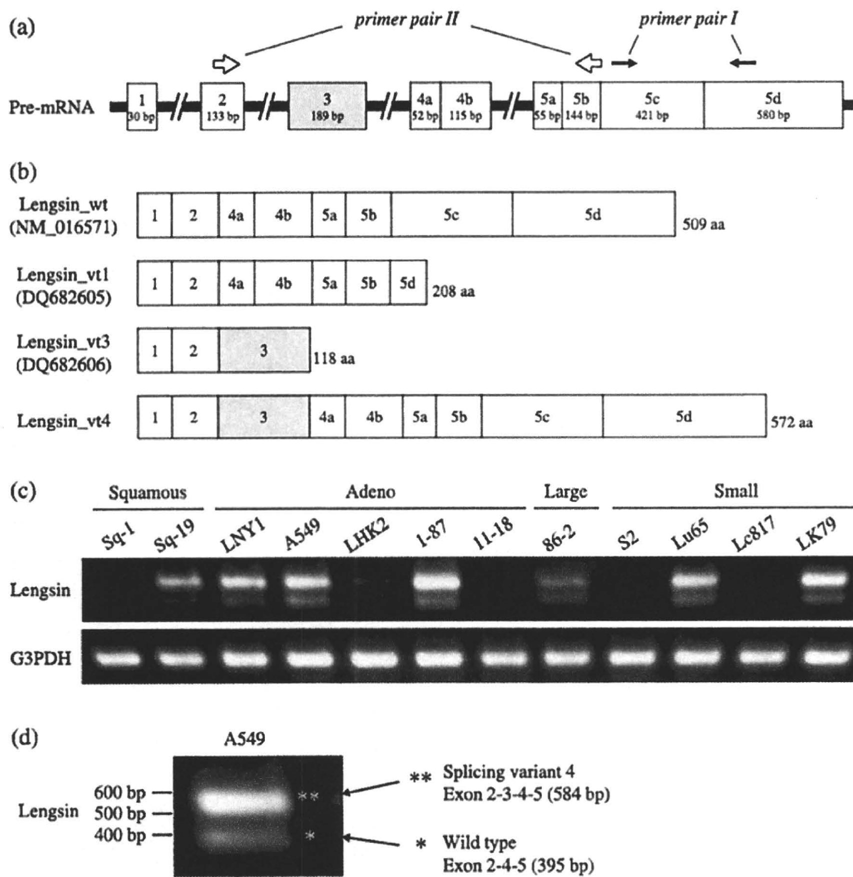

Fig. 2. Splicing variant 4 is more highly expressed than the wild type in lung cancer cell lines. (a) Diagram of premRNA showing five exons of Lengsin. Black arrow indicates PCR primer pair I and white arrow indicates PCR primer pair II. (b) Diagram of the derived protein of each splicing variant. Brackets indicate GenBank accession number. wt, wild type; vt1, splicing variant 1; vt3, splicing variant 3; vt4, splicing variant 4; aa, amino acid. (c) Expression profiles of Lengsin as assessed by RT-PCR with primer pair II in lung cancer cell lines. Squamous, squamous cell carcinoma lines; Adeno, adenocarcinoma lines; Large, large cell carcinoma lines; Small, small cell carcinoma lines. G3PDH was used as an internal control. (d) Results for two PCR products with primer pair II in A549.

surgically resected lung adenocarcinoma, 21 squamous cell carcinoma, two large cell carcinoma and four small cell carcinoma tissues were evaluated the expression of Lengsin proteins with mAb #517. Seventeen of 34 adenocarcinoma, 11 of 21 squamous cell carcinoma, two of two large cell carcinoma and two of four small cell carcinoma tissues showed positive staining (Table 1). In positive cases, Lengsin proteins could be detected in the cytoplasm of the cancer cells, but not in adjacent normal cells (Fig. 3d). To examine the expression of Lengsin protein in major normal tissues, we performed immunohistochemical staining with mAb #517. Lengsin protein was undetectable in liver and placenta, which expressed Lengsin mRNA at very low levels (Fig. 3e), or other organs including the heart, brain, lung, kidney, pancreas and large intestine (data not shown). These data suggest that Lengsin protein was preferentially expressed in lung carcinoma cells and secondary lens fibers, but not in major normal tissues including liver and placenta.

Effect of Lengsin siRNA on cell viability in lung cancer cells. To assess the functions of Lengsin protein in lung cancer cells, we investigated the effects of Lengsin siRNA on the survival of 1-87 cells, which expressed Lengsin, by WST-1 assay and flow cytometric analysis. Introducing Lengsin-specific siRNA significantly reduced expression of Lengsin mRNA compared with control siRNA (Fig. 4a). WST-1 assay revealed that treatment with Lengsin siRNA significantly decreased the cell viability compared with control siRNA (Fig. 4b,c). In addition, we measured the percentage of sub-G1 cells, which represents the percentage of apoptotic cells, by flow cytometric analysis using PI staining of DNA. The percentage of apoptotic cells was found to be increased in Lengsin siRNA-treated cells (Fig. 4d). These data indicate that Lengsin might be essential for cell viability in Lengsin-positive lung cancer cells.

Table 1. Clinical characteristics of patients with lung cancer and detection of Lengsin protein by immunohistochemical staining

Histology	Positive/total			
	Adeno	Squamous	Large	Small
Patients	17/34 (50%)	11/21 (52%)	2/2 (100%)	2/4 (50%)
Age (years)				
< 65	8/16	2/6	1/1	2/3
> 65	9/18	9/15	1/1	0/1
Sex				
Male	7/14	10/20	2/2	0/2
Female	10/20	1/1	ND	2/2
UICC Stage				
Stage I	10/27	4/11	1/1	1/2
Stage II	1/1	3/3	ND	ND
Stage III	6/6	4/7	1/1	1/1
Stage IV	ND	ND	ND	0/1

Adeno, adenocarcinoma; Squamous, squamous cell carcinoma; Large, large cell carcinoma; Small, small cell carcinoma; ND, not determined.

Detection of anti-Lengsin autoantibodies by ELISA. Since Lengsin protein is expressed only in cancerous tissue and the normal lens, which is an immunologically privileged site, we hypothesized that Lengsin might be one of the immunogenics for immune systems. Thus, to assess the immune response against Lengsin *in vivo* we investigated anti-Lengsin autoantibodies in sera from 23 lung cancer patients and 19 healthy donors by ELISA using recombinant Lengsin protein. The cutoff value was settled as the

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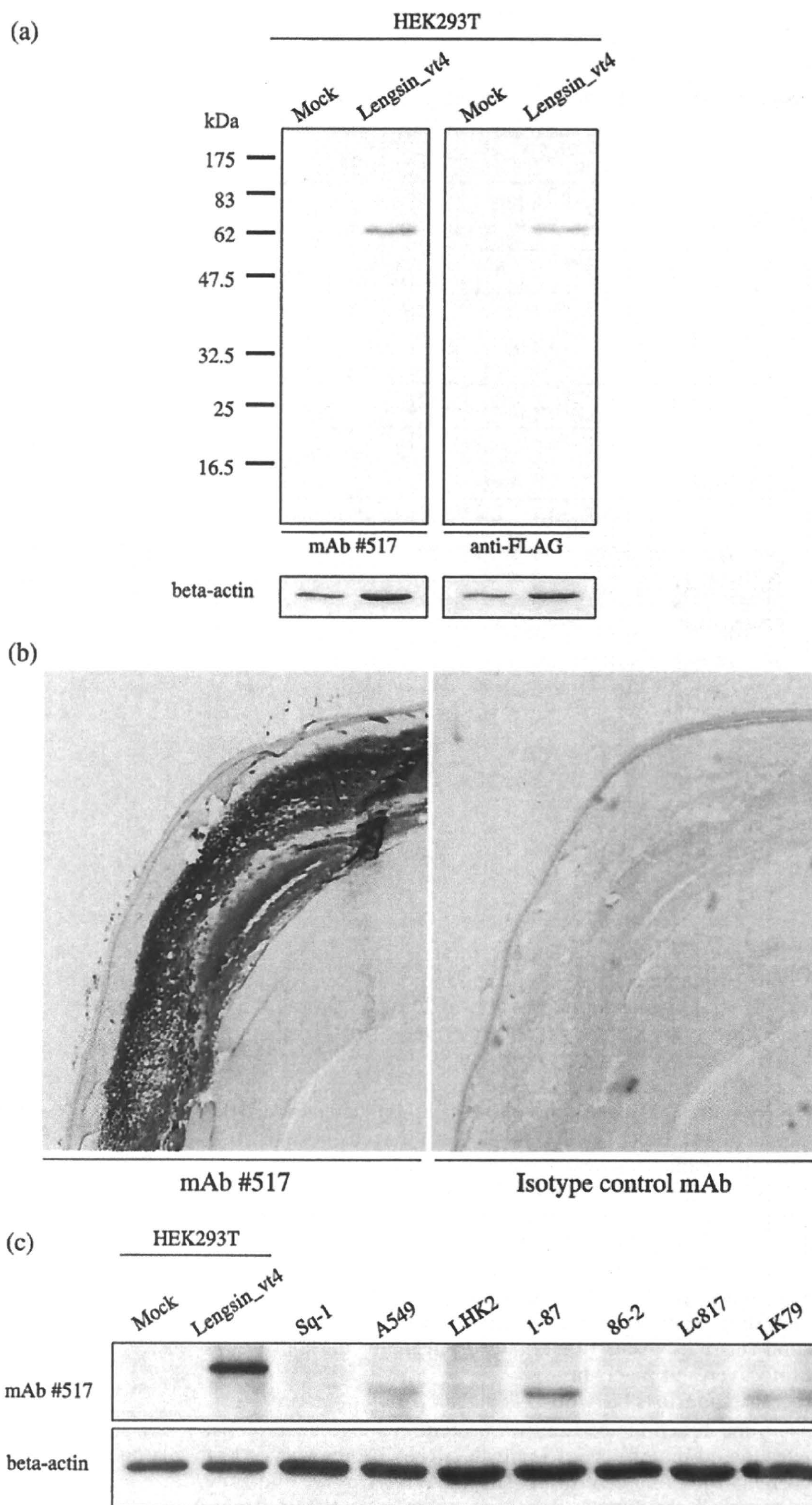

Fig. 3. Detection of Lengsin protein as assessed by Western blot analysis and immunohistochemical staining with anti-Lengsin mAb #517. (a) Specific detection of Lengsin protein in HEK293T cells transfected with expression vectors of FLAG-epitope-tagged Lengsin_vt4 as assessed by Western blot analysis with mAb #517 and an anti-FLAG mAb. Beta-actin was used as a protein loading control. (b) Detection of Lengsin in human lens by immunohistochemical staining with mAb #517. Magnification 40×. (c) Expression of Lengsin in lung cancer cell lines as assessed by Western blot analysis. HEK293T cells transfected with Lengsin_vt4 or mock-transfected were used as a control sample. (d) Representative immunohistochemical staining with mAb #517 in primary lung adenocarcinoma, squamous cell carcinoma, large cell carcinoma and small cell carcinoma tissues. Magnification 200×. (e) Representative immunohistochemical staining with mAb #517 in normal liver and placenta. Magnification 200×.

mean plus two SD of healthy donor samples. The clinical characteristics and results of 23 lung cancer patients are summarized in Table 2. There is no significant difference of anti-Lengsin antibodies between healthy donors and lung cancer patients; sera from 6 of the 23 lung cancer patients (26.1%) were positive. These data indicated that the anti-Lengsin immune response was elicited with Lengsin protein ectopically expressed in lung cancer cells. Moreover, all six anti-Lengsin autoantibody-positive patients had no oculopathy including any disease of the lens, indicating that anti-Lengsin antibodies might not be relevant to a lens-related pathologic state. Anti-Lengsin autoantibodies in serum might have no adverse effect on the ocular compartment, which is presumed to be an immune-privileged site. Taken together, these results strongly suggest that ectopically expressed Lengsin could cause immunological reactions for lung cancer cells, but not for the lens; hence, Lengsin might be a novel target molecule for cancer immunotherapy as well as for a diagnostic marker.

