen, although reactivity with one or both of the 26–28 and 7–8 kDa antigens was evident. In this paper, we report on the five registered AE patients with interesting serological characteristics and discuss the importance of making diagnoses based on the overall reactivity against plural antigens.

Materials and methods

Serum samples

A total of 278,696 serum samples were collected for the primary screening (ELISA) process undertaken between 2001 and 2006. From these samples, 848 individuals were recalled for a secondary examination involving WB, ultrasound, computed tomography and/or magnetic resonance imaging. In addition, 897 sera were also tested as part of a routine examination of hospital-suspected cases.

Antigen production

ELISA and WB tests were undertaken employing a crude antigen extracted from *E. multilocularis* cysts. To prepare the antigen, protoscoleces were collected from cysts in cotton rat liver which had been experimentally inoculated with metacestodes of *E. multilocularis*. The protoscoleces were repeatedly washed with phosphate-buffered saline (PBS, pH 7.4) and treated with 0.2% Triton X-100 in PBS. This solution was then centrifuged for 15 min at 500 rpm and the supernatant dialysed against PBS for 24 h at 4°C to remove detergent. The total protein concentration of the antigen solution was set to 12 $\mu g\, ml^{-1}$.

ELISA protocol

ELISAs were performed as described previously by Sato *et al.* (1983a, b) with some minor modifications. Flat-bottomed microplates (EIA/RIA 8-well strip, high-bind type, Corning, New York, USA) were coated with crude antigen (100 μl/well) for 4 h at 37°C. Serum samples diluted at 1:250 in 0.05% Tween–PBS (100 μl/well) were then added to the wells of the microplates and incubated overnight at 4°C. After washing, 100 μl of anti-human IgG alkaline phosphatase conjugated antibody (Sigma, St Louis, Missouri, USA; 1:2500 in 0.05% Tween–PBS) was added and incubated for 4 h at 37°C. After further washing, bound antibodies were detected by the presence of a reaction for alkaline phosphatase on *p*-nitrophenyl-phosphate (0.1 mg/well) in 10% diethanolamine buffer (pH 9.8) following an incubation period of 8 min at 37°C (100 μl/well). The reaction was stopped with the addition of 50 μl of 3N NaOH and the optical density (OD) values read at 405 nm on a microplate reader (Model 680, BIORAD, Hercules, California, USA). Between each incubation step, plates were washed three times with 0.05% Tween-PBS (300 μl/well).

Preliminary experiments undertaken in 1983 gave a mean OD value of 0.94 ± 0.21 for 46 confirmed AE patients. Therefore, the diagnostic criteria for the current study were defined based on the mean OD and mean OD minus 2 standard deviations as follows: OD 0–0.50 = negative; 0.51–0.99 = quasi-positive; over 1.00 = positive (Sato *et al.*, 1983a). A negative mean OD value ($n = 471$) was defined as 0.29 under these conditions.

Western blot protocol

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was undertaken as described by Laemmli (1970), utilizing a crude *E. multilocularis* antigen preparation. Antigens were separated electrophoretically on SDS–15% polyacrylamide gels and the separated antigens were electroblotted on to a nitrocellulose membrane. The membrane was subsequently blocked in 5% skimmed milk in PBS for 1 h at room temperature (RT) and then incubated with patient sera at 1:100 in PBS containing 0.05% Tween 20 and 5% skimmed milk for 1.5 h at RT. After washing, the membrane was treated with an anti-human IgG alkaline phosphatase conjugated antibody (Sigma), diluted at 1:2500 in 0.05% Tween–PBS, for 1 h at RT. After further washing, the membrane was exposed to BCIP/NBT substrate solution (PerkinElmer Life Sciences, Gaithersburg, Maryland, USA) for colour development. We adopted multiple markers including 26–28, 18 (the main marker utilized) and 7–8 kDa bands in our WB testing. The criteria for judgement are presented in table 1.

In addition, the commercially available immunoblot assay kit, ECHINOCOCCUS Western Blot IgG (Ldbio Diagnostics, Lyon, France) was also utilized in this study. The operation of this kit and the interpretation of subsequent results were undertaken in accordance with the manufacturer's recommendations.

Results

An outline of examination results and echinococcosis detection

Table 1 presents an outline of the examination results and echinococcosis detection. Confirmative WB tests were carried out on a total number of 1745 sera between 2001 and 2006, including both secondary examinations undertaken as part of the Hokkaido serological mass screening system and the regular examination of suspected outpatients in hospitals. Under the 'law concerning prevention of infectious diseases and medical care for patients of infections', 103 patients from

Table 1. Results of Western blot (WB) testing and a summary of echinococcosis detection between 2001 and 2006.

Number of sera examined at the Hokkaido Institute of Public Health (HIPH)			
Secondary examination	848	*Primary screening: 278,696	
Regular examination for outpatients	897		
Total	1745		
Number of registered patients			
Detected by WB in HIPH (including a CE patient)	81	Type A (18, 26–28, 7–8 kDa)[a]	76
		Type B (26–28, 7–8 kDa)[a]	3
		Type C (26–28 kDa)[a]	2
	1	CE (against 7–8 kDa antigen and AgB)	
Not detected by WB in HIPH	21	Diagnosed by other system Only primary examination by ELISA Found at operation for other diseases	
Total	103		

CE, cystic echinococcosis.
[a] Type A and B are positive and Type C is quasi-positive.
* The first step of the screening process by ELISA.

