# Novel spliced form of a lens protein as a novel lung cancer antigen, Lengsin splicing variant 4

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**A glutamine synthetase I family protein, Lengsin, was previously identified as a novel lens-specific transcript in the vertebrate eye. In this report, we show for the first time that Lengsin is a novel tumor-associated antigen expressed ectopically in lung cancer. Interestingly, a novel spliced form of human Lengsin termed 'splicing variant 4', gaining exon 3 that codes extra 63 amino acids, is the dominant transcript form in lung cancer cells. Lengsin mRNA could be detected in 7 of 12 (58%) lung cancer cell lines and 7 of 7 (100%) surgically resected lung cancer tissues. On the other hand, Lengsin transcripts could not be detected in normal major tissues or in other cancer cell lines, including melanoma, colorectal carcinoma, breast carcinoma and hepatocellular carcinoma. In addition, knockdown of Lengsin mRNA with RNAi caused cell death and a decrease of cell viability, suggesting that Lengsin has some essential role in cell survival. Since the lens is an immune-privileged site, we regard Lengsin as a highly immunogenic cancer antigen. Anti-Lengsin autoantibodies were detectable in sera of lung cancer patients, although these patients did not show any lens-related disturbances. Hence, Lengsin splicing variant 4 might be an immunogenic lung cancer-specific antigen that is suitable as a diagnostic marker and for molecular targeting therapy, including immunotherapy. (*Cancer Sci* 2009; 100: 1485–1493)**

Lung cancer is one of the most common malignancies and has high mortality rates in industrial countries.[1] Despite recent progress in chemotherapeutic, radiotherapeutic and surgical treatments, the five-year survival rate of lung cancer patients still remains low, especially in advanced cases. Recently, it was reported that antigen-specific cancer immunotherapy had a partial antitumor effect against lung cancer, and that antigen-specific cancer immunotherapy might be a possible novel treatment for the disease.[2,3] However, candidates for potent immunogenic lung cancer antigens are few at present, and exploitation of such immunogenic lung cancer antigens is highly needed.

Several methods to identify tumor-associated antigens (TAAs) have been reported; among them, microarray screening is a powerful tool to screen tumor-specific genes.[4] We identified several genes expressed preferentially in cancer tissues, but not in normal tissues, with gene chip microarray screening using the GeneChip Human Genome U133 Array Set (Affymetrix, Inc., Santa Clara, CA), which contains approximately 39 000 genes. With this screening, we isolated several genes, including Lengsin, that were overexpressed ectopically and specifically in lung cancers. Lengsin, in the glutamine synthetase I (GSI) superfamily, was previously reported to be a constitutive lens-specific protein with unknown function, although some studies suggested it might have chaperone-like activity.[5–7]

In this study, to evaluate the potency of Lengsin as a molecular target for immunotherapy of lung cancer, we examined expression profiles of Lengsin in lung cancers and normal tissues. We also analyzed cell viability in Lengsin knockdown cells and anti-Lengsin autoantibodies in sera from lung cancer patients. Taken together, our present data suggest that Lengsin may act as a novel immunogenic tumor antigen in lung cancer. We will discuss the immunobiological significance of the lens-related antigen in ocular disease and cancer immunotherapy.

## Materials and Methods

**Human cell lines and culture media.** Lung adenocarcinoma cell lines LHK2 and LNY1 and breast carcinoma cell line HMC2 were established in our laboratory. Lung squamous cell carcinoma cell lines Sq-1 and Sq-19, lung adenocarcinoma cell lines 1-87 and 11-18, lung large cell carcinoma cell line 86-2 and lung small cell carcinoma cell lines Lu65, S2 and LK79, were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). Colon carcinoma cell line HCT15, pancreatic carcinoma cell lines PK8, PK45, CFPAC and Panc-1 were kind gifts from Dr K Imai (Sapporo, Japan). Lung small cell carcinoma cell line Lc817 and hepatocellular carcinoma line CHC32 were purchased from the Japanese Cancer Research Resources Bank (Osaka, Japan). Colon carcinoma cell line KM12LM was a kind gift from Dr K Itoh (Kurume, Japan). Colon carcinoma cell lines Colo205 and WiDr, lung adenocarcinoma cell line A549, breast carcinoma cell line MCF7 and embryonic kidney cell line HEK293T were purchased from American Type Culture Collection (Manassas, VA). Melanoma cell lines 888mel and 1102mel were kind gifts from Dr FM Marincola (National Cancer Institute, Bethesda, MD). All of these cells were cultured in 90% DMEM (Sigma-Aldrich, St. Louis, MO) with 10% heat-inactivated fetal bovine serum (Filtron, Brooklyn, NSW, Australia) at 37°C in a humidified 5% $CO_2$ atmosphere.

**Tissue and serum samples.** Seven pairs of lung cancers and the corresponding non-neoplastic lung tissues were obtained from surgically resected tissues removed at Kushiro City General Hospital. The histological types of the seven cancer tissues were: squamous cell carcinoma, cases #1, #4, #7; adenocarcinoma, cases #2, #5, #6; and large cell carcinoma, case #3. Thirty-four formalin-fixed, paraffin-embedded lung adenocarcinoma tissues for immunohistochemical staining were obtained from surgically resected specimens at Sapporo Medical University Hospital. Forty-two serum samples for enzyme-linked immunosorbent assay (ELISA) were collected from 23 lung cancer patients and 19 healthy donors at Sapporo Medical University Hospital,

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Sapporo Railway Hospital, Sapporo Tokushukai Hospital and Kitahiroshima Hospital. Tumor staging was determined according to the UICC classification.[8] We obtained informed consent from all patients and healthy donors according to the guidelines of the Declaration of Helsinki.