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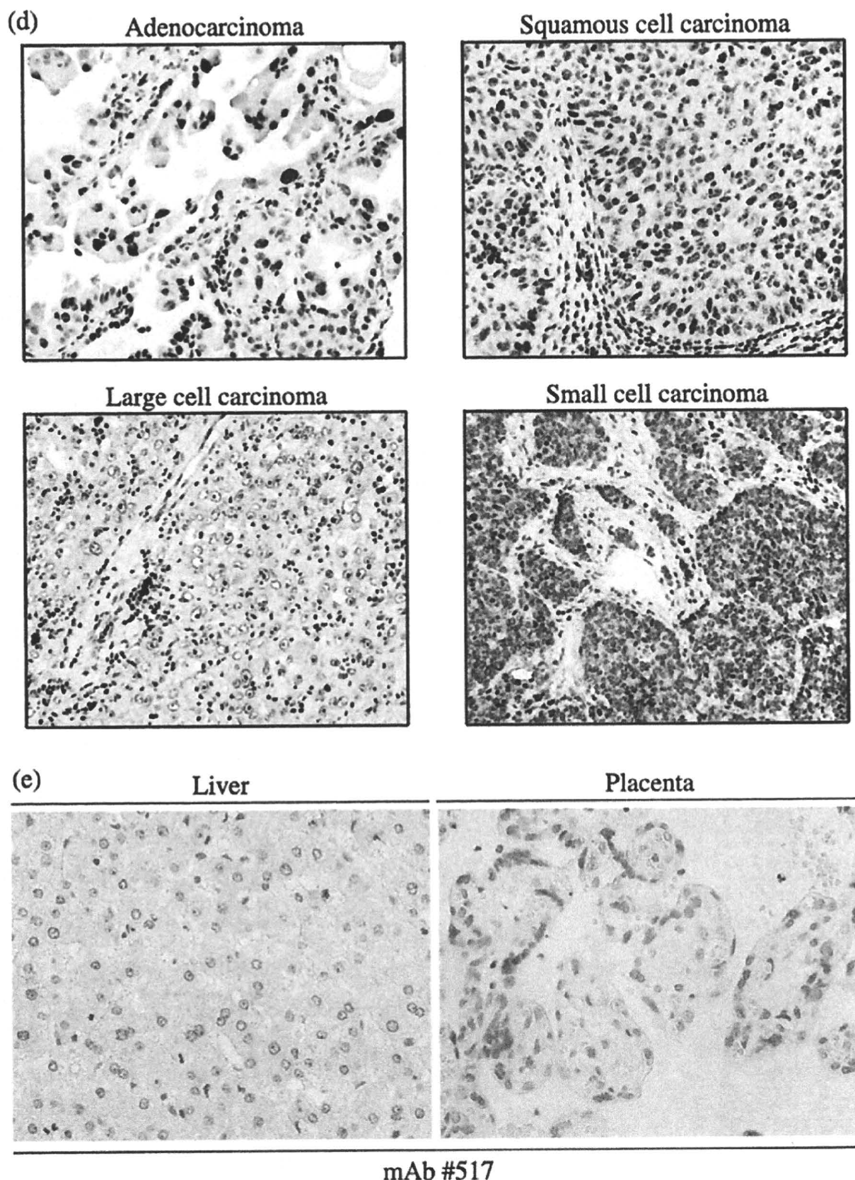

Fig. 3. *Continued.*

Discussion

Wistow *et al.* reported that Lengsin was an abundant transcript in the human lens, and had a sequence similar to glutamine synthetase.[13] However, Lengsin did not catalyze glutamine synthesis, yet cross-reacted with antiglutamine synthetase antibodies assembled into the same dodecameric structure as prokaryotic class I glutamine synthetase.[5,6]

Lengsin is a highly specific protein for the lens.[5–7] Lengsin and lens intermediate filament proteins colocalize at the plasma membrane in maturing lens fiber cells and expression of Lengsin correlates with the reorganization of the lens fiber cell cytoskeleton. Thus, it may act as a component of the cytoskeleton in the lens.[7] In addition, Lengsin was expressed at high levels in the transparent but not the cataractous human lens, indicating that it may be related to the maintenance of lens transparency. Moreover, Lengsin relieves cellular toxicity caused by amyloid-beta expression, and thus, may have a chaperone-like role.[5]

In this study, we reported for the first time that Lengsin, a novel lung cancer antigen, was overexpressed ectopically in the four major histological types of lung cancer. Furthermore, we could immunohistochemically detect Lengsin protein strongly in the human lens with anti-Lengsin mAb #517 generated in our study. Lengsin protein was detected in 50–100% of primary lung carcinoma tissues with mAb #517, but was not detectable in any normal tissues except for lens. Thus, mAb #517 might be a fine marker to diagnose lung carcinoma and define the indication for molecular targeting therapy using Lengsin.

Two splicing variants of Lengsin, variants 1 and 3, were already reported to be expressed in the human lens.[5] Analysis of the gene structure of Lengsin in lung cancer cells revealed that a new splicing variant of human Lengsin mRNA, which was termed splicing variant 4 (Lengsin_vt4), was the major transcript in lung cancer cells. Lengsin_vt4 retains exon 3 that codes 63 amino acids between exon 2 and exon 4 without the frame shift, but the wild type of human Lengsin does not contain exon 3. Lengsin protein retains exon 3 in mammals other than primates.[6] Exon 3 might be evolutionarily eliminated in the human lens; however, our data indicated that Lengsin_vt4 retaining exon 3 was expressed dominantly in human lung cancer cells.

In addition, knockdown of Lengsin expression caused a decrease of cell viability in 1-87 cells, which expressed Lengsin.

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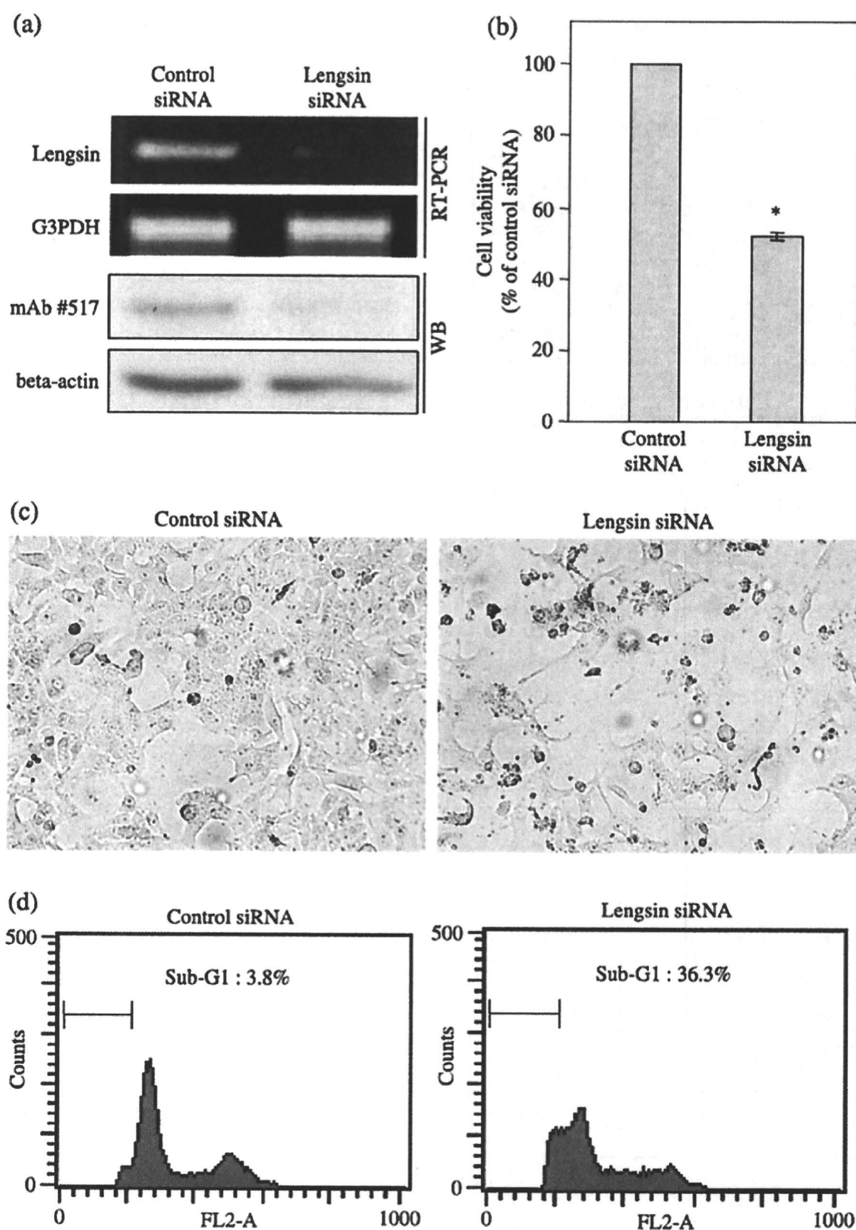

Fig. 4. Effect of Lengsin on cell viability in lung cancer cell line 1-87. (a) Gene silencing was performed using Lengsin siRNA. RT-PCR analysis was done using Lengsin primer pair I. G3PDH was used as an internal control. (b) WST-1 assay shows a decrease in the numbers of viable cells after knockdown of Lengsin expression in 1-87 cells. Statistical analysis was done using a Student's *t*-test. *, $P < 0.01$ compared with the control siRNA. The assay was performed in triplicate; bars, SD. (c) 1-87 cells transfected with control siRNA (right panel) and Lengsin siRNA (left panel). Magnification, ×200. (d) Percentage of cells in the sub-G1 phase indicated by bars was determined by flow cytometry using propidium iodide staining of DNA.