Hokkaido were newly reported to the Infectious Disease Surveillance Center as having echinococcosis in these 6 years. From our review study, it was identified that HIPH directly participated in the detection of at least 82 cases (79.6%), including one cystic echinococcosis (CE) patient. Among these 81 AE patients, 79 cases were found to be positive and two quasi-positive in our WB testing procedure. In addition, 21 of the newly registered 103 patients included cases that were diagnosed by other systems undertaken by some facilities, and were detected only *via* primary screening using ELISA, or were identified during surgery associated with other diseases.

As outlined in table 1, 81 AE patients detected by our WB testing were classified into three groups. Type A comprised 76 positive cases that demonstrated reactivity against the 18, 26–28 and 7–8 kDa antigens, Type B consisted of three positive cases showing reactivity against the 26–28 and 7–8 kDa antigens, and Type C included two quasi-positive cases that reacted only to the 26–28 kDa antigen. The Type A cases, that demonstrated reactivity to the 18 kDa antigen, are thought to represent typical positive AE patterns. In contrast, Types B and C ($n = 5$) represented patients with active AE and with characteristic sero-negativity for antibody directed to the 18 kDa antigen.

Five atypical cases

Five atypical patients of Types B ($n = 3$) and C ($n = 2$) were comprehensively diagnosed based on serological examination, diagnostic imaging and histopathological analysis. These patients characteristically lacked the presence of an 18 kDa band on their immunoblots (fig. 1), and similar results were also obtained using a commercially available immunoblot kit.

Case 1 was a 70-year-old Japanese male of Type B, the details of whom have already been reported (Yamano *et al.*, 2005).

Case 2 was a 49-year-old Japanese male of Type B, who was detected via the Hokkaido screening system in 2003. His ELISA result was quasi-positive (OD 0.658) in the primary screening process, and a WB test undertaken as part of a secondary confirmation step was judged to be positive for the 26–28 and 7–8 kDa bands. When he consulted a physician for a thorough examination, several cysts were identified in S2/3, S5 and S7 of the liver using ultrasonography and computed tomography. He subsequently underwent surgery in September 2003, and histopathological confirmation was carried out.

Case 3 was a 43-year-old Japanese male of Type B. In this patient, several cysts with calcification were identified in S6/7 and S8 of the liver using ultrasonography and computed tomography following a medical examination. A serum sample for serological testing was collected in September 2004. The subsequent ELISA test demonstrated an OD value within the negative range (OD 0.417). Further WB tests identified the presence of 26–28 and 7–8 kDa protein bands and were judged to be positive. The cysts were surgically removed in December 2004 and histopathological confirmation was carried out.

Case 4 was a 71-year-old Japanese female of Type C. Upon consultation with a physician for ureteral calculus, a phyma of 5 cm in diameter was identified in S8 of the liver using computed tomography. A subsequent serological test was undertaken on a serum sample collected in February 2005. Both ELISA and WB analysis gave a quasi-positive result, with an OD value of 0.731 and the presence of a 26–28 kDa band, respectively. This patient received a resection of phyma in March 2005, and a diagnosis of AE was confirmed following histopathological analysis.

Case 5 was a 19-year-old Japanese male of Type C. Following a medical examination for stomach ache, a phyma with calcification was identified in the left lobe of the liver using ultrasonography, computed tomography and magnetic resonance imaging. A serological test was undertaken on a serum sample collected in February 2005. An ELISA test demonstrated an OD value within the positive range (OD 1.51); however, a WB test identified the presence of only the 26–28 kDa band and was thus judged to be quasi-positive. The patient underwent surgery in May 2006 and a diagnosis of AE was confirmed following histopathological analysis.

Discussion

AE is prevalent in the forest and farming areas of the northern hemisphere and is endemic to the Hokkaido region of Japan. CE, which is caused by infection with

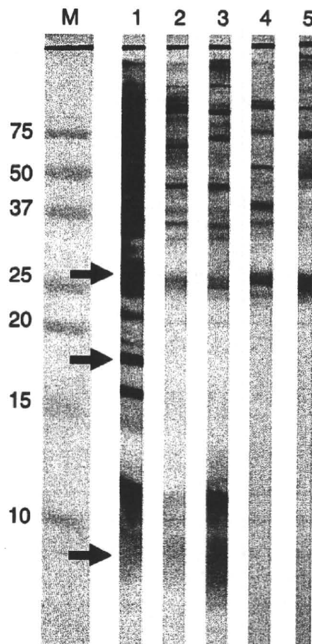

Fig. 1. Immunoblot analysis of patients showing sero-negativity against the 18 kDa antigen. Lanes: M, the molecular size marker; 1, a positive control (Type A); 2 and 3 are sera from Type B patients (Cases 2 and 3); 4 and 5 are sera from Type C patients (Cases 4 and 5). The diagnostic bands are indicated by arrows.

E. granulosus, occurs most often around areas of the world involved in the raising of livestock and is generally regarded to be of minor importance in imported cases in Japan (Furuya *et al.*, 2000). Therefore, the accurate diagnosis of AE is a major priority in Japan, especially in Hokkaido.

A serological screening system using ELISA (Sato *et al.*, 1983a, b) and WB (Furuya *et al.*, 1987, 1989, 1990; Nagano *et al.*, 1995) tests has been established previously to detect patients with AE residing in Hokkaido. These methods employ a crude antigen extracted from *E. multilocularis* cysts which detects numerous antibodies directed specifically against *E. multilocularis* antigens.