**RT-PCR analysis.** Human Multiple Tissue cDNA Panels I and II, and the Human Fetal Multiple Tissue cDNA Panel (Clontech, Mountain View, CA, USA) were used as templates of normal tissue cDNA and normal fetal tissue cDNA. Total RNA was isolated from cultured cells and tumor tissues using the RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNAs were synthesized as described previously.[9] PCR amplification was done in 20 μL of PCR mixture containing 0.25 μL of the cDNA mixture, 0.1 μL of Taq DNA polymerase (Qiagen), and 12 pmol of primers. The PCR mixture was initially incubated at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. We designed two primer pairs for specific detection of Lengsin. The sequences of primer pair I were 5′-CCCTGCTTTCTGCTTTCATC-3′ as a sense primer and 5′-AATAACGCTTTCGGCAGCTA-3′ as an antisense primer. The expected size of the PCR product with primer pair I was 507 bp. The sequences of primer pair II were 5′-GGGAGAAA CGGATATGTCCA-3′ as the sense primer and 5′-CAGTCAC AGTGAAGGTATCA-3′ as the antisense primer. The expected size of the PCR product with primer pair II for Lengsin_wild type (Lengsin_wt) was 395 bp and that for Lengsin splicing variant 4 (Lengsin_vt4) was 584 bp. As an internal control, G3PDH expression was detected using sense primer 5′-ACCACAGTCCATGCCATCAC-3′ and antisense primer 5′-TCCACCACCCTGTTGCTGTA-3′ with an expected PCR product of 452 bp.

**Western blot analysis and immunohistochemical staining.** Western blot analysis using mouse antihuman Lengsin mAb clone #517 (established in our laboratory) was performed as described previously.[10]

Immunohistochemical staining was done on formalin-fixed, paraffin-embedded sections as described previously.[11]

**Small interfering RNA transfection.** Lengsin small interfering RNA (siRNA) duplexes were designed and synthesized using the BLOCK-it RNAi designer system (Invitrogen, Palo alto, CA, USA). The oligonucleotide encoding Lengsin siRNA was 5′-CCTAATGCCAGAGTTATCAACCTTT-3′. It targeted a common sequence between Lengsin_wt and Lengsin_vt4 transcripts. Cells were seeded at 50% confluence, and transfections were carried out using Lipofectamine 2000 (Invitrogen) in Opti-MEM according to the manufacturer's instructions.

**WST-1 assay.** WST-1 assay (Wako Chemicals, Osaka, Japan) was performed according to manufacturer's instructions. Forty-eight hours after post transfection of Lengsin siRNA, the cells were seeded in 96-well flat-bottomed plates ($1 \times 10^4$ in 100 μL of culture medium per well) followed by an additional 72-h incubation. Then, 10 μL of WST-1 solution was added into each wells, and the plates were incubated at 37°C for another 2 h. Absorbance was measured by a microplate reader at a wavelength of 450 nm with a reference wavelength of 655 nm. Each experiment was done independently in triplicate.

**Flow cytometry.** Five days after siRNA transfection, the cells were harvested and washed with PBS, followed by fixation with 70% ethanol overnight at –20°C. After washing with PBS, the cells were re-suspended in PBS containing 250 μg/mL RNase A (Sigma-Aldrich) for 30 min at 37°C and stained with 50 μg/mL propidium iodide (PI) (Invitrogen) for 10 min at 4°C in the dark. To calculate the percentage of cells in the sub-G1 phase, the results were analyzed by flow cytometry (FACS Calibur, Becton-Dickinson, Franklin Lakes, NJ) with CellQuest software analysis. The apoptotic cell rate was determined as the percentage of cells in the sub-G1 phase.

**ELISA.** Preparation of purified recombinant Lengsin was performed according to the method described previously.[12] To coat a 96-well plate with capture protein, purified recombinant Lengsin was diluted in 50 mM bicarbonate buffer (pH 9.5) to a final protein concentration of 5 μg/mL and placed in each well of the 96-well plates (Corning, NY) and incubated overnight at 4°C. After removing antigen solutions and three washes with PBS including 0.05% Tween 20 (PBS–T), plates were blocked with 1% BSA in PBS for 2 h at room temperature (RT). After emptying the wells and three washes with PBS–T, 100 μL of serum sample diluted (1:100) in PBS–T including 0.5% of BSA was added to each well and incubated for 1 h at RT. Then, samples were removed and the wells were washed three times with PBS–T, followed by incubation for 30 min with two thousand dilution of rabbit antihuman IgG conjugated with horseradish peroxidase (Dako, Carpinteria, CA). After removing the antibody solution, the wells were washed three times with PBS–T, then each well was developed with ABTS peroxidase substrate (KPL, Gaithersburg, MD). After incubation for 15 min, absorbance was measured at a wavelength of 405 nm.

**Statistical analysis.** A Student's *t*-test was used to compare two groups. $P < 0.05$ was considered significant.

## Results

**Lengsin is preferentially expressed in lung cancer cell lines and human primary lung cancer tissues.** Novel TAAs are essential for the establishment of cancer vaccine therapies. For the identification of novel TAAs, we initially screened the gene chip microarray expression profile database of more than 700 malignant tissues including breast, colon, pancreas, renal cell, lung and gastric carcinomas. We chose 30 cancer overexpressed genes as TAA candidates (data not shown). Then, the mRNA expression profiles of the TAA candidates were confirmed by RT-PCR, and one of the lung cancer-associated antigens proved to be the lens-specific GSI superfamily member, Lengsin. For precise analysis of Lengsin mRNA expression in various types of cancer cells RT-PCR analysis was performed with Lengsin primer pair I. As shown in Fig. 1(a), Lengsin was expressed in only three of the six lung cancer cell lines, but not in the two melanoma, four colorectal carcinoma, two breast carcinoma, four pancreas carcinoma and one hepatocellular carcinoma cell lines. To test the expression of Lengsin in the four major histological types of lung cancer, 12 lung cancer cell lines were examined by RT-PCR (Fig. 1b). Lengsin was expressed in one of the two squamous cell carcinoma lines (Sq-19), three of the five adenocarcinoma lines (LNY1, A549, 1-87), one large cell carcinoma line (86-2) and two of the four small cell carcinoma lines (Lu65, LK79). Then, we analyzed Lengsin expression in primary lung cancer tissue specimens. As shown in Fig. 1(c), we could detect Lengsin mRNA in primary lung cancerous tissues in 7 of the 7 (100%) cases, but not in normal counterpart tissues. The expression profile of Lengsin mRNA was also assessed in normal adult and fetal tissue panels including heart, brain, placenta, lung, liver, kidney, skeletal muscle, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, large intestine and PBMCs (Fig. 1d). Lengsin mRNA could not be detected in mature adult tissues and fetal tissues with the exception of adult liver and placenta, although at very low levels. Thus, these data indicated that Lengsin mRNA was overexpressed specifically in primary lung cancer tissues as well as lung cancer cell lines with considerable frequency independent of the histological type, but not in major normal tissues.