Although the mechanism remains unclear, Lengsin might play an essential role for cell viability in Lengsin-expressing cancer cells.

It remains to be explained why a lens-specific protein is expressed in lung cancers. The eye, including the retina and lens, is considered an immune-privileged site and is protected from immune responses by a variety of mechanisms including the blood–organ barrier, lack of lymphatic drainage, low expression of MHC molecules, local production of immunosuppressive cytokines such as TGF-beta and constitutive expression of Fas ligand.[14,15] However, recoverin, a calcium-binding protein localized specifically in the retina, is expressed in various cancers,[16,17] and it is reported that antirecoverin autoantibodies may cause retina cells to degenerate and cause cancer-associated retinopathy.[18,19] This suggests the retina is an incompletely immune-privileged organ. Immunization of recoverin-positive cancer-bearing mice with recoverin-derived antigenic peptide caused both an antitumor effect and dysfunction of the retina.[20] On the other hand, no lens-related disease caused by an autoimmune response against any cancer antigen has been reported to date. Our data also showed that antilengsin antibody positive lung cancer patients had no lens troubles, suggesting that the lens is completely immune-privileged, which is different from the retina. As Lengsin localizes to the cytosol, anti-Lengsin antibodies might not have biological significance; however, Lengsin protein derived from necrotic or apoptotic cancer cells can make immune complexes with anti-Lengsin antibody, which can potentially cause serial immunological responses including CTL activity and subsequent injury of the lens. However, our data suggest that the anti-Lengsin immunological response is not harmful for the lens, and support the feasibility of lung cancer immunotherapy targeting the Lengsin molecule.

The testis is also an immune-privileged site.[21] It is well known that cancer–testis (CT) antigens, including the MAGE gene family and NY-ESO-1, are expressed exclusively in cancers and normal testis tissue. Hence, it is difficult to induce immune tolerance toward CT antigens.[22] Therefore, CT antigens are highly immunogenic and are promising targets for cancer immunotherapy.[23–25]

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Table 2. Clinical characteristics of serum donors with lung cancers and detection of anti-Lengsin autoantibodies by ELISA

Patient No.	Sex	Age	Histology	UICC Stage	Anti-Lengsin autoantibodies†	Lengsin‡
1	Male	61	Ad	IIIB	+	+
2	Male	79	Ad	IA	+	+
3	Female	79	Ad	IV	+	ND
4	Male	76	Sq	IB	+	+
5	Female	65	Ad	IA	+	+
6	Male	60	Sq	IIIA	+	+
7	Male	67	Ad	IV	–	ND
8	Male	59	Sq	IA	–	–
9	Male	65	Ad	IA	–	+
10	Female	62	Ad	IIIB	–	ND
11	Male	63	Ad	IV	–	ND
12	Male	87	Ad	IV	–	ND
13	Male	70	Ad	IA	–	+
14	Female	64	Sm	IB	–	+
15	Male	65	Sq	IIIB	–	ND
16	Male	69	Sq	IA	–	+
17	Male	66	Ad	IIIB	–	+
18	Male	62	Sm	IA	–	–
19	Male	73	Sq	IIA	–	–
20	Male	74	Ad	IA	–	+
21	Male	56	Ad	IA	–	–
22	Male	73	Sm	IV	–	–
23	Female	56	Ad	IA	–	–

Ad, adenocarcinoma; ELISA, enzyme-linked immunosorbent assay; Sq, squamous cell carcinoma; Sm, small cell carcinoma; ND, not determined.
†The cutoff value is the mean plus two SD for healthy donor samples. Antibody levels for upper or lower cutoff values are evaluated as either positive (+) or negative (–), respectively.
‡Positive (+) or negative (–) indicate that expression of Lengsin protein in lung cancer tissues assessed by immunohistochemical staining is either detected or not detected, respectively.

Lengsin is expressed exclusively in lung cancers and the immune-privileged normal lens; thus, we consider Lengsin to be not only a risk-free but also a highly immunogenic target for immunotherapy. We are now investigating and identifying Lengsin epitopes recognized by cytotoxic T lymphocytes. Some cancer–testis antigens have been isolated by analyzing a testis cDNA expression library with cancer patients' sera.[26–28] Possible new cancer antigens like Lengsin, which is exclusively expressed in the lens and cancer, may be found by studying a lens cDNA library. Lengsin is obviously the first such reported cancer antigen, a 'cancer–lens antigen', which might play a role in molecular targeting therapy, including antigen-specific immunotherapy like cancer–testis antigens.

In summary, we identified that lens-specific antigen Lengsin is expressed ectopically in lung cancer cells. The predominant transcript form was a novel splicing variant, termed Lengsin_vt4. Lengsin plays an essential role in lung cancer cell survival. Anti-Lengsin humoral immune reactions could be detected in lung cancer patients' serum, but not in healthy donors'. These data suggest that Lengsin_vt4 might be a novel biomarker of lung cancers, and also a molecular target including immunotherapy.

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Disclosure statement

There is no financial interest with regard to the submitted manuscript that might be construed as a conflict of interest.

Abbreviations

mAb	monoclonal antibody
PBMCs	peripheral blood mononuclear cells
RT-PCR	reverse transcription-PCR
TAA	tumor-associated antigen
UICC	International Union Against Cancer

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Research **Open Access**

HLA-A*0201-restricted CTL epitope of a novel osteosarcoma antigen, papillomavirus binding factor

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Abstract

Background: To develop peptide-based immunotherapy for osteosarcoma, we previously identified papillomavirus binding factor (PBF) as a CTL-defined osteosarcoma antigen in the context of HLA-B55. However, clinical application of PBF-based immunotherapy requires identification of naturally presented CTL epitopes in osteosarcoma cells in the context of more common HLA molecules such as HLA-A2.

Methods: Ten peptides with the HLA-A*0201 binding motif were synthesized from the amino acid sequence of PBF according to the BIMAS score and screened with an HLA class I stabilization assay. The frequency of CTLs recognizing the selected PBF-derived peptide was determined in peripheral blood of five HLA-A*0201$^+$ patients with osteosarcoma using limiting dilution (LD)/mixed lymphocyte peptide culture (MLPC) followed by tetramer-based frequency analysis. Attempts were made to establish PBF-specific CTL clones from the tetramer-positive CTL pool by a combination of limiting dilution and single-cell sorting. The cytotoxicity of CTLs was assessed by ^{51}Cr release assay.

Results: Peptide PBF A2.2 showed the highest affinity to HLA-A*0201. CD8+ T cells reacting with the PBF A2.2 peptide were detected in three of five patients at frequencies from 2×10^{-7} to 5×10^{-6}. A tetramer-positive PBF A2.2-specific CTL line, 5A9, specifically lysed allogeneic osteosarcoma cell lines that expressed both PBF and either HLA-A*0201 or HLA-A*0206, autologous tumor cells, and T2 pulsed with PBF A2.2. Five of 12 tetramer-positive CTL clones also lysed allogeneic osteosarcoma cell lines expressing both PBF and either HLA-A*0201 or HLA-A*0206 and T2 pulsed with PBF A2.2.

Conclusion: These findings indicate that PBF A2.2 serves as a CTL epitope on osteosarcoma cells in the context of HLA-A*0201, and potentially, HLA-A*0206. This extends the availability of PBF-derived therapeutic peptide vaccines for patients with osteosarcoma.

Background

Osteosarcoma is the most common primary malignant tumor of bone. The survival rate of patients with osteosarcoma was under 20% before 1970. The introduction of neoadjuvant chemotherapy, establishment of guidelines for adequate surgical margins, and development of post-excision reconstruction raised the five-year survival rate to 60–70% [1,2]. These advances overshadowed the pioneering adjuvant immunotherapy trials using autologous tumor vaccines for patients with osteosarcoma, despite their having some therapeutic efficacy [3-5]. However, the survival rate of patients with osteosarcoma has reached a plateau in the last decade [6,7], which has reignited interest in immunotherapeutic approaches [8-10].

We previously identified papillomavirus-binding factor (PBF) as a novel osteosarcoma antigen, using an osteosarcoma cell line and an autologous CTL (cytotoxic T lymphocyte) clone restricted by HLA-B*5502 [11,12]. PBF is a DNA-binding transcription factor and a regulator of apoptosis [13-15]. PBF protein is expressed in 92% of osteosarcomas. Moreover, PBF-positive sarcomas have a significantly worse prognosis than PBF-negative sarcomas [16,17]. Development of PBF-based immunotherapy requires identification of naturally presented CTL epitopes in osteosarcoma cells in the context of common HLA molecules such as HLA-A2 and HLA-A24. The present study was designed to determine HLA-A*0201-restricted CTL epitopes from PBF.

Methods

This study was approved under institutional guidelines for the use of human subjects in research. The patients and their families as well as healthy donors gave informed consent for the use of blood samples and tissue specimens in our research.

Cells

The osteosarcoma cell lines OS2000 and KIKU were established in our laboratory [11,18]. The osteosarcoma cell lines U2OS, Saos-2 and HOS, human lymphoblastoid cell line T2, and erythroleukemia cell line K562 were purchased from ATCC (Manassas, VA). OS2000, KIKI, U2OS, Saos-2, HOS and K562 are PBF-positive [12]. U2OS, Saos-2, and T2 are HLA-A*0201 positive. The HLA genotypes of the osteosarcoma cell lines were as follows: OS2000, *A*2402*, *B*5502*, *B*4002*, *Cw*0102*; U2OS, *A*0201*, *A*3201*, *B*4402*, *Cw*0501*, *Cw*0704*; Saos-2, *A*0201*, *A*2402*, *B*1302*, *B*4402*, *Cw*0602*, *Cw*0704*; HOS, *A*0211*, *B*5201*, *Cw*1202*; KIKU, *A*0206*, *A*2402*, *B*4006*, *B*5201*, *Cw*0802* and *Cw*1202*. Epstein-Barr virus-transformed B cell line NS-EBV-B was established from a healthy donor in our laboratory. Another Epstein-Barr virus-transformed B cell line, LCL-OS2000, was established from a patient with osteosarcoma [11].