In our original WB testing procedures, protein bands of 55–66 kDa and a broad smear band including polysaccharides ranging from 30–35 kDa were identified as markers for AE when separated by SDS–PAGE (8% polyacrylamide gel). More recently, Em18, which is located in the 18 kDa region of immunoblots, has been reported to be a good marker for AE detection (Ito *et al.*, 1993, 1995, 2003). It was subsequently demonstrated that the Em18 antigen is the breakdown product of EM10 (55–66 kDa antigen) following proteolysis by cysteine proteinases (Sako *et al.*, 2002). These results confirmed that Em18 and 55–66 kDa antigens were basically equal in the diagnosis of AE with WB testing. In addition, a commercially available immunoblot kit that detects bands ranging from 7 to 28 kDa has been developed and manufactured in France (Liance *et al.*, 2000) for the detection of echinococcosis. Thus, we began to investigate the use of low molecular weight antigens in the identification of AE (Yamano *et al.*, 2005). Our improved WB method monitors the presence of three marker bands ranging from 7 to 28 kDa. This concept is similar to that utilized by the above-mentioned commercially available kit. However, in the utilization of crude antigen, the 7–8 kDa band is usually accompanied by an approximately 12 kDa band which appears to be related to antigen B (AgB) (Leggatt *et al.*, 1992). Moreover, some CE cases may be detected due to cross-reaction with the 26–28 and/or 7–8 kDa antigens, a result that may also occur with the commercially available kits (see table 1).

In the previously utilized WB testing procedures, we identified patients demonstrating sero-negativity for the 55/66 kDa antigens. Similarly, we detected five patients showing sero-negativity for the 18 kDa antigen over a period of 6 years, using the application of low molecular weight markers. The prognosis for these five patients is good as a result of surgical treatment, suggesting that precise serodiagnosis aids in the improvement of curative effects.

It has recently become popular to use low molecular weight antigens as a diagnostic marker for AE. However, 6.2% (5 of 81) of patients showed sero-negativity against the 18 kDa antigen, a main marker for AE. Unfortunately this frequency is quite significant. We have also reported previously that AE patients demonstrating sero-negativity against the 18 kDa antigen were detected at a frequency of 21.7%, due to the presence of the 26–28 kDa and/or 7–8 kDa bands when using the commercially available immunoblot kits (Furuya *et al.*, 2004). Similar findings regarding sero-negativity against the 18 kDa antigen were also reported for Chinese and French AE patients (Nirmalan & Craig, 1997; Liance *et al.*, 2000). These findings may indicate that the positive rate against the 18 kDa antigen in sera from AE patients is not sufficiently high and that the detection of multiple bands on an immunoblot test may be required for the correct diagnosis of AE.

The recent development of recombinant antigens has also contributed to an improvement in serodiagnostic examinations. These antigens include EmII/3 (Vogel *et al.*, 1988; Felleisen & Gottstein, 1994; Kouguchi *et al.*, 2005), EM10 (Frosch *et al.*, 1991; Helbig *et al.*, 1993; Hubert *et al.*, 1999) and Em18 (Ito *et al.*, 2002). Unfortunately, no single antigen identified to date has proven successful in detecting all AE patients. Therefore, it is concluded that we need to pay particular attention to the specific reactions to plural different diagnostic antigens such as the 26–28 and 7–8 kDa proteins to minimize the risk of inaccurate diagnosis of AE.

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Scythe/BAT3 regulates apoptotic cell death induced by papillomavirus binding factor in human osteosarcoma

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Papillomavirus binding factor (PBF) was first identified as a transcription factor regulating the promoter activity of human papillomavirus. We previously demonstrated that PBF is an osteosarcoma-associated antigen and 92% of osteosarcoma tissues express PBF in the nucleus. Moreover, PBF-positive osteosarcoma has a significantly poorer prognosis than that with negative expression of PBF. In the present study, we assessed the biological role of PBF in cell survival. Overexpression of PBF induced cell death-mediated lactate dehydrase (LDH) release from 293EBNA cells. Cleaved poly(ADP-ribose) polymerase and active caspase-3 were also detected. However, PBF-induced apoptosis did not affect caspase-9 activity. Next, to identify the apoptosis regulator of PBF, we screened a cDNA library constructed from mRNA of the osteosarcoma cell line OS2000 using a yeast two-hybrid system and isolated Scythe/BAT3. Scythe/BAT3 mRNA was detected in 56% of osteosarcoma tissues and ubiquitously in various normal tissues. Although Scythe/BAT3 was localized to the cytoplasm in normal tissue, it was localized to the nucleus in osteosarcoma tissue. PBF and Scythe/BAT3 also colocalized to the cytoplasm in 293T cells and the nucleus in OS2000. Furthermore, overexpression of Scythe/BAT3 suppressed cell death events that resulted from overexpression of PBF in OS2000, but not in 293EBNA cells. Thus, our results support the ideas that: (i) PBF could induce apoptotic cell death via a caspase-9-independent pathway; (ii) the apoptosis regulator Scythe/BAT3 is a PBF-associated molecule acting as a nucleus–cytoplasm shuttling protein; and (iii) colocalization of PBF and Scythe/BAT3 in the nucleus might be an important factor for survival of osteosarcoma cells. (*Cancer Sci* 2009; 100: 47–53)

Papillomavirus binding factor (PBF) was first identified as a transcription factor regulating promoter activity of the human papillomavirus type 8 genome.[1] We demonstrated that PBF is an osteosarcoma-associated antigen recognized by an autologous cytotoxic T lymphocyte clone.[2] Immunohistochemical analysis revealed that 92% of biopsy specimens of osteosarcoma express PBF. Moreover, PBF-positive osteosarcoma has a significantly poorer prognosis than that with negative expression of PBF.[3] Generally, conventional osteosarcoma is a malignant neoplasm of mesenchymal origin and there is no specific cause such as viral infection.[4] Therefore, it is suggested that PBF has certain functions not only in transcription of the human papillomavirus genome, but also in the cell survival and apoptosis of osteosarcoma.