**Novel Lengsin splicing variant 4 is the dominant form in lung cancer cell lines.** For precise analysis of the structure of Lengsin mRNA, we performed further RT-PCR analysis with an additional Lengsin primer pair located in the upstream of primer pair I (primer pair II, white arrow in Fig. 2a). As described above, we could detect a single band with primer pair I, whereas we detected two bands with primer pair II in lung cancer cell lines



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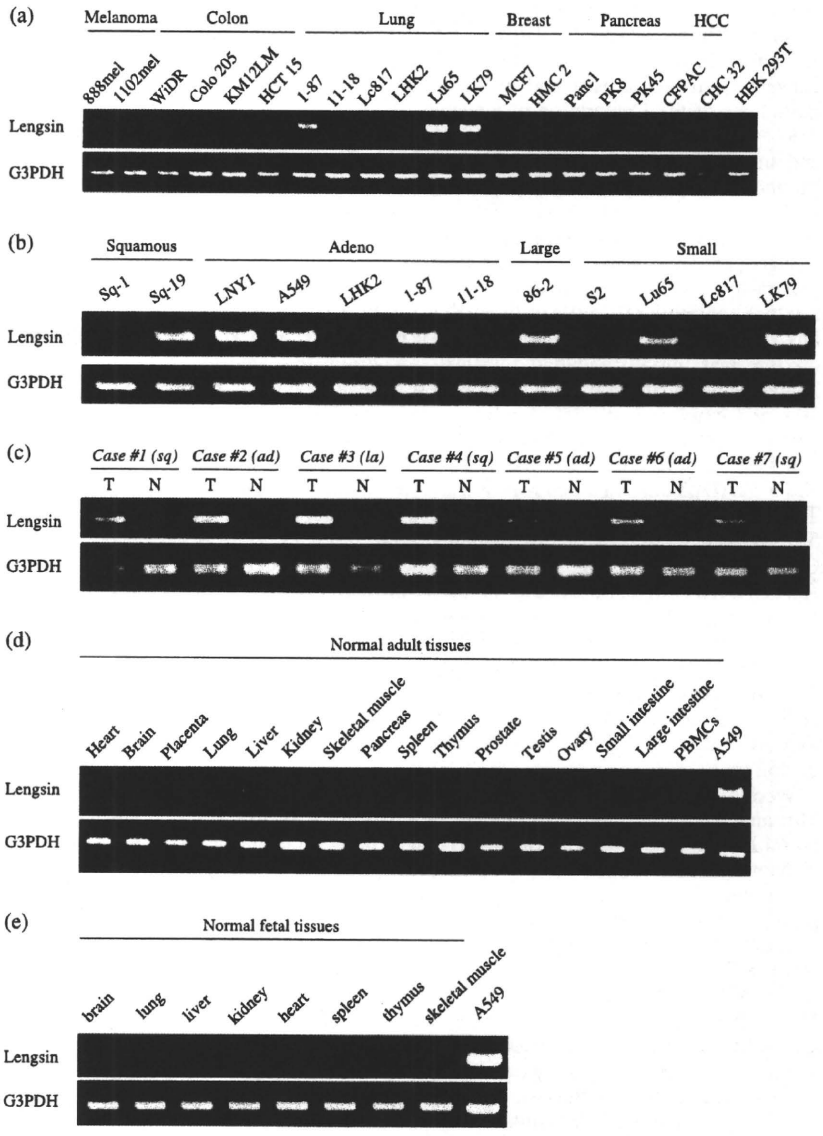


**Fig. 1.** Expression profiles of Lengsin as assessed by RT-PCR with Lengsin primer pair I in various cancer cell lines, lung cancer cell lines, human primary lung cancer tissues, normal adult tissues and fetal tissues. (a) Expression of Lengsin in various cancer cell lines. Cells include two melanoma cell lines (888mel, 1102mel), four colon cancer cell lines (WiDR, Colo205, KM12LM, HCT15), six lung cancer cell lines (1-87, 11-18, Lc817, LHK2, Lu65, LK79), two breast cancer cell lines (MCF7, HMC2), four pancreas cancer cell lines (Panc1, PK8, PK45, CFPAC), one hepatocellular carcinoma line (CHC32), and HEK293T. The expression of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was analyzed as an internal control. Melanoma, melanoma lines; Colon, colon cancer cell lines; Lung, lung cancer cell lines; Breast, breast cancer cell lines; Pancreas, pancreas cancer cell lines; HCC, hepatocellular carcinoma line. (b) Expression of Lengsin in lung cancer cell lines. Cells include two squamous cell carcinoma lines (Sq-1, Sq-19), five adenocarcinoma lines (LNY1, A549, LHK2, 1-87, 11-18), one large cell carcinoma line (86-2), and four small cell carcinoma lines (S2, Lu65, Lc817, LK79). Squamous, squamous cell carcinoma lines; Adeno, adenocarcinoma lines; Large, large cell carcinoma lines; Small, small cell carcinoma lines. (c) Expression of Lengsin in primary lung cancer (T) and non-cancerous tissues (N) including three squamous cell carcinomas (cases #1, #4, #7), three adenocarcinomas (cases #2, #5, and #6), and one large cell carcinoma (case #3). sq, squamous cell carcinoma; ad, adenocarcinoma; la, large cell carcinoma. (d) Expression of Lengsin in normal adult tissues and (e) fetal tissues, including heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, large intestine, and PBMCs. Lung adenocarcinoma line A549 was used as a positive control for Lengsin.