Autologous tumor cells were developed from fresh frozen biopsy specimens of osteosarcoma. The specimens were thawed in Iscove's modified Dulbecco's modified Eagle's medium containing 10% FCS at room temperature, minced into small pieces and filtrated with a 70 I m Cell Strainer (BD Biosciences, Bedford, MA). The cells were used immediately for cytotoxicity assay.

Design and synthesis of PBF-derived peptides

Based on the entire amino acid sequence of PBF, peptides with the ability to bind to HLA-A*0201 class I molecules were searched for through the World Wide Web site Bioinformatics and Molecular Analysis Section (BIMAS) HLA Peptide Binding Predictions http://www-bimas.cit.nih.gov/molbio/hla_bind/[19]. Based on the binding scores, ten peptides were selected and synthesized [see Additional file 1].

HLA class I stabilization assay

The affinity of peptides for HLA-A*0201 molecules was evaluated by T2 cell surface HLA class-I stabilization assay as described previously [20,21]. An HLA-A*0201-binding influenza matrix protein-derived peptide (Inf-MP A2; GILGFVFTL) [22] was used for positive control. Mouse H-2Kb-restricted peptide VSV8 (RGYVYQGL) [23] was used for negative control. Assays were performed in triplicate. The affinity of each peptide for HLA-A*0201 molecules was evaluated by the percent mean fluorescence intensity (%MFI) increase of the HLA-A*0201 molecules detected by staining with an anti-HLA-A2 monoclonal antibody (BB7.2, purchased from ATCC) using the following calculation. %MFI increase: [(MFI with the given peptide – MFI without peptide)/(MFI without peptide)] × 100.

Limiting dilution/mixed lymphocyte peptide culture

Prior to frequency analysis and cytotoxicity assays, PBMC of patients were subjected to mixed lymphocyte peptide culture under limiting dilution conditions (LD/MLPC) according to the method described by Karanikas et al. [24] with some modifications [17]. LD/MLPC aims to seed at most one CTL precursor cell per well and induces proliferation of the precursor cell by subsequent mixed lymphocyte peptide culture. For this purpose, the appropriate number of PBMC and $CD8^+$ cells per well is considered to be 1×10^5–2×10^5 [17,24].

PBMCs were used as a source of responder cells in the initial five subjects (Patients 1 and 2 and three healthy donors) and $CD8^+$ cells were used in the following three patients (Patients 3–5) [see Additional file 2].

PBMC obtained from peripheral blood samples (50 ml) of Patients 1 and 2 and three healthy donors were suspended in AIM-V (Invitrogen Corp., Carlsbad, CA) supplemented with 1% human serum (HS). These cells were

incubated for 60 min at room temperature with peptide PBF A2.2 (50 I g/ml). Peptide-pulsed PBMC were seeded at 2 × 10^5 cells/200 I l/well into round-bottom 96-microwell plates in AIM-V with 10%HS, IL-2 (20 U/ml; a kind gift from Takeda Chemical Industries, Ltd., Osaka Japan) and IL-7 (10 ng/ml; R&D Systems, Minneapolis, Minnesota, USA), and incubated. On day 7, half of the medium was replaced with fresh AIM-V containing IL-2, IL-7 and the same peptides. The cell cultures were maintained by adding fresh AIM-V containing IL-2. On days 14–21, they were subjected to tetramer-based frequency analysis.

PBMC obtained from Patients 3–5 were separated into $CD8^+$ cells and $CD8^-$ cells using magnetic anti-CD8 microbeads (Miltenyi Biotec, Gladbach, Germany). $CD8^-$ cells were pulsed with the PBF A2.2 peptide for 60 min. Half of the $CD8^-$ cells were cryopreserved at -80°C for the second stimulation. $CD8^+$ cells (1.0–2.1 × 10^5/well) and irradiated PBF A2.2 peptide-pulsed $CD8^-$ cells (1–5 × 10^5/well) were cocultured in 48-well cell culture plates in 500 I l of AIM-V with 10%HS, IL-2 and IL-7. On day 7, the second stimulation was performed by adding irradiated peptide-pulsed $CD8^-$ cells to each culture well in 500 I l of AIM-V with 10%HS, IL-2 and IL-7. On days 13–23, they were subjected to tetramer-based frequency analysis.

Tetramer-based frequency analysis

An FITC-conjugated HLA-A*0201/HIV tetramer (here termed the control tetramer) and a PE-conjugated HLA-A*0201/PBF A2.2 tetramer (A2/PBF A2.2 tetramer) were constructed by Medical & Biological Laboratories Co., Ltd. (Tokyo, Japan). PBMCs from patients were stimulated with the PBF A2.2 peptide by using the LD/MLPC procedure as described above. From each microwell containing 200 I l of the microculture pool, 100 I l was transferred to a V-bottom microwell and washed. To the spin-down pellets, the control tetramer and A2/PBF A2.2 tetramer (10 nM in 25 I l of PBS) were added in combination and incubated for 15 min at room temperature. Then a PE-Cy5-conjugated anti-CD8 antibody (eBioscience, San Diego, California, USA) was added (dilution of 1:30 in 25 I l of PBS containing the control tetramer and A2/PBF A2.2 tetramer) and incubated for another 15 min. The cells were washed in PBS twice, fixed with 0.5% formaldehyde, and analyzed by flow cytometry using FACScan and CellQuest software (Becton Dickinson, San Jose, California, USA). $CD8^+$ living cells were gated and the cells labeled with the A2/PBF A2.2 tetramer were referred to as tetramer-positive cells. Tetramer-positive cells in each well are theoretically derived from a single CTL precursor, regardless of the number (percentage) of tetramer-positive cells. Accordingly, the number of tetramer-positive wells represents the number of CTL precursors. The frequency of anti-PBF A2.2 CTLs was evaluated using the following calculation: (number of tetramer-positive wells)/[(total number of tested wells) × (initial number of CD8+ cells per well)].

Development of CTL line and CTL clones

Attempts to establish CTL clones were made by a limiting dilution procedure and subsequent single-cell sorting procedures.

In the limiting dilution procedure, cells from a tetramer-positive T cell pool derived from Patient 4 were replated into a 96-well round-bottom microplate at one cell per well. In each well, a T cell was cocultured with irradiated A*0201+ NS-EBV-B cells (2 × 10^4) pulsed with the PBF A2.2 peptide and irradiated allogeneic PBMCs (8 × 10^4) in 200 I l of AIM-V containing 10%HS, IL-2 (200 U/ml) and IL-7 (10 ng/ml). On days 7, 14 and 21, the stimulation was repeated by adding irradiated peptide-pulsed NS-EBV cells (1 × 10^4), LCL-OS2000 cells (1 × 10^4), and allogeneic PBMCs (8 × 10^4) to each culture well in 100 I l of freshly replaced AIM-V with 10%HS, IL-2 and IL-7. On day 35, tetramer staining of all wells was performed. The tetramer-positive population was selected and further expanded. These cells were seeded at 2 × 10^3 per well with irradiated allogeneic PBMCs (1 × 10^5) in 100 I l of AIM-V containing 10% HS, IL-2 (200 U/ml) and phytohemagglutinin-P (PHA; 7.5 I g/ml, Wako Chemicals, Osaka, Japan) in a total of 192 wells of 96-well round-bottom microplates. On day 7, 100 I l of AIM-V containing 10% HS and IL-2 was added. On day 14, all proliferated cells were collected, washed and replaced with fresh AIM-V containing 10% HS and IL-2, followed by maintenance in a 48-well microplate at 0.5–1 × 10^6 cells per well. The established oligoclonal cell line was designated CTL 5A9.

Subsequently, a frozen stock of the oligoclonal CTL 5A9 was reactivated and subjected to single-cell sorting. In the reactivation procedure, thawed CTL 5A9 cells were cultured with allogeneic PBMCs in AIM-V containing 10% HS, IL-2 (200 U/ml) and PHA (7.5 I g/ml) for 27 days. The reactivated CTL 5A9 cells were stained by the A2/PBF A2.2 tetramer and the control tetramer. The tetramer-positive cells (0.82%) were sorted at one cell per well using FACS Aria II (Becton Dickinson) with allogeneic PBMCs (1 × 10^5) to each culture well in 200 I l of AIM-V with 10% HS, IL-2 (200 U/ml) and PHA (7.5 ug/ml) in a total of 384 wells of 96-well microplates. On days 7, 10 and 14, half of each medium was replaced with fresh medium without PHA. On days 20–34, tetramer staining was performed. Single-cell sorting was repeated until tetramer staining showed single clone populations.

Cytotoxicity assay

CTL-mediated cytolytic activity was measured by a 6 h-^{51}Cr-release assay [25]. Osteosarcoma cell lines (U2OS, OS2000, Saos-2, KIKU and HOS), K562, T2, and autolo-

gous osteosarcoma cells obtained from Patient 4 were used as target cells. T2 cells were treated with or without peptides at the indicated concentrations for 1 h at room temperature after ^{51}Cr-labeling. An HIV peptide (SLYNTVATL)[26] was used as a negative control peptide. Target cells were labeled with 100 I Ci of ^{51}Cr for 1 h at 37°C. The labeled target cells were suspended in RPMI without serum and seeded to microwells (2–5 × 10^3 cells/well).

CTL 5A9 and CTL clones were used as the effector cells. The effector cells were transferred to V-bottom microwells, suspended in AIM-V and mixed with the labeled target cells. After a 6 h incubation period at 37°C, the ^{51}Cr level in the culture supernatant was measured using an automated gamma counter. The percentage of specific cytotoxicity was calculated as follows: the percentage of specific ^{51}Cr release = 100 × (experimental release – spontaneous release)/(maximum release – spontaneous release).

Results

Affinity of PBF-derived synthetic peptides to HLA-A*0201 molecules

To determine HLA-A*0201-restricted epitopes of PBF, we synthesized 10 peptides from the amino acid sequence of PBF in accordance with the BIMAS scores for HLA-A*0201 affinity [see Additional file 1]. Subsequently we evaluated the affinity of these peptides to HLA-A*0201 molecules by HLA class I-stabilization assay [see Additional file 1]. Peptide PBF A2.2 showed the highest %MFI increase among the peptides. Peptide titration experiments (Fig. 1) revealed dose-dependent increases of %MFI by PBF A2.2 and the positive control Inf-MP A2 peptide, but not the VSV8 negative control peptide.