Apoptosis is tightly controlled programmed cell death. Generally, it is induced by two major pathways, an extrinsic pathway and an intrinsic pathway.[5] The initiation of the extrinsic pathway of apoptosis is triggered by extracellular death signals. The binding of extracellular ligands to the death receptors of the tumor necrosis factor receptor superfamily leads to formation of the death-inducing signaling complex, which is capable of activating the initiator caspase-8.[6–8] Meanwhile, initiation of the intrinsic pathway of apoptosis is triggered by cellular stress. Cellular death signals induce mitochondrial membrane permeabilization, mitochondrial cytochrome *c* release and activation of the initiator caspase-9.[5,8,9] Apart from these two major pathways, mitochondrial membrane permeabilization also leads to the release of apoptosis-inducing factor (AIF), which induces caspase-independent cell death.[10] Recently, Sichtig *et al.* reported that PBF is an inducer of cell death and that 14-3-3 regulates the localization of PBF and PBF-induced cell death in skin keratinocytes.[11] 14-3-3 is associated with apoptotic cell death, the cell cycle, and regulation of various oncogenes and tumor-suppressor genes.[12,13] Moreover, 14-3-3 binds to the Bcl-2 family member Bax and inhibits Bax-induced cytochrome *c* release from the mitochondria upon apoptotic stimuli.[14] Although these findings suggest a certain function of PBF as an apoptosis regulator, the function of PBF in osteosarcoma cells remains unknown.

In the present study, we demonstrated that PBF-induced cell death results from apoptosis via a caspase-9-independent pathway. Next, we identified Scythe/BAT3[15,16] as a PBF-associated molecule using a yeast two-hybrid system. Finally, we showed that PBF-induced apoptotic cell death is inhibited by Scythe/BAT3 colocalized to the nuclei of osteosarcoma cells. Taken together, these findings suggest that both PBF and Scythe/BAT3 might play important roles in apoptosis of osteosarcoma cells.

Materials and Methods

The present study was approved under institutional guidelines for the use of human subjects in research. The patients and their families gave informed consent for the use of tissue specimens in our research.

Cell lines. The osteosarcoma cell lines OS2000, HOS, Saos-2, and U2OS, as well as the human embryonic kidney cell lines 293EBNA and 293T, were used. 293EBNA and 293T cells did not express endogenous PBF protein (data not shown). OS2000 was established in our laboratory.[17] The other cell lines were purchased from American Type Culture Collection (Manassas, VA, USA).

Antibodies. The mouse monoclonal antibodies used in the present study were anti-cleaved poly(ADP-ribose) polymerase (PARP) (D216, 1:1000 dilution; Cell Signaling, Danvers, MA,

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USA), anti-caspase-9 (clone 5B4, :1000 dilution; MBL International, Woburn, MA, USA), anti-β-actin (clone AC-15, 1:1000 dilution; Sigma-Aldrich, St Louis, MO, USA), anti-myc (clone 9E10, 1:1000 dilution; American Type Culture Collection), and anti-human influenza hemagglutinin (HA) (HA-7, 1:10 000 dilution and 1:2000 in western blotting and immunofluorescence analysis, respectively; Sigma-Aldrich, St Louis, MO, USA). Rabbit polyclonal antibodies used were anti-Scythe/BAT3, kindly provided by Dr Peter J. McKinnon (St Jude Children's Research Hospital, Minneapolis, TN, USA; #23, used at 1:8000 dilution and 1:200 in western blotting and immunofluorescence analysis, respectively),[18] and anti-PBF (used at 1:800 dilution and 1:200 in western blotting and immunofluorescence analysis, respectively; SigmaGenosys, Sapporo, Japan).[2]

Screening of a cDNA library using the yeast two-hybrid system. A cDNA library was constructed from OS2000 mRNA using a FastTrack 2.0 mRNA Isolation Kit (Invitrogen, Carlsbad, CA, USA) and the Superscript Choice System (Invitrogen). The cDNA was ligated to *Eco*RI–*Xho*I adapters and digested with *Xho*I. The resultant cDNA was cloned into the pACT2 vector (Clontech Laboratories, Mountain View, CA, USA). Recombinant plasmids were electroporated into Electromax DH10B cells (Invitrogen) and selected with ampicillin (100 μg/mL). The resultant 30 000 clones were amplified and plasmid DNA was extracted using a Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany). Full-length PBF subcloned into the pGBKT7 vector (Clontech) was used as bait to screen the cDNA library. Screening of the cDNA library was carried out according to the Mammalian Matchmaker Two-hybrid Assay Kit protocol (Clontech).