as shown in Fig. 2(c,d). With primer pair II, the estimated size of wild type Lengsin mRNA should be 395 bp, but the major band was located around 600 bp (Fig. 2d), suggesting the existence of a splicing variant. Therefore, we performed DNA direct sequencing of these two bands and found that the upper band corresponded to a novel spliced form, which we named splicing variant 4 (Lengsin_vt4), containing an additional exon 3 compared with the wild type (Fig. 2b). The lower weak band proved to be the wild-type form of Lengsin mRNA (Fig. 2b,d). Thus, these data indicated that a novel spliced form, Lengsin_vt4 was the major transcript in lung cancer cell lines.

**Detection of Lengsin protein in human lens and lung cancers by Western blot analysis and immunohistochemical staining.** To assess the Lengsin expression in lung cancer cells and tissues at the protein level, we generated novel anti-Lengsin mAb #517 suitable for Western blot analysis and immunohistochemical staining. The Lengsin-specific reactivity of mAb #517 was confirmed by Western blot analysis. We could detect a specific band with both anti-Lengsin mAb #517 and an anti-FLAG mAb in the HEK293T cell line transfected with expression vectors of FLAG-epitope-tagged Lengsin_vt4 (Fig. 3a), suggesting mAb #517 recognized Lengsin_vt4 protein specifically. The epitope of mAb #517 was located within exon 1 or exon 2 with further Western blot analysis using several deletion mutants (data not shown). Furthermore, we could detect Lengsin protein in human lens with mAb #517, but not with an isotype control mAb by immunohistochemical staining (Fig. 3b). Strong staining was seen in layers of secondary lens fibers, but not in the central region containing primary lens fibers (the lens nucleus). These findings are compatible with previous data of mouse Lengsin expression profiles.[7] The endogenously expressed Lengsin protein was also analyzed with Western blot analysis (Fig. 3c). The double-FLAG-tagged Lengsin_vt4 transfected 293T cells showed a specific band with mAb #517 as a positive control. A549 and 1-87 lung adenocarcinoma cell lines and LK79 small cell carcinoma line also showed mAb #517 specific band. Since these bands are located slightly lower than double FLAG tagged Lengsin_vt4 band, this difference might depend on the difference of double-FLAG-tag, which represents around 3 kDa. This protein expression profile was consistent with the mRNA expression. In addition, we performed immunohistochemical staining to assess the Lengsin protein expression *in vivo*. Thirty-four