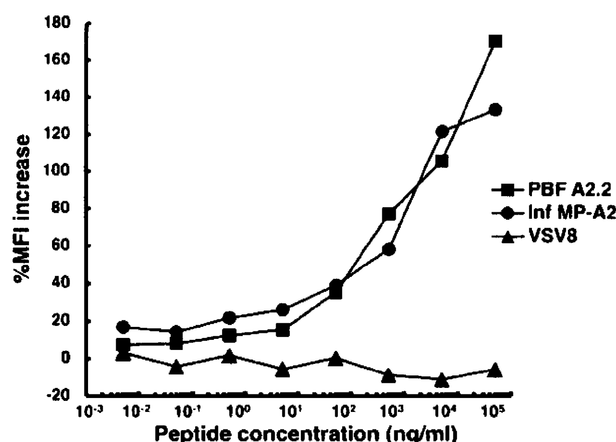

Figure 1

Binding affinity of PBF A2.2 peptide to HLA-A*0201 molecules. The affinities of three peptides, PBF A2.2, Inf MP-A2 and VSV8, were determined by HLA class I stabilization assay at the indicated concentrations.

Frequency of anti-PBF A2.2-specific T cells in HLA-A*0201+ patients with osteosarcoma and healthy donors

We then examined the frequency of peripheral CD8$^+$ T-lymphocytes that recognized the PBF A2.2 peptide in five HLA-A*0201$^+$ patients with PBF-positive osteosarcoma by LD/MLPC/tetramer analysis. A2/PBF A2.2 tetramer-positive T cells were detected in three of the five patients [see Additional file 2]. Fig. 2 presents the results of flow cytometric analysis of Patient 4, showing two tetramer-positive wells and 12 of 34 tetramer-negative wells. This indicated the presence of at least two CTL precursor cells (PBF A2.2-specific CD8$^+$ T cells) in 5.4 × 10^6 CD8+ T cells examined. The frequencies of the PBF A2.2-specific CD8$^+$ T cells ranged from 2 × 10^{-7} to 5 × 10^{-6} (2 × 10^{-6} on average) in three tetramer-positive patients. In the three healthy donors, the PBF A2.2-specific CD8$^+$ T cells ranged from 1 × 10^{-7} to 3 × 10^{-7} (2 × 10^{-7} on average).

Establishment of A2/PBF A2.2 tetramer-positive CTL oligoclonal line and CTL clones

Attempts to establish CTL clones were made by a combination of limiting dilution and repeated single-cell sorting. Limiting dilution of one of the tetramer-positive T cell pools from Patient 4 yielded a cell population (designated CTL 5A9) with more than 80% tetramer-positive CD8$^+$ cells (Fig. 3). RT-PCR analysis of TCR expression in CTL 5A9 revealed four V alpha mRNAs (V alpha 3, 5, 8 and 12) and clonal V beta mRNA (V beta 13.1) (data not shown), indicating the oligoclonal nature of CTL 5A9.

We then performed single cell sorting of CTL 5A9 (Fig. 3). The first single-cell sorting resulted in 11 tetramer-positive oligoclonal populations. Two of these 11 oligoclones were subsequently subjected to the second single cell sorting. From one oligoclone (clone 140), 12 single clones were established. Of these, five clones (1B1, 1D7, 1E1, 1F4 and 1F7) showed cytotoxic activity to PBF A2.2-pulsed T2 cells.

Cytotoxicity of A2/PBF A2.2 tetramer-positive CTL oligoclonal line and CTL clones

Finally we examined the cytotoxic properties of the oligoclonal line, 5A9, and five CTL clones. As shown in Fig. 4A, CTL 5A9 lysed PBF A2.2 peptide-pulsed T2 cells in an effector:target ratio-dependent manner. In contrast, such cytotoxic activity of CTL 5A9 was not seen against T2 cells without peptide pulsation or K562 cells. Cytotoxic activity of CTL 5A9 against PBF A2.2-pulsed T2 cells was dependent on the concentration of the PBF A2.2 peptide (Fig. 4B). Given the oligoclonal nature of CTL 5A9, we also examined the peptide-specific cytotoxicity of their tetramer-negative subpopulation. The tetramer-negative

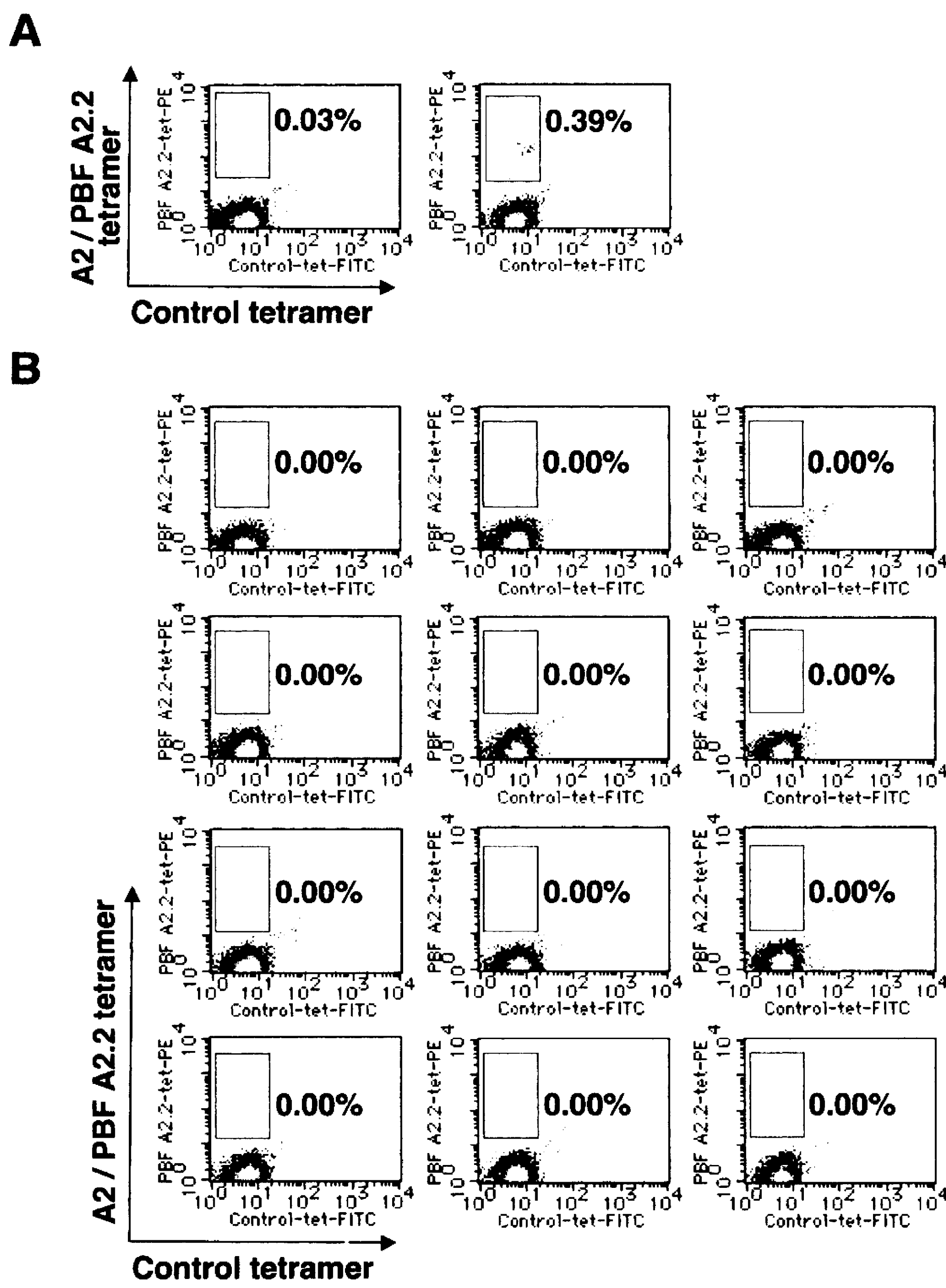

Figure 2
Tetramer-based detection of PBF A2.2-specific T cells. CD8+ T cells (5.4×10^6) collected from Patient 4 were seeded into 36 wells at the concentration of 1.5×10^5 per well and cultured with peptide PBF A2.2 and cytokines. On day 21, tetramer analysis was performed. This analysis showed that 2 of 36 wells were positive, containing 0.03% and 0.39% tetramer-positive cells, respectively (A). The remaining 34 wells were negative with 0.00% reactivity. Here, 12 of 34 tetramer-negative wells are shown (B). Each of the 2 positive wells contained at least 1 CTL precursor, indicating that there were at least 2 CTL precursors in a total of 5.4×10^6 CD8+ cells. The frequency was calculated as $2/5.4 \times 10^6 = 3.7 \times 10^{-7}$.