Reverse transcription–polymerase chain reaction analysis. Expression of Scythe/BAT3 was determined by reverse transcription–polymerase chain reaction (PCR). Normal-tissue cDNA was purchased (Multiple Tissue cDNA Panels; Clontech). Total RNA was extracted from nine osteosarcoma biopsy specimens and reverse transcribed. PCR was carried out with KOD dash DNA polymerase (Toyobo, Tokyo, Japan), using the forward primer (XhoI-Scythe/BAT3-FW) 5′-GGGGGGCTCG-AGAAATGGAGCCTAATGATAGTACCAGT-3′ and the reverse primer (Scythe/BAT3-NotI-Rv) 5′-AAAAAAGCGGCCGCC-TAAGGATCATCAGCAAAGGCCCGC-3′. The mixture was denatured at 98°C for 2 min, followed by 30 cycles at 98°C for 15 s, 56°C for 2 s, and 74°C for 3 min. Reaction products were analyzed by electrophoresis on 1.0% agarose gels with ethidium bromide. Full-length Scythe/BAT3 cDNA, amplified from OS2000 cDNA by PCR, was digested with *Xho*I and *Not*I and subcloned into the pCMV-HA tag vector (Clontech).

Transfection. 293EBNA or 293T cells were transfected with the indicated cDNA or small interfering RNA (siRNA) using Lipofectamine 2000 (Invitrogen) in Dulbecco's modified Eagle's medium containing 10% fetal calf serum in six-well plates according to the manufacturer's protocol. OS2000 or U2OS cells were transfected with cDNA using Cell Line Nucleofector Kit V (Amaxa, Cologne, Germany) according to the manufacturer's protocol.

Western blotting. Cell lines and biopsy specimens were homogenized and suspended in ice-cold Nonidet P-40 (NP-40) buffer for 20 min as described previously.[19] The lysates were mixed with 2× sample buffer and boiled for 5 min. Then the lysates were separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 7 or 13% gels and transferred to polyvinylidene fluoride membranes (Millipore, Billercia, MA, USA). The membranes were blocked and probed with the indicated antibodies for 40 min at room temperature. The membranes were then stained with peroxidase-labeled secondary antibody and visualized using an enhanced chemiluminescence detection system (Amersham Life Science, Arlington Heights, IL, USA).

Silencing of Scythe/BAT3. Three Stealth siRNA targeting Scythe/BAT3 and a negative control siRNA were purchased from Invitrogen. Three micrograms of each siRNA was transfected into 293EBNA cells and OS2000 cells as described above. After screening of their silencing effect using western blotting (data not shown), one of the three siRNA (BAT3-HSS111846) was selected for further analysis.

Immunoprecipitation. The cells were lysed on ice with NP-40 lysis buffer. Lysates were precleared with protein A–sepharose beads (Phadia, Uppsala, Sweden) before immunoprecipitation. The anti-HA-7 monoclonal antibody and aliquots of lysates were mixed and incubated at 4°C for 24 h. Immunoprecipitates were recovered from the mixture using protein A–sepharose beads, washed three times with phosphate-buffered saline (PBS) containing 0.1% NP-40, mixed with SDS-PAGE gel electrophoresis running buffer, and boiled for 5 min. Supernatants containing immunoprecipitates were fractionated on 13% SDS-PAGE, dried, and exposed to X-ray films with an intensifying screen.

Immunofluorescence analysis. OS2000, U2OS and 293T cells cultured on glass coverslips (Asahi Techno Glass, Tokyo, Japan) were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton X-100. After washing with PBS, cells were incubated with the polyclonal anti-PBF antibody and monoclonal anti-HA antibody to detect the subcellular localization of PBF protein and HA-tagged Scythe/BAT3 protein. The monoclonal anti-myc antibody and polyclonal anti-Scythe/BAT3 antibody were used to detect exogenous myc-tagged PBF protein and endogenous Scythe/BAT3 protein. 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) was used for counterstaining nuclei. After washing with PBS, cells were immunostained with secondary antibodies (Alexa Fluor 488- or Alexa Fluor 594-conjugated goat anti-mouse or anti-rabbit antibodies; Invitrogen). After staining, cells were visualized by confocal laser microscopy (R2100AG2; Bio-Rad Laboratories, Hercules, CA, USA). Formalin-fixed paraffin-embedded sections of one osteosarcoma biopsy specimen and autopsy specimens of liver, pancreas, and kidney (obtained from a brain infarction patient) were deparaffinized and incubated with the polyclonal anti-Scythe/BAT3 antibody overnight at 4°C. After washing with PBS, cells were immunostained with Alexa Fluor 488-conjugated goat anti-rabbit IgG, followed by visualization using confocal laser microscopy. DAPI was used for counterstaining of nuclei.

Lactate dehydrase (LDH) release assay. 293EBNA, 293T or OS2000 cells were transfected with cDNA as described above. After a 24-h incubation period at 37°C, the supernatant was replaced with AIM-V. The amount of LDH in the supernatant was measured by colorimetric assay using an LDH Cytotoxicity Detection Kit (Takara, Ohtsu, Japan) after an additional 24 h incubation period. LDH release was calculated using the following equation: LDH release =([sample release] – [spontaneous release]).

Caspase-3 colorimetric assay. 293EBNA cells were transfected with cDNA as described above. After a 24-h incubation period at 37°C, the amount of active caspase-3 in the supernatant was measured by colorimetric assay using an APOPCYTO Caspase-3 Colorimetric Assay Kit (Medical and Biological Laboratories, Nagoya, Japan) according to the manufacturer's protocol.