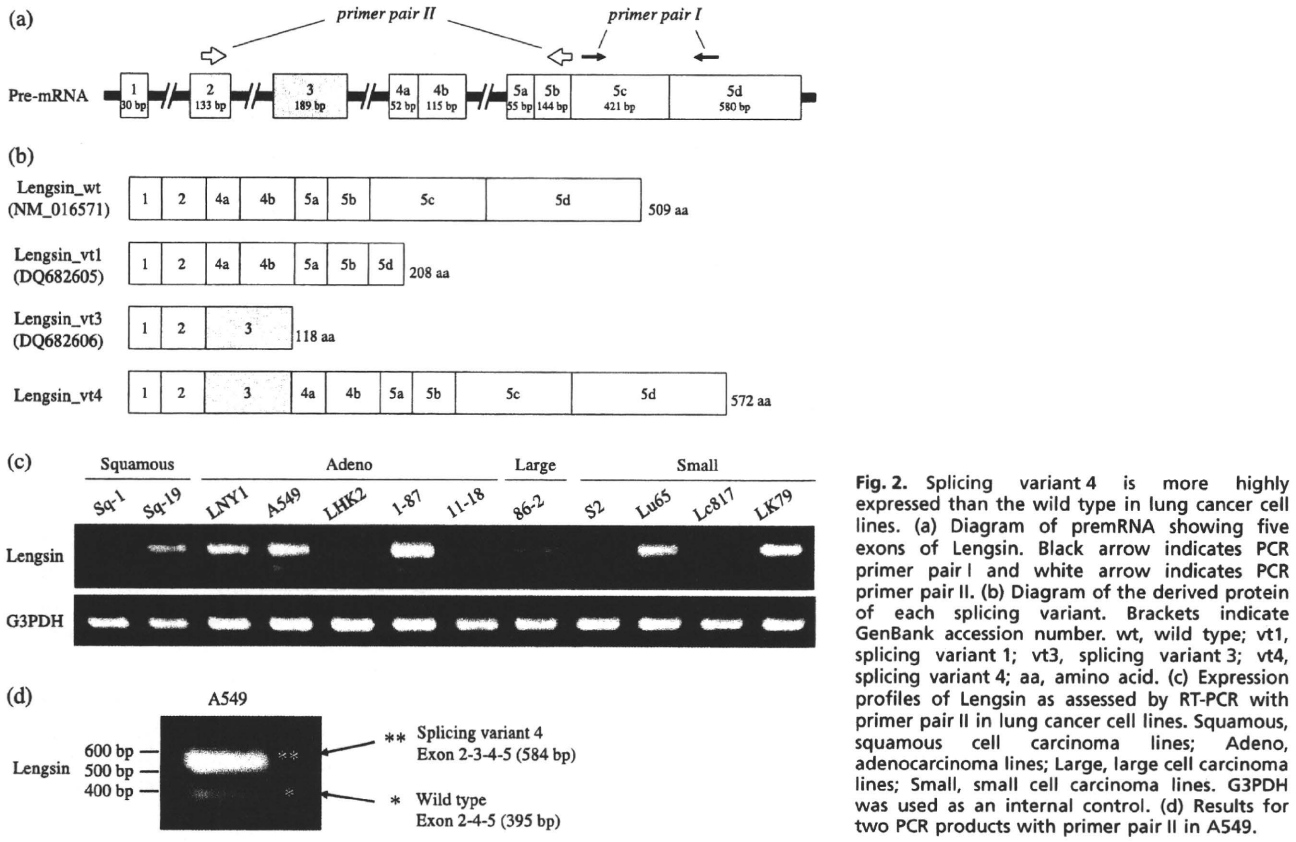


**Fig. 2.** Splicing variant 4 is more highly expressed than the wild type in lung cancer cell lines. (a) Diagram of premRNA showing five exons of Lengsin. Black arrow indicates PCR primer pair I and white arrow indicates PCR primer pair II. (b) Diagram of the derived protein of each splicing variant. 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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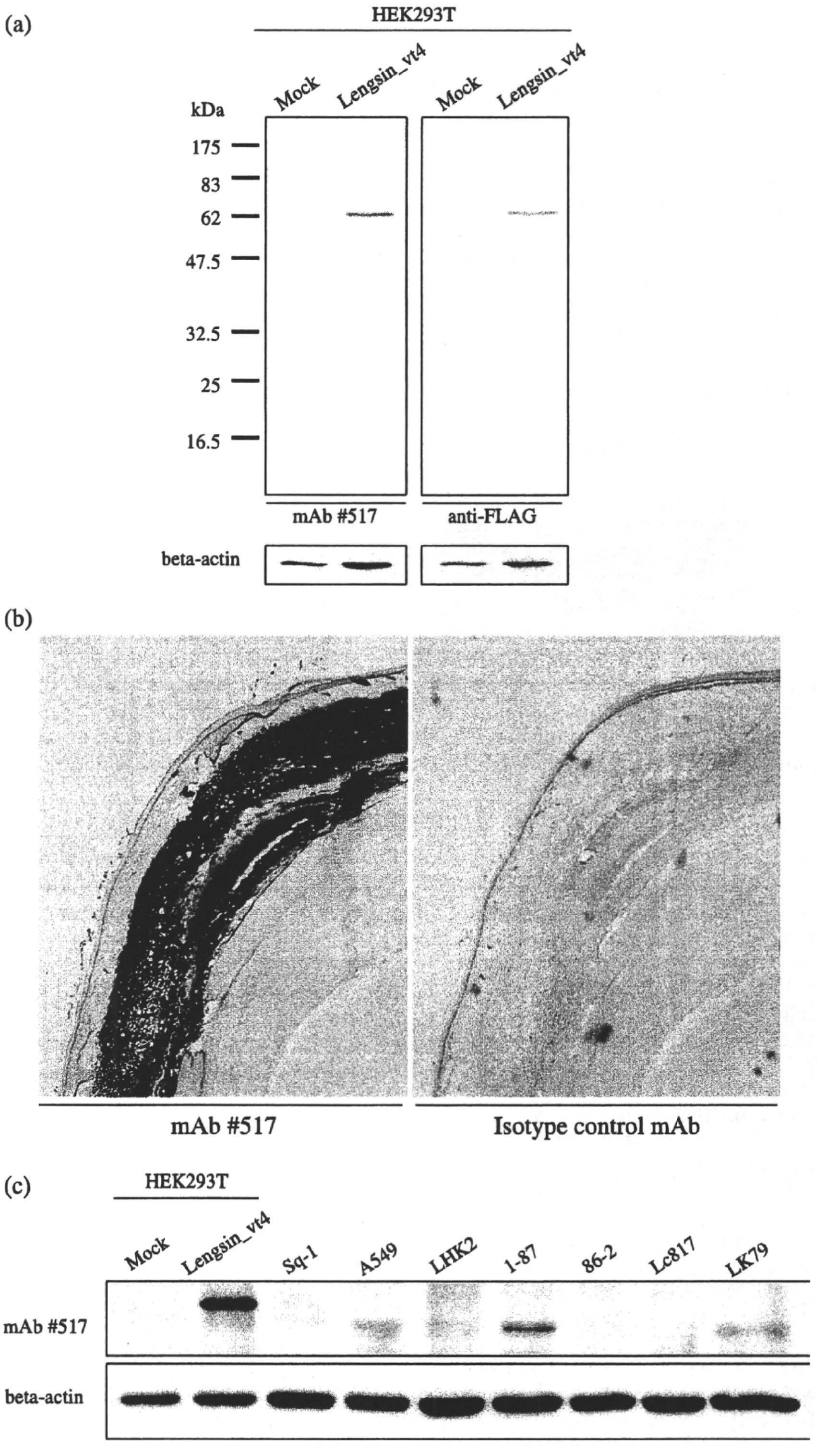


**Fig. 3.** Detection of Lengsin protein as assessed by Western blot analysis and immunohistochemical staining with anti-Lengsin mAb #517. (a) Specific detection of Lengsin protein in HEK293T cells transfected with expression vectors of FLAG-epitope-tagged Lengsin_vt4 as assessed by Western blot analysis 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.