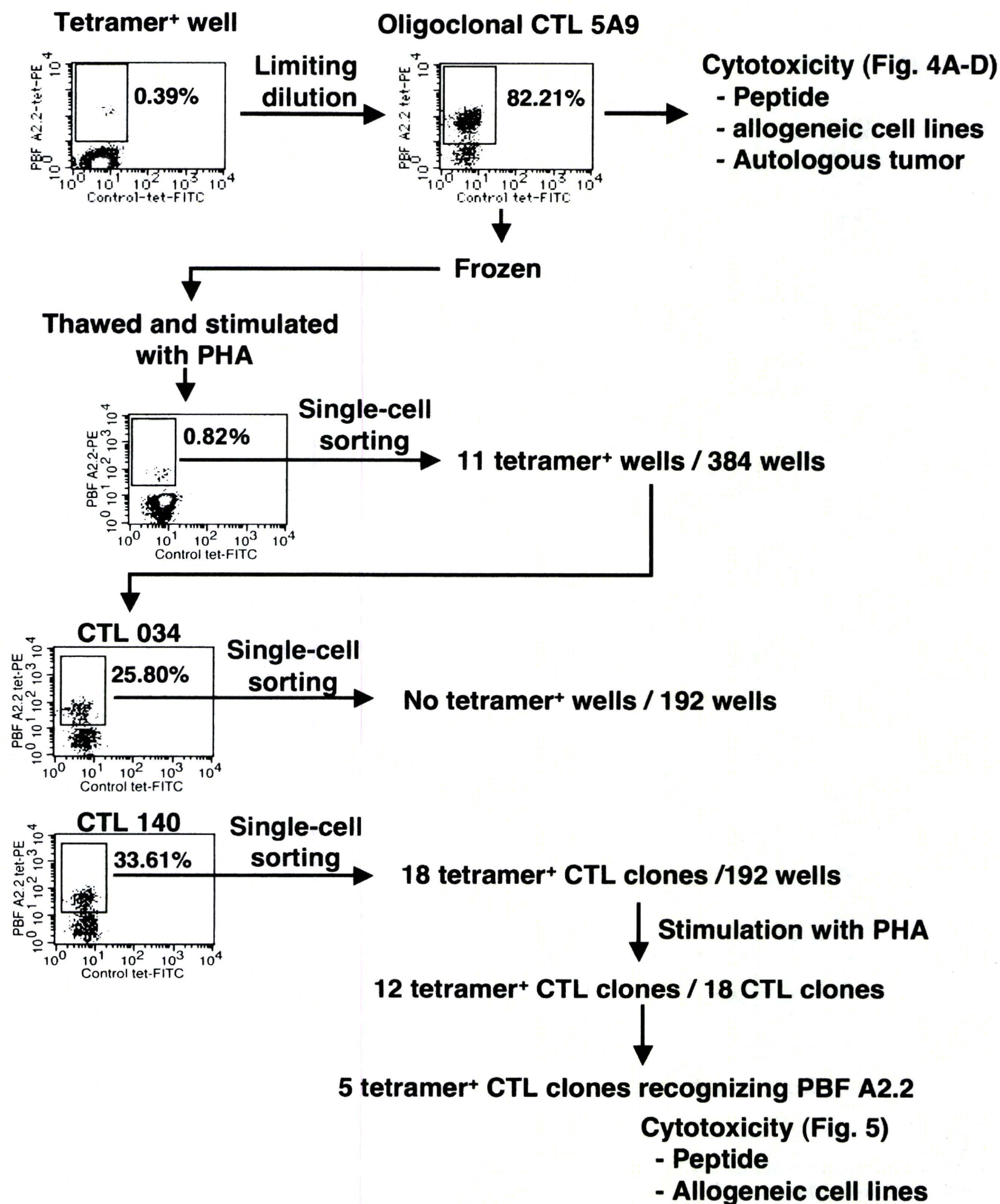

Figure 3
Establishment of PBF A2.2-specific CTL line and CTL clones.

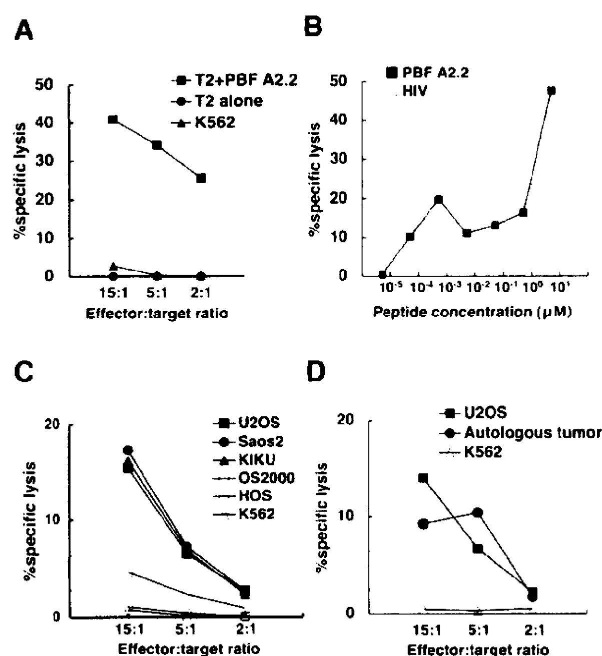

Figure 4
Cytotoxic activity of A2/PBF A2.2 tetramer-positive CTL line 5A9. A. The peptide-specific cytotoxicity of CTL 5A9 was determined using T2 and K562 cells in a 6 h standard ^{51}Cr release assay. T2 cells were pulsed with 50 I g/ml peptide PBF A2.2 or medium for 1 h at room temperature after labeling with ^{51}Cr. CTL 5A9 lysed PBF A2.2 peptide-pulsed T2 cells in an effector:target ratio-dependent manner, but not K562 or T2 cells without peptide pulsation. **B**. T2 cells were incubated with various concentrations of the PBF A2.2 peptide and 5 I M HIV control peptide. The cytotoxicity of CTL 5A9 against peptide-pulsed T2 cells was determined at an effector to target ratio of 30:1. Dotted lines indicate half maximum lysis. **C**. The cytotoxicity of CTL 5A9 against allogeneic osteosarcoma cell lines U2OS, Saos-2, KIKU, OS2000 and HOS. All cell lines express PBF. U2OS and Saos-2 are HLA-A*0201-positive. KIKU is HLA-A*0201-negative, HLA-A*0206-positive. OS2000 and HOS are HLA-A*0201-negative. **D**. Autologous tumor cells were derived from fresh-frozen biopsy specimens of Patient 4, from whom CTL 5A9 was also developed. U2OS and K562 were used as positive control target cells and natural killer target cells, respectively.

5A9 subpopulation did not react against T2 cells, PBF A2.2 peptide-pulsed T2 cells, or K562 cells (data not shown).

Fig. 4C shows the cytotoxic activity of CTL 5A9 against osteosarcoma cells. CTL 5A9 exhibited cytotoxicity against U2OS (PBF-positive, HLA-A*0201-positive), Saos-2 (PBF-positive, HLA-A*0201-positive), and KIKU (PBF-positive, HLA-A*0201-negative, HLA-A*0206-positive) in an effector:target ratio-dependent manner. In contrast, CTL 5A9 showed marginal cytotoxicity against OS2000 (PBF-positive, HLA-A*0201-negative), and undetectable levels of cytotoxicity against HOS (PBF-positive, HLA-A*0201-negative) and K562 cells (PBF-positive, HLA-null). To assess the possibility of an allogeneic reaction for the cytotoxicity of CTL 5A9, we developed autologous tumor cells from fresh-frozen biopsy specimens of Patient 4 and used them as target cells. As shown in Fig. 4D, CTL 5A9 also lysed autologous tumor cells as well as the positive control, U2OS cells, but not K562 cells.

To further determine the specificity of A2/PBF A2.2 tetramer-positive CTLs against osteosarcoma cells in the context of HLA-A2, we analyzed the cytotoxicity of five CTL clones derived from CTL 5A9 (Fig. 5). All five CTL clones lysed PBF A2.2 peptide-pulsed T2 cells and osteosarcoma cell lines U2OS and KIKU. In contrast, none of five clones recognized OS2000, HOS or K562.

Discussion

In the present study, we examined the immunogenicity of an HLA-A*0201-binding peptide derived from a novel tumor-associated antigen PBF. The peptide PBF A2.2 was recognized by CD8+ T cells in three of five HLA-A*0201-positive patients with osteosarcoma and induced an oligoclonal CTL line and five CTL clones from these CD8+ T cells. The CTL line, CTL 5A9, and five CTL clones all exhibited specific cytotoxic activity against PBF A2.2-pulsed T2 cells and allogeneic osteosarcoma cell lines expressing both HLA-A*0201 and PBF. In addition, CTL 5A9 lysed autologous osteosarcoma cells derived from fresh biopsy specimens. These findings indicated that PBF A2.2 served as a CTL epitope on osteosarcoma cells in the context of HLA-A*0201.

Interestingly, CTL 5A9 and the five CTL clones lysed an allogeneic osteosarcoma cell line (KIKU) that expressed PBF and HLA-A*0206, but not HLA-A*0201. This suggested that the peptide PBF A2.2 might also be presented on osteosarcoma cells in the context of HLA-A*0206, as seen for other tumor-associated antigens [27,28]. Alternatively, CTL 5A9 and the five CTL clones might cross-react with an allogeneic antigen presented by HLA-A*0206, B*4006, or -Cw*0802, that was not shared by OS2000 and HOS, on KIKU cells. To determine these possibilities, cytotoxicity assays with other target cells that express both PBF and HLA-A*0206 will be required. Thus far, the proof of immunogenicity of PBF has been limited to an HLA-B55-positive patient [12] and HLA-A24-positive patients with osteosarcoma [17]. Our findings in the present study

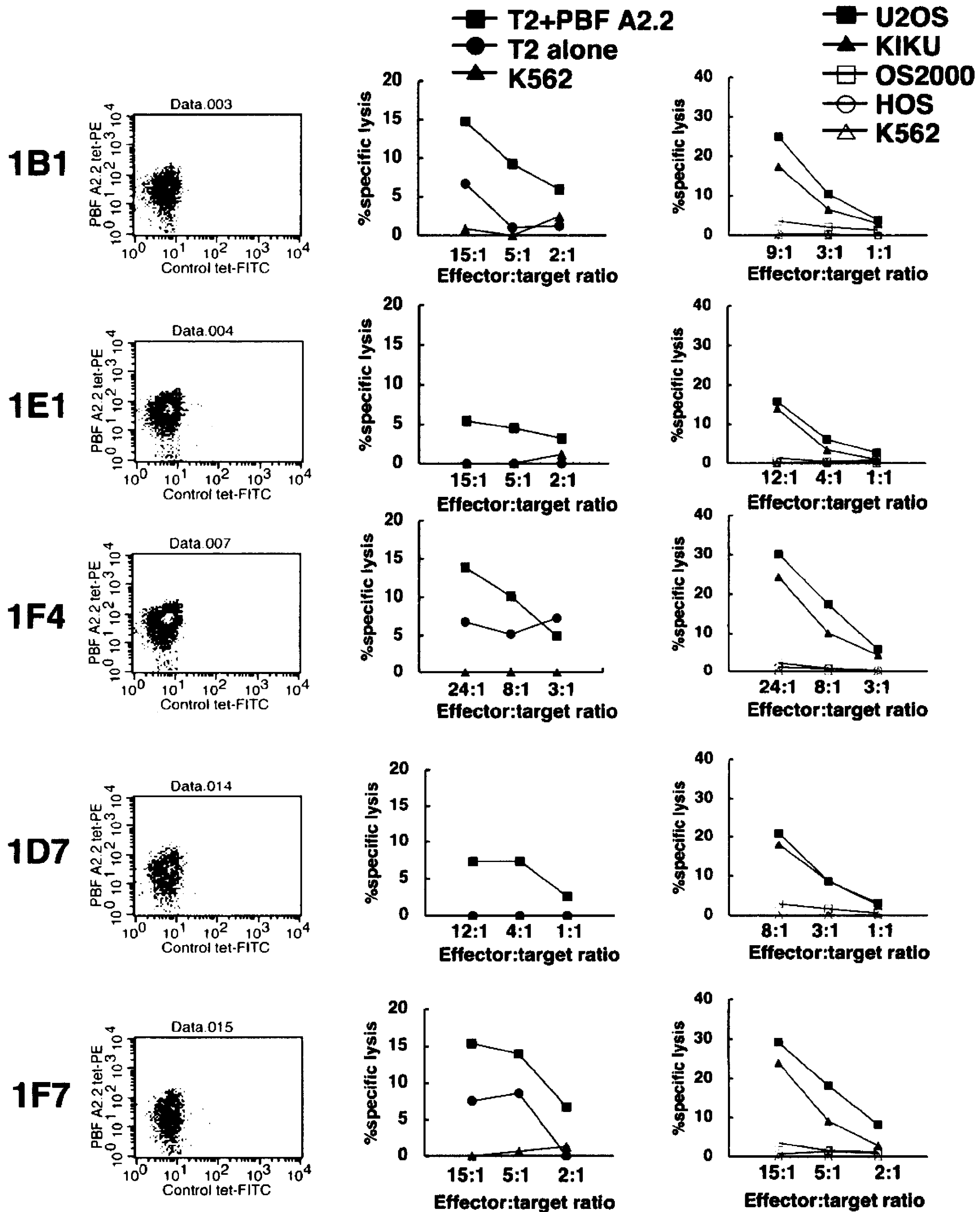

Figure 5

Cytotoxic activity of CTL clones derived from CTL 5A9. Five CTL clones were established from CTL 5A9. Left panels indicate tetramer staining of CTL clones. $CD8^+$ cells were gated. X-axis and Y-axis indicate the fluorescence intensity of control tetramer-FITC and A2/PBF A2.2 tetramer-PE, respectively. Middle panels indicate CTL-mediated cytotoxicity against T2 cells with or without PBF A2.2 peptide-pulsation. Right panels indicate CTL-mediated cytotoxicity against allogeneic osteosarcoma cell lines.

extend the application of PBF-targeting immunotherapy towards patients with HLA-A*0201 and potentially those with HLA-A*0206.