Statistical analysis. Significant differences among samples in the LDH release assay and caspase-3 colorimetric assay were determined by Welch's *t*-test using StatMate III for Macintosh v3.10 (ATMS, Tokyo, Japan). A probability of less than 0.05 was considered statistically significant.

Results

Overexpression of PBF induces apoptotic cell death via a caspase-independent pathway. To investigate the function of PBF as an apoptosis regulator, we analyzed the cell death and apoptotic events that occurred in 293EBNA cells transfected transiently with PBF. As shown in Figure 1a, overexpression of PBF induced cell death-mediated LDH release from 293EBNA and

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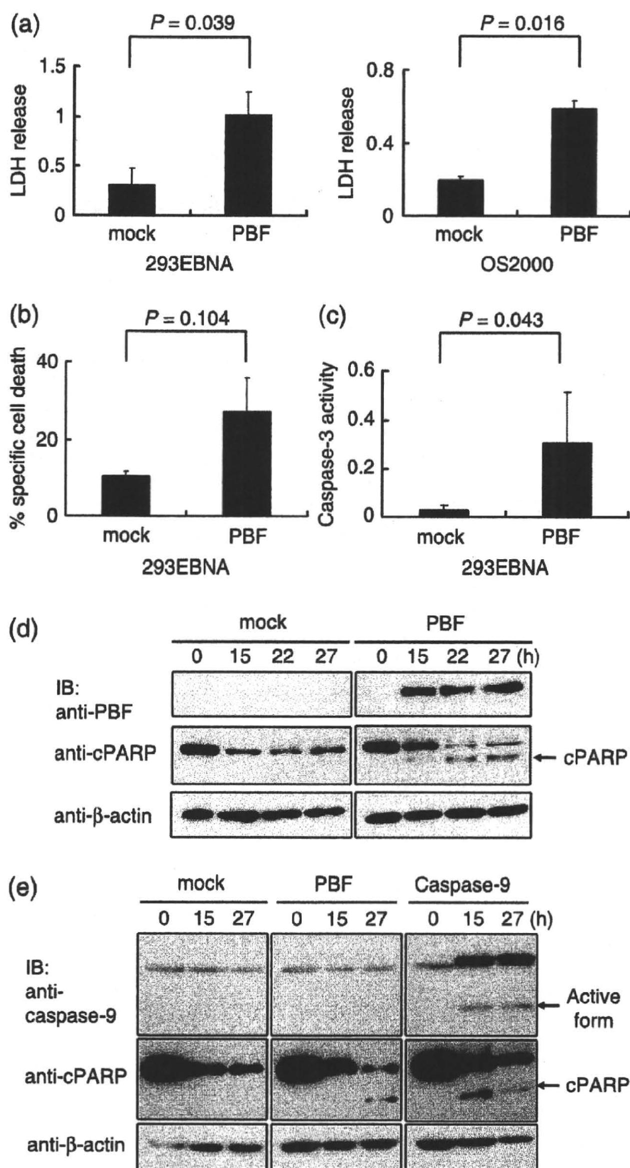

Fig. 1. Apoptotic cell death induced by overexpressed papillomavirus binding factor (PBF). (a) LDH release from 293EBNA cells and OS2000 cells transiently transfected with PBF-myc/pcDNA3.1⁺ or pcDNA3.1⁺ as mock transfection. (b) The proportion of cell death in 293EBNA cells transiently transfected with PBF-myc/pcDNA3.1⁺ or mock transfection. (c) Expression of PBF and cleaved poly(ADP-ribose) polymerase (cPARP) in 293EBNA cells was assessed from 0 to 27 h after transient transfection with PBF-myc/pcDNA3.1⁺ or mock transfection. (d) Caspase-3 activity in 293EBNA cells transiently transfected with PBF-myc/pCMV or pCMV as mock transfection. (e) Expression of PBF, caspase-9, and cPARP in 293EBNA cells was assessed after transient transfection with the indicated plasmids. IB, immunoblotting.

OS2000 cells. As depicted in Figure 1b, the proportions of mock-induced and PBF-induced cell death in 293EBNA cells were 10.6 ± 1.02 and 27.3 ± 8.57%, respectively. Next, we examined whether PBF overexpression could induce major intrinsic apoptosis via a caspase-9-dependent pathway.[5] Overexpressed PBF and cleaved PARP, which is a classical substrate for caspase-3 during the end stage of the caspase cascade, were detected from 12 h after transfection, followed by an increase in cleaved PARP products (Fig. 1d). Moreover,