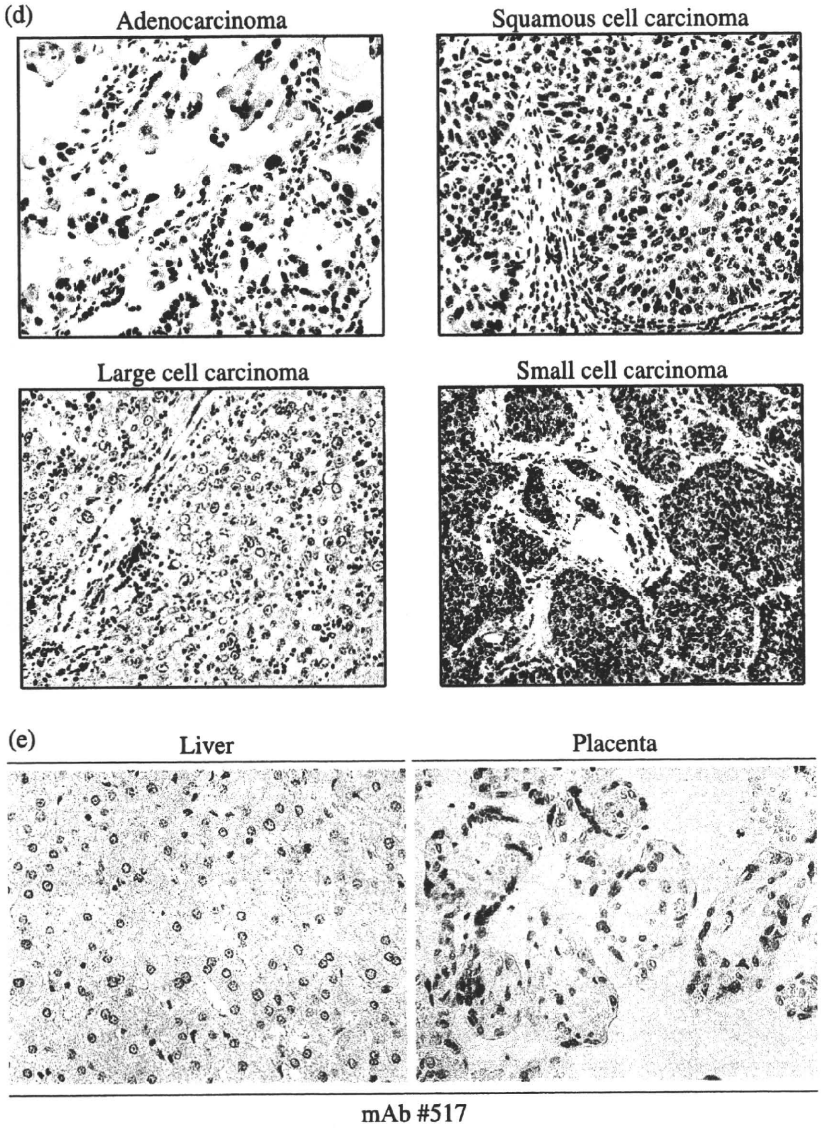


**Fig. 3.** *Continued.*

## 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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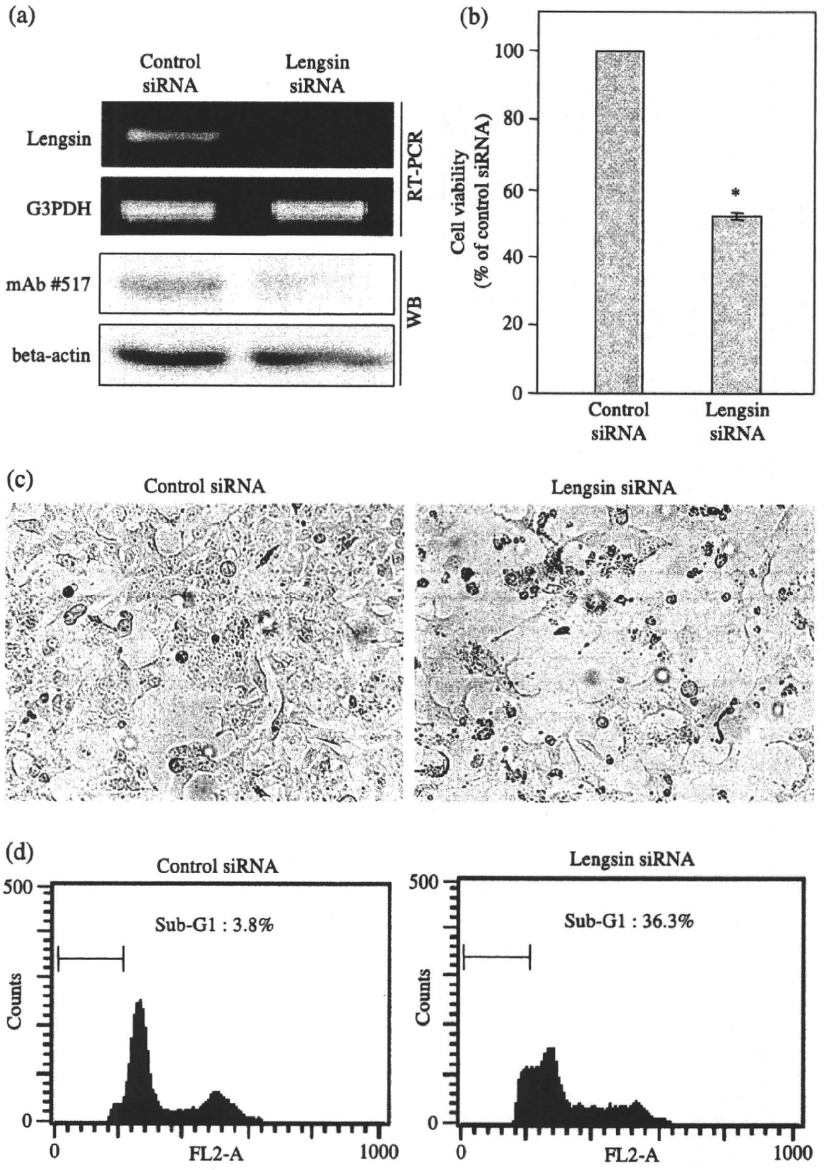


**Fig. 4.** Effect of Lengsin on cell viability in lung cancer cell line 1-87. (a) Gene silencing was performed using Lengsin siRNA. RT-PCR analysis was done using Lengsin primer pair I. G3PDH was used as an internal control. (b) WST-1 assay shows a decrease in the numbers of viable cells after knockdown 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]

| 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 | – | – |

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

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

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# 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 $2 \times 10^{-7}$ to $5 \times 10^{-6}$. 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 μm 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 $1 \times 10^5$–$2 \times 10^5$ [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 μg/ml). Peptide-pulsed PBMC were seeded at 2 × $10^5$ cells/200 μl/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 μl 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 μl 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 μl of the microculture pool, 100 μl 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 μl 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 μl 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 μl 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 μl 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 μl of AIM-V containing 10% HS, IL-2 (200 U/ml) and phytohemagglutinin-P (PHA; 7.5 μg/ml, Wako Chemicals, Osaka, Japan) in a total of 192 wells of 96-well round-bottom microplates. On day 7, 100 μl 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 μg/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 μl 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 μCi 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.