The frequency of the PBF A2.2-specific CTL precursors ranged from 2×10^{-7} to 5×10^{-6} in patients with osteosarcoma. On the other hand, the frequency of the PBF A2.2-specific CTL precursors in healthy donors ranged from 1×10^{-7} and 3×10^{-7}. In our previous study [17], the frequency of PBF A24.2-specific CTL precursors was between 5×10^{-7} and 7×10^{-6}. In melanoma patients, the MAGE3.A1-specific CTL precursor frequency was less than 10^{-7} in normal individuals and non-vaccinated patients as determined by the LD/MLPC/tetramer procedure [29]. Notably the frequency of MAGE3.A1-specific CTL precursors rose to 10^{-6} following vaccination [29]. Therefore the significance of measuring the frequency of peptide-reactive CTL precursors is to determine the baseline frequency in non-vaccinated patients for forthcoming clinical vaccination trials.

The frequency of CTL precursors is generally under the detection limit of the standard tetramer analysis [30-33] so the LD/MLPC/tetramer procedure was developed. The presence of false-positive wells is a concern in the LD/MLPC/tetramer procedure. To reduce this, we double-stained cells with A2/PBF A2.2 tetramer-PE and control tetramer-FITC (this detects cells that nonspecifically bind tetramers). In tetramer-positive wells, percentages of tetramer-positive cells varied from 0.03% to 0.39% in the present study. The variation of the percentages of tetramer-positive cells conceptually reflects the differing proliferation activities of a single CTL precursors seeded in each well, but does not affect calculation of the frequency of CTL precursors. Therefore, it is critical in the LD/MLPC/tetramer procedure to detect cells that react with the A2/PBF A2.2 tetramer despite the quite low percentages.

Conclusion
The present study demonstrates the immunogenicity of peptide PBF A2.2 in HLA-A*0201-positive patients with osteosarcoma. The PBF A2.2 peptide is a novel antigenic peptide naturally presented on osteosarcoma cells in the context of HLA-A*0201 and, potentially, HLA-A*0206. This extends the availability of PBF-derived therapeutic peptide vaccines for patients with osteosarcoma.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
TT designed the study, carried out most experiments and drafted the manuscript.

SK made a substantial contribution to critical reading. AT performed single-cell sorting. MM and MK participated in the preparation of patients' samples. SK, TW, MK and SN contributed to collecting patients' samples with the informed consent. SK, TT, TW, TY and NS participated in its design and coordination. All authors read and approved the final manuscript.

Additional material

Additional file 1
*Sequences and binding affinities of PBF-derived peptides with HLA-A*0201 binding motif. *Binding score was determined by BIMAS HLA Peptide Binding Predictions. †The affinity of each peptide (50 I g/ml) was evaluated by a HLA class I stabilization assay.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1479-5876-7-44-S1.xls]

Additional file 2
Clinical picture and frequency of anti-PBF A2.2 peptide CTLs in PBMC of patients with osteosarcoma. P: primary tumor, M: metastatic tumor. †Frequency of anti-PBF A2.2 CTLs among CD8+ cells. ‡Parentheses indicate that the tumor had been resected at the time of blood sampling. §Magnetically separated CD8+ cells. Irradiated peptide-pulsed CD8- cells were used as stimulator.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1479-5876-7-44-S2.xls]

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Review Article

Molecular pathological approaches to human tumor immunology

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Research on human tumor immunology has greatly advanced in the past two decades. Many immunogenic tumor antigens have been identified, and some of these antigens entered in clinical trials. Consequently, it has been shown that these antigens can inhibit tumor growth in patients to some extent, indicating that they act as potent immunogenic therapeutic vaccines in cancer patients with malignancies originating from various tissues. These patients had antigen-specific cytotoxic T-lymphocyte (CTL) responses when assessed on tetramer, enzyme-linked immunospot (ELISPOT), T-cell clonotype and CTL induction efficiency. Thus, it has become clear that human tumor vaccines can evoke clinical and immunological anti-tumor responses in patients. The tumor regression effects of tumor vaccines, however, are generally low, and it is obvious that current vaccination protocols are generally too weak to provide substantial and satisfactory clinical benefits. This means that other drastic and more potent clinical and immunological protocols are required in cancer immunotherapy. To find such efficient protocols the basic immunological and biological properties of cancers must be investigated. In the present review the identification of human tumor antigens recognized on CTL and the clinical trials are introduced. Next, the most recent analysis of human cancer-initiating cell (cancer stem cell)-associated antigens is described. These antigens might be able to act as 'universal, general and fundamental' tumor antigens. Also present is the authors' recent study for increasing cross-presentation efficiency in dendritic cells and subsequent enhancement of human leukocyte antigen (HLA)-class I-restricted peptide antigenicity by using HSP90 and ORP150 molecular chaperones that act as endogenous Toll-like receptor ligands. In addition to the aforementioned manipulation of the positive loop of tumor immunity, it is necessary to regulate and intervene in the negative loop. In particular, the potential of the expression of HLA class I molecule regulation by epigenetic mechanisms will be discussed. Finally, the type of basic and clinical tumor immunology research highly required currently, and in the very near future, are described.

Key words: antigenic peptide, cross-presentation, epigenetics, human leukocyte antigen, heat shock protein, T cell, tumor immunology, tumor immunotherapy

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The exploitation of human cancer vaccines has been one of the main aims in basic cancer research and clinical studies. Although a huge number of immunological studies using animal tumor models has been reported, human tumor immunology research has advanced since the first human melanoma tumor antigen recognized by CD8 (+) cytotoxic T lymphocytes (CTL) was identified in 1992 by van der Bruggen *et al.*[1] In the past decade many such melanoma tumor antigens and their peptides presented by each human leukocyte antigen (HLA) allele have been discovered, and subsequently many tumor antigens of epithelial cancer origin have also been identified. These antigens were found using molecular cloning of tumor antigens with human autologous pairs of established tumor lines and CTL clones, reverse immunology and complementary DNA microarray devices. Using antigenic peptides derived from tumor protein antigens or protein antigens themselves as anti-tumor vaccines, clinical trials for tumor immunotherapy were subsequently performed in many institutes and hospitals in the USA, Europe and Japan.[2–11]

When these vaccine candidates were injected into patients without any addition of adjuvants, there were no overt side-effects or toxicity, but clinical responses were generally not strong except for a few cases.[5] Immunological monitoring using tetramer, enzyme-linked immunospot (ELISPOT), CTL induction efficiency, and T-cell receptor clonotype expansion has indicated that in some cases, but not many, there is a positive correlation between clinical and immunological responses.

These observations suggested that tumor antigenic peptides could work as anti-cancer vaccines in tumor immunotherapy as well as for immunoprophylaxis without severe adverse effects. It is also highly likely that these anti-cancer vaccines can be used for strong anti-cancer therapeutic regimens if combined with adequate immunostimulatory adjuvants, including dendritic cells (DC). Along with these trends in human tumor immunology research, a certain European Union-based pharmaceutical company has already begun to undertake commercialization of tumor vaccines for patients with diseases such as lung cancers and gliomas.

Immune responses generally involve both positive and negative regulation, and this has proved to be true for tumor immunology. Current and future tumor immunology studies require much deeper understanding of these conflicting aspects. Obviously, there needs to be an efficient link between innate and acquired (specific) immunity. Efficient activation of antigen-presenting cells (APC), particularly DC, is indispensable for the ample induction of antigen-specific CTL. Meanwhile, although the role of CD4+, CD25+, Foxp3+ regulatory T cells (Treg) was investigated to a certain extent in human cancers, a large part of the negative regulation of tumor immunity remains to be elucidated.[12–15]

In our laboratory at Sapporo Medical University, for the past 10 years we have principally investigated the molecular nature and pathological characteristics of human tumor antigens that are recognized by CTL. In collaboration with clinical departments, phase I clinical trials for assessing the toxicity and immunotherapeutic potential of antigenic peptides have been performed.[3,4,10,11] Along with these studies, others were conducted on positive regulation of tumor immunity with chaperone-assisted peptide vaccination and on negative regulation, namely, immunoescape by downregulating HLA class I. In the present article, the current status of these studies is reviewed, and future perspectives for human cancer immunotherapy and prophylaxis are discussed from the viewpoint of molecular pathology.

POSITIVE REGULATION OF HUMAN TUMOR IMMUNITY

Melanoma antigens recognized by CTL and immunotherapy

Various human melanoma antigens that are recognized by CTL have been identified since the discovery in 1992,[1] and more than 20 melanoma antigens have been reported, as shown in Table 1.[16,17] Some antigens and HLA class I-restricted antigenic peptides underwent clinical trials, and their side-effects and clinical and immunological responses were assessed. At the first stage of the trials there were positive clinical results in Europe and the USA. In 2003, however, Rosenberg *et al.* reported on a large number of melanoma patients and found that <5% of patients who received peptide vaccines such as gp100, Mart1 and tyrosinase plus interleukin (IL)-2 had a complete response.[5]

This led to skepticism about immunotherapy with peptide cancer vaccines. Furthermore, immunological monitoring for peptide-specific CTL using tetramers and ELISPOT, which can detect peptide-specific precursor CTL and activated functional CTL, respectively, showed that the immunological response was not always parallel to the clinical response. Regardless of such problems, however, in 2006 a UK-based pharmaceutical company announced that a 3 year-long observation after melanoma antigen family A, 3 (MAGE-A3) vaccine inoculation indicated a 33% reduction of the postoperative recurrence in non-small lung cancers as compared with a placebo group. This observation provides strong hope for current and future immunotherapy, and has accelerated many different investigations for the establishment of human tumor immunotherapy and immunoprophylaxis.