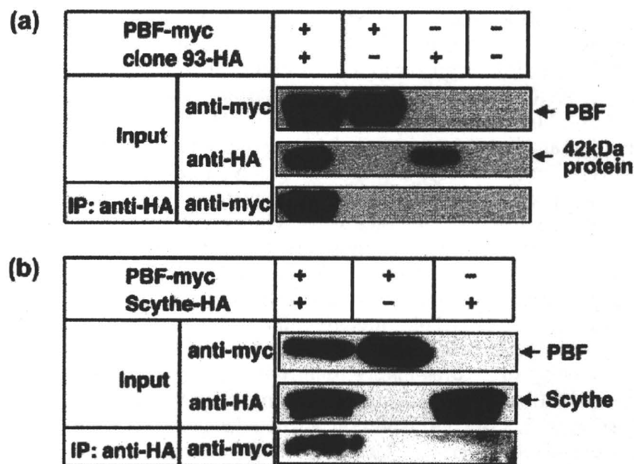

Fig. 2. Association of papillomavirus binding factor (PBF) and Scythe/BAT3 in mammalian cells. Lysates of 293T cells transiently transfected with the indicated plasmids (PBF-myc/pCMV, Scythe/BAT3-HA/pCMV, and mock plasmid) were immunprecipitated with an anti-HA monoclonal antibody and immunoblotted with an anti-myc monoclonal antibody. Expression of each transfected cDNA in the input lysate was assessed by western blotting with anti-HA or anti-myc monoclonal antibodies. IP, immunoprecipitation.

overexpressed PBF also activated caspase-3 (Fig. 1c). However, overexpression of PBF in 293EBNA cells did not affect caspase-9 activity (Fig. 1e). These results suggest that overexpression of PBF could induce apoptotic cell death via a capsase-9-independent pathway.

Identification of PBF-associated molecules in osteosarcoma cells. For identification of PBF-associated molecules as apoptotic regulators in osteosarcoma cells, we screened a cDNA library constructed from OS2000 mRNA using a yeast two-hybrid system. After screening of 30 000 cDNA clones, 106 positive clones were identified. Among them, cDNA clone 93 (Fig. 2a) was identical to the longest open reading frame of Scythe/BAT3. Clone 93 contained 852 bp of the 3′ sequence of Scythe/BAT3 with deletion of the 5′ sequence. There were no frameshifts or point mutations. Subsequently, the association of PBF with Scythe/BAT3 was confirmed by coimmunoprecipitation experiments in mammalian cells. As depicted in Figure 2b, exogenous myc-tagged PBF was precipitated specifically with the anti-HA antibody from the cell lysate of 293T cells cotransfected with myc-tagged PBF and HA-tagged Scythe/BAT3.

Expression and subcellular localization of Scythe/BAT3 in osteosarcoma and normal tissues. Expression of Scythe/BAT3 mRNA was analyzed by reverse transcription-PCR. Scythe/BAT3 mRNA was detected in five of nine primary osteosarcoma tissues, both osteosarcoma cell lines examined, and ubiquitously in various normal tissues (Fig. 3a,b). Comparing the expression levels in osteosarcoma cell lines, mRNA expression in primary osteosarcoma tissue seemed to be lower. Next, expression of Scythe/BAT3 protein was assessed by western blotting and immunofluorescence analysis. Although Scythe/BAT3 protein was similarly detected in all four osteosarcoma cell lines and 293EBNA cells (Fig. 4a), the subcellular localization of Scythe/BAT3 was clearly different between osteosarcoma and normal tissues (Fig. 4b). In normal liver, pancreas, and kidney, Scythe/BAT3 was localized to the cytoplasm, not the nucleus. In contrast, Scythe/BAT3 was localized to both the cytoplasm and nucleus in one osteosarcoma tissue.

Colocalization of PBF and Scythe/BAT3 in the nucleus suppresses PBF-induced cell death in osteosarcoma cells. PBF has been reported as a nuclear-shuttling transcription factor, and the subcellular localization of PBF might be important to regulate apoptotic

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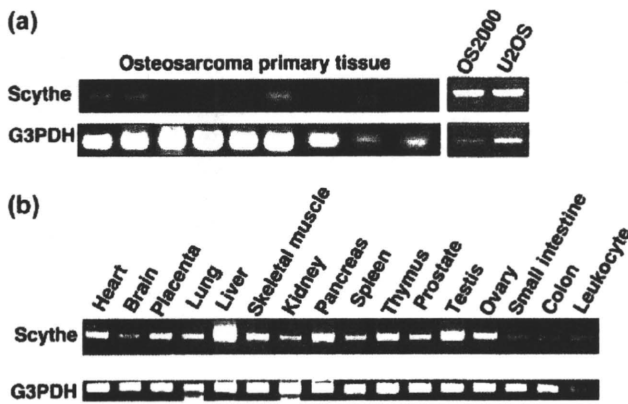

Fig. 3. Expression of Scythe/BAT3 mRNA by reverse transcription–polymerase chain reaction analysis. The expression of Scythe/BAT3 was determined in (a) nine osteosarcoma primary tissues and two osteosarcoma cell lines and (b) 16 adult normal tissues. The glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) housekeeping gene was used as a positive control.