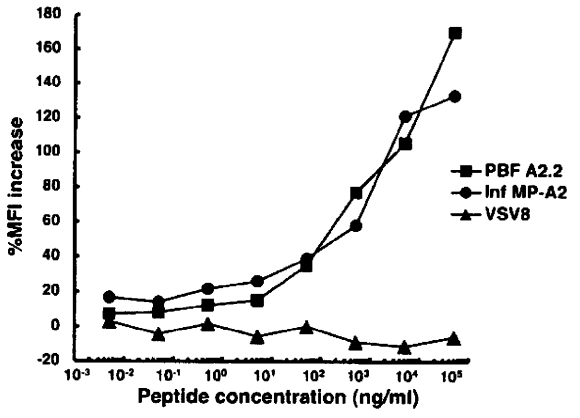


**Figure 1**
**Binding affinity of PBF A2.2 peptide to HLA-A*0201 molecules.** The affinities of three peptides, PBF A2.2, Inf MP-A2 and VSV8, were determined by HLA class I stabilization assay at the indicated concentrations.

### *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

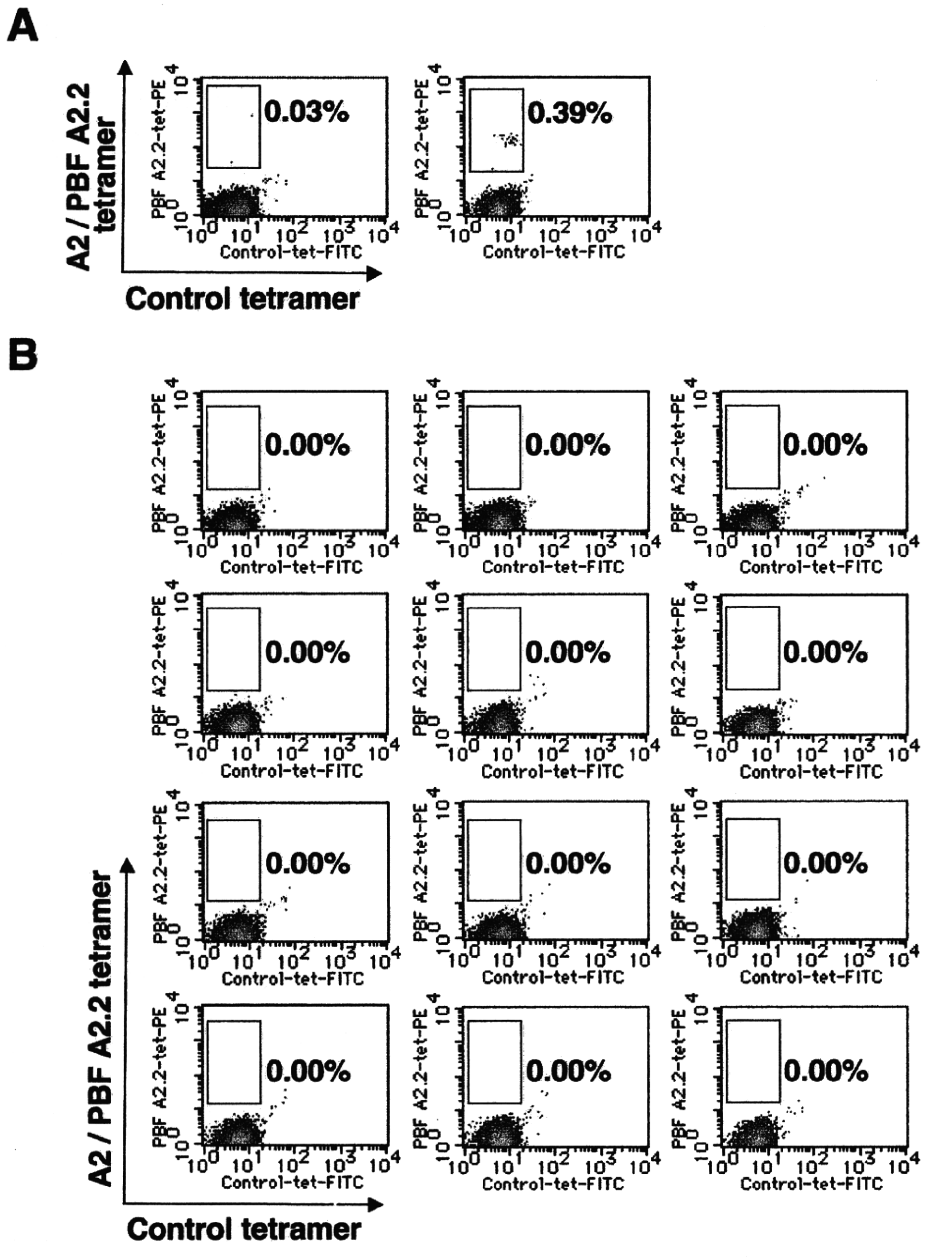


**Figure 2**
**Tetramer-based detection of PBF A2.2-specific T cells.** CD8+ T cells ($5.4 \times 10^6$) collected from Patient 4 were seeded into 36 wells at the concentration of $1.5 \times 10^5$ per well and cultured with peptide PBF A2.2 and cytokines. On day 21, tetramer analysis was performed. 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 $5.4 \times 10^6$ CD8+ cells. The frequency was calculated as $2/5.4 \times 10^6 = 3.7 \times 10^{-7}$.