Table 1 Melanoma antigens/peptides recognized by autologous CTL

Antigens	HLA	Peptides
Cancer-testis antigens		
BAGE	Cw16	AARAVFLAL
GAGE	Cw6	YRPRPRRY
MAGE-1	A1	EADPTGHSY
MAGE-3	A1	EVDPIGHLY
NY-ESO-1	A31	ASGPGGGAPR
Melanoma-melanocyte differentiation antigens		
MAERT-1/Melan-A	A2	AAGIGILTV
gp100 (pmel-17)	A2	LLDGTATLRL
Tyrosinase	A1	SSDYVIPIGTY
TRP-1 (gp75)	A31	MSLQRQFLR
TRP-2	A31	LLPGGRPYR
Mutated (unique) antigens		
β-Catenin	A24	SYLDSGIHF
MUM-1	B44	EEKLIVVLF
MUM-2	B44	SELFRSGLDSY
	C6	FRSGLDSYV
MUM-3	A28	EAFIQPITR
CDK-4	A2	ACDPHSGHFY
MART-2	A1	FLGGNEVGKTY
Over-expression antigens		
PRAME	A24	LYVDSLFFL
P15	A24	AYGLDFYIL

CTL, cytotoxic T lymphocyte; HLA, human leukocyte antigen.

Tumor antigens identified in Sapporo

In addition to melanomas, human tumor antigens of non-melanoma tumors such as colon, breast, lung, urinary tract, head and neck cancers and soft-tissue sarcomas have been analyzed extensively in various laboratories. Although the immunogenicity of these non-melanoma antigens was

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Table 2 Candidates for cancer vaccines identified in Sapporo

Tumors	Peptides	Proteins	HLA	Clinical study
Autologous system				
Stomach	YSWMDISCWI (F4.2)	c98	A31	
Osteosarcoma	CTACRWKKACQR	PBF	B55	
	AYRPVSRNI		A24	Scheduled
			A2	Scheduled
Reverse immunology				
Apoptosis-related				
Various	AYACNTSTL (survivin 2B80-88)			
		Survivin	A24	Phase I
Various	KWFPSCQFLL (L7)	Livin	A24	Phase I
Chromosome translocation				
Synovial sarcoma				
	GYDQIMPKK (B)	SYT-SSX	A24	Phase I
	GYDQIMPKI (K9I)	SYT-SSX	A24	Phase I
Bioinformatic immunology				
Various	RYAMTVWYF (HIFPH3-8)	HIFPH3		Scheduled
	VYVKGLLAKI (Cep55-10)	CEP55		
	NMVEGTAYL (AMARCR2)	AMACR		
	QYFKKIPIL (STEAP-B)	STEAP		
	N. D.	Lengsin		

AMACR, α-methylacyl-coenzyme A racemase; Cep55, centrosome protein 55 kDa; HIFPH3, hypoxia-inducible factor prolyl hydroxylase 3; HLA, human leukocyte antigen; Lengsing, lens protein with glutamine synthetase domain; STEAP, six-transmembrane epithelial antigen of prostate.

relatively weak as compared with that of melanoma-associated tumor antigens, a certain number of tumor antigens from these non-melanoma tumors were identified.

In our laboratory at Sapporo Medical University, as shown in Table 2, we have identified tumor antigens using several different experimental systems, namely (i) autologous established tumor line-CTL pairs; (ii) reverse immunology; and (iii) bioinformatics. In established autologous tumor line-CTL pairs, two tumor antigen genes, C98[18,19] and papilloma binding factor (PBF),[20–24] were identified from gastric signet ring cell cancer and osteosarcoma, respectively. Using reverse immunology the inhibitor of apoptosis protein family members survivin and livin were shown to be highly immunogenic tumor antigens in addition to that fact that these two antigens were selectively expressed in tumor tissues of different tissue origins but not in normal counterparts.[25–30] In particular, the expression of survivin protein was very high: >90% of colon, lung, pancreas and breast primary cancers had high expression of this protein.[25] Furthermore, HLA-A24-restricted survivin 2B80-88 nonamer peptide, which was derived from the survivin splicing variant survivin 2B from cancer patients, appeared to have strong immunogenicity when assessed for CTL induction efficiency, tetramer CTL frequency and on ELISPOT.[26]

Meanwhile, HLA-A24-restricted immunogenic peptide from immunologically non-self SYT-SSX fusion protein of synovial sarcomas was also identified. This peptide, designated SYT-SSX B peptide, is derived from the fusion point at the SYT-SSX t(X;18) (p11;q11) chromosomal translocation. When assessed in a peptide-specific tetramer study, it was confirmed that this peptide-specific CTL was found in SYT-SSX chromosomal translocation (+) synovial sarcoma patients' peripheral blood lymphocytes with a relatively higher frequency than in non-synovial sarcoma patients.[31,32] Subsequently, we also identified variant antigenic peptide K9I, in which the C-terminal ninth agretope residue required for the binding to HLA class I molecules was substituted to isoleucine from lysine.[33]

Very recently, using bioinformatics approaches, several interesting, highly immunogenic tumor antigens have been found. Hypoxia-inducible factor prolyl hydroxylase 3 (HIFPH3), centrosome protein 55 kDa (Cep55), α-methylacyl-coenzyme A racemase (AMACR), six-transmembrane epithelial antigen of prostate (STEAP) and lens protein with glutamine synthetase domain (Lengsin) are expressed most preferentially in renal cell, colon, prostate, oral and lung cancers, respectively.[34,35] We routinely developed mAb to each of all these tumor antigens, through work on paraffin-embedded tissue sections, and subsequent immunohistochemical data indicated that the expression status of antigens in tumors was highly parallel to the induction efficiency of CTL from the patients. Therefore it is highly likely that these tumor antigens could work as tumor vaccines.

Clinical trials

In 2003 the General Surgery, Oral Surgery, Urology and Orthopedic Surgery Departments of Sapporo Medical University Hospital and affiliated hospitals began phase I clinical trials with survivin 2B80-88 peptide and SYT-SSX B peptide.

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Table 3 Summary of phase I clinical trials

Tumors	No. patients	Side-effect	Clinical response	
			Tumor marker/SD *n* (%)	RECIST *n* (%)
Survivin 2B peptide				
Colon	15	no	7/15 (47)	1/15 (7)
Breast	12	no	1/17 (6)	0/17 (0)
Lung	10	no	5/10 (50)	0/10 (0)
Oral cavity	9	no	3/11 (27)	1/11 (9)
Urinary bladder	3	no	2/3 (67)	1/3 (33)
Lymphoma	2	no	1/2 (50)	1/2 (50)
SYT-SSX B peptide				
Synovial sarcoma				
	6	no	1/6 (17)	0/6 (0)

RECIST, Response Evaluation Criteria in Solid Tumors; SD, stable disease.

As shown in Table 3, the HLA-A24-restricted survivin2B peptide was given s.c. to patients six times or more at biweekly intervals for colon, breast, lung, oral cavity, urinary bladder cancers and lymphomas. There were no severe side-effects and, clinically, certain patients with colon and lung cancers showed reductions in tumor markers (minor response) and growth arrest (stable disease) as assessed on CT.[9–11] These effects, however, were not strong enough for the clinical requirements as judged by the criteria of cancer chemotherapy. When assessed with the Response Evaluation Criteria in Solid Tumors (RECIST), which requires >30% regression of tumors on CT, only one patient each out of 15 with colon cancers, three with urinary bladder cancers and two with lymphomas had a positive clinical response. Meanwhile, breast cancer patients exhibited rare clinical responses to the current protocol of survivin2B80-88 administration. Interestingly, however, these breast cancer patients showed enhancement of peptide-specific CTL frequency, indicating a high possibility that certain negative immunoregulatory mechanisms existed in the breast tumors.

Our group studied immunological characteristics of products derived from chromosomal translocation that occurred in synovial sarcomas, and identified immunogenic SYT-SSX B peptide in the context of the HLA-A24 molecule. This peptide was also entered into clinical trials. Consequently, administration of this SYT-SSX translocation-derived B peptide to six synovial sarcoma patients also produced no side-effects, but resulted in little clinical response.[7,8]

These data strongly indicated that the current protocol of single use of survivin 2B80-88 peptide alone or SYT-SSX B peptide alone was not sufficient for clinical application. We also studied the immunological responses of almost all the patients. With survivin 2B80-88 peptide vaccinations, certain patients exhibited approximately 10–15-fold increases in the CTL precursor frequency and number of functional CTL as determined on peptide-specific tetramer and ELISPOT, respectively. There was no clear statistical correlation, however, between immunological and clinical responses.[10,11] Despite such observations it was also evident in colon cancer patients given the survivin 2B80-88 peptide vaccine that the tetramer-detected survivin 2B80-88 peptide-specific CTL precursor frequency was obviously increased in accordance with peptide plus incomplete Freud adjuvant (IFA) compared to the peptide alone, and it was interesting that the peptide plus IFA plus interferon (IFN)-α resulted in the strongest enhancement of CTL precursor frequency. In this protocol we used IFN-α for maturating DC,[36,37] and on this protocol patients displayed more overt clinical responses as well, suggesting that additional clinical trials should be undertaken for exact assessment in a larger number of patients.

Side population technology and identification of cancer-initiating cell tumor antigens

It has long been disputed whether tumor cells are homologous. Many recent studies indicated that tumor cells are not homogeneous but rather different in several biological aspects such as growth potential, differentiation status and invasive or metastatic characteristics. Histologically it is obvious that tumors are composed of very different cellular and structural morphologies with diverse neoplastic characteristics. This complex nature has also been one of the main interests for pathologists for many years, and two models for the origin of tumor cell populations, stochastic and hierarchic, have been advocated, as shown in Fig. 1. The stochastic model indicates that the tumor is composed of homogeneous cells, whereas the hierarchic model is of heterogeneous cells, and in this latter model there must be 'cancer stem' or 'cancer-initiating' cells in tumors.[38–40]

The concept of 'cancer stem cells' (CSC) or 'cancer-initiating cells' (CIC) is highly intriguing and important for development of efficient cancer immunotherapy and immunoprophylaxis protocols, because tumor antigens expressed in these cells must be the most suitable immunological

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