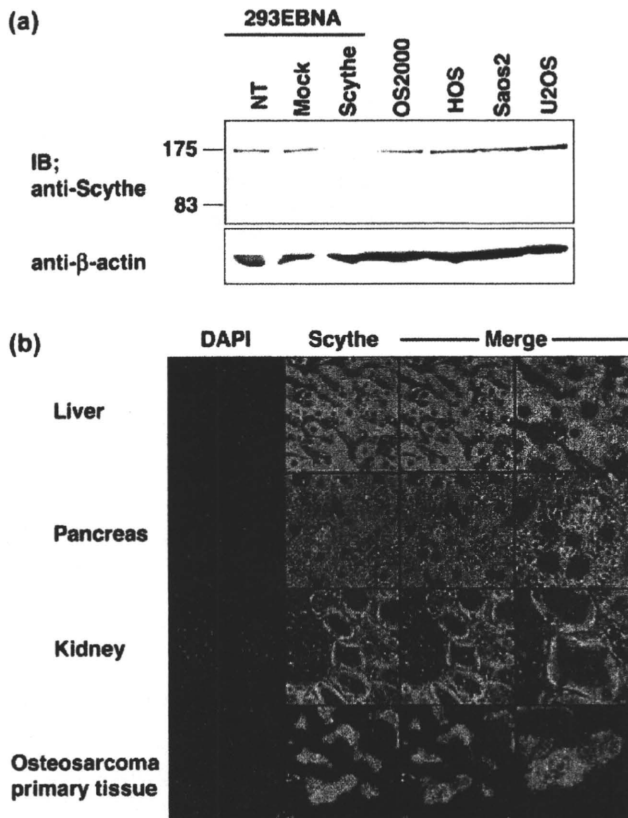

Fig. 4. Expression of Scythe/BAT3 protein. (a) Lysates of 293EBNA cells transiently transfected with the indicated plasmids and four osteosarcoma cell lines were immunoblotted with an anti-Scythe/BAT3 antibody. (b) Immunofluorescence analysis. Formalin-fixed paraffin-embedded sections of liver, pancreas, kidney, and an osteosaroma primary tissue were stained with an anti-Scythe/BAT3 antibody and assessed by confocal laser microscopy. DAPI, 4′,6-diamidine-2′-phenylindole dihydrochloride; IB, immunoblotting; NT, no treatment.

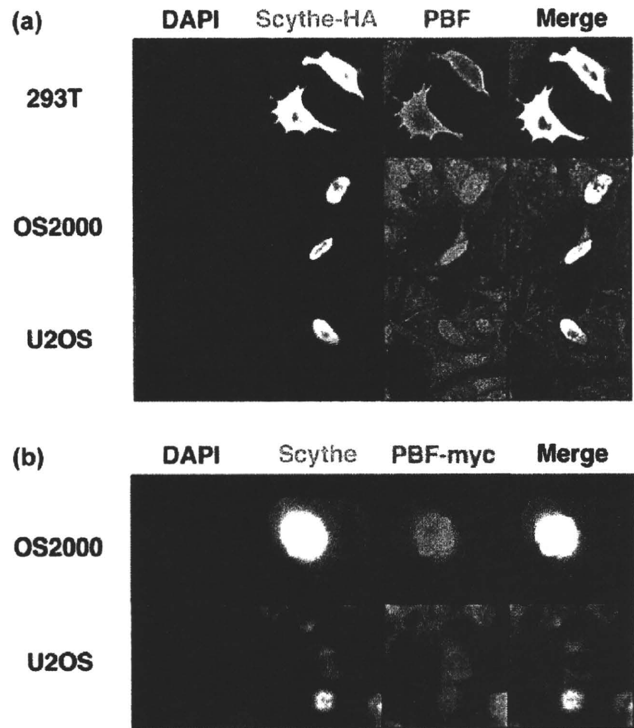

Fig. 5. Cellular localization of papillomavirus binding factor (PBF) and Scythe/BAT3. (a) Immunofluorescence analysis using anti-PBF antibody and anti-HA antibody. Upper column: 293T cells were transiently transfected with PBF-myc/pCMV and Scythe/BAT3-HA/pCMV. 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) was used for counterstaining of nuclei. Middle and lower columns: OS2000 (middle) or U2OS (lower) cells were transiently transfected with Scythe/BAT3-HA. (b) Immunofluorescence analysis using anti-myc antibody and anti-Scythe/BAT3 antibody. OS2000 (upper) or U2OS (lower) cells were transiently transfected with PBF-myc. DAPI was used for counterstaining of nuclei.

function.[11] Therefore, we analyzed the colocalization of PBF and Scythe/BAT3 in 293T, OS2000, and U2OS cells. As shown in Figure 5a, immunofluorescence analysis revealed that exogenous PBF and exogenous Scythe/BAT3 were colocalized to the cytoplasm in 293T cells. However, the majority of both of these proteins was colocalized to the nucleus in OS2000 and U2OS cells. We also observed that exogenous PBF and Scythe/BAT3 were similarly colocalized to the nucleus in OS2000 and U2OS cells (Fig. 5b).

In Figure 1 we already showed that overexpressed PBF induced apoptotic cell death. Therefore, we assessed the apoptosis regulatory function of Scythe/BAT3 in non-cancerous and osteosarcoma cells. As shown in Figure 6a, overexpression of Scythe/BAT3 did not affect cell death events that resulted from overexpression of PBF in 293EBNA cells. However, Scythe/BAT3 significantly suppressed PBF-induced cell death events in OS2000 cells (Fig. 6b). Next, we assessed the effects of the downregulation of Scythe/BAT3 on PBF-induced cell death using siRNA. In 293EBNA cells, the downregulation of Scythe/BAT3 did not change cell death events, the same as with overexpression of Scythe/BAT3 (Fig. 6c). In contrast, downregulation of Scythe/BAT3 significantly increased LDH release in OS2000 with the mock control but did not change it in OS2000 with PBF (Fig. 6d). The expression status of transfected PBF-myc, Scythe/BAT3-HA, and endogenous Scythe/BAT3 was confirmed by western blotting, as shown in Figure 6e,f. Considering these observations, we can briefly summarize the relationship between PBF and Scythe/BAT3 in 293EBNA and OS2000 cells as follows:

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