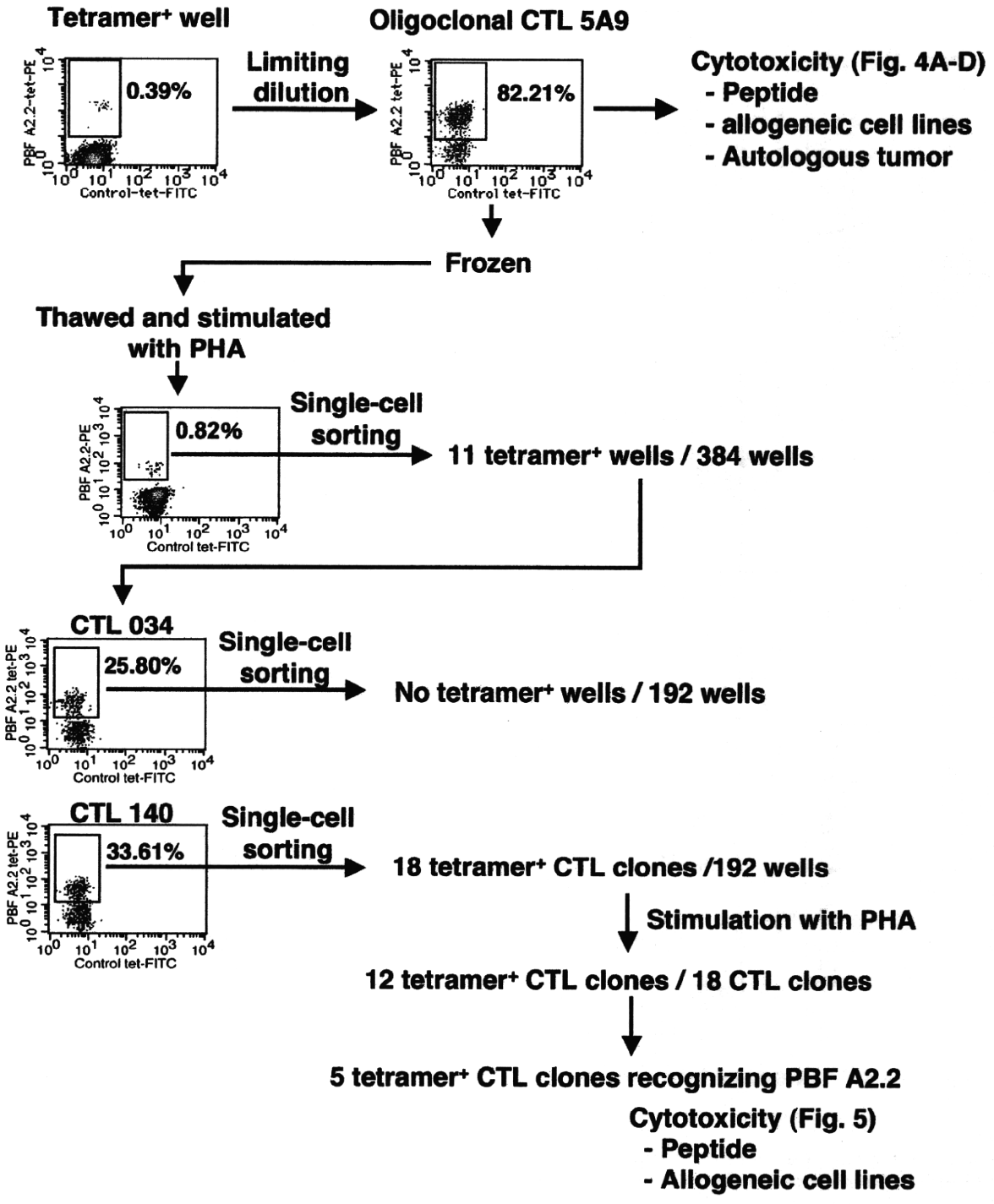


**Figure 3**
**Establishment of PBF A2.2-specific CTL line and CTL clones.**

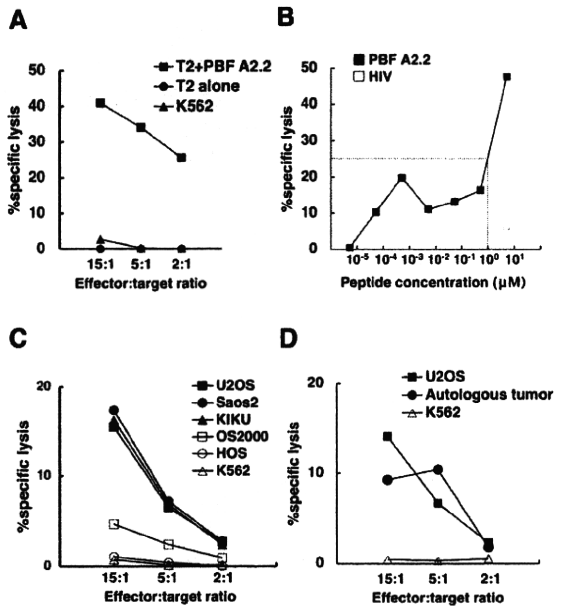


**Figure 4**
**Cytotoxic activity of A2/PBF A2.2 tetramer-positive CTL line 5A9. A.** The peptide-specific cytotoxicity of CTL 5A9 was determined using T2 and K562 cells in a 6 h standard $^{51}Cr$ release assay. T2 cells were pulsed with 50 μg/ml peptide PBF A2.2 or medium for 1 h at room temperature 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 μM 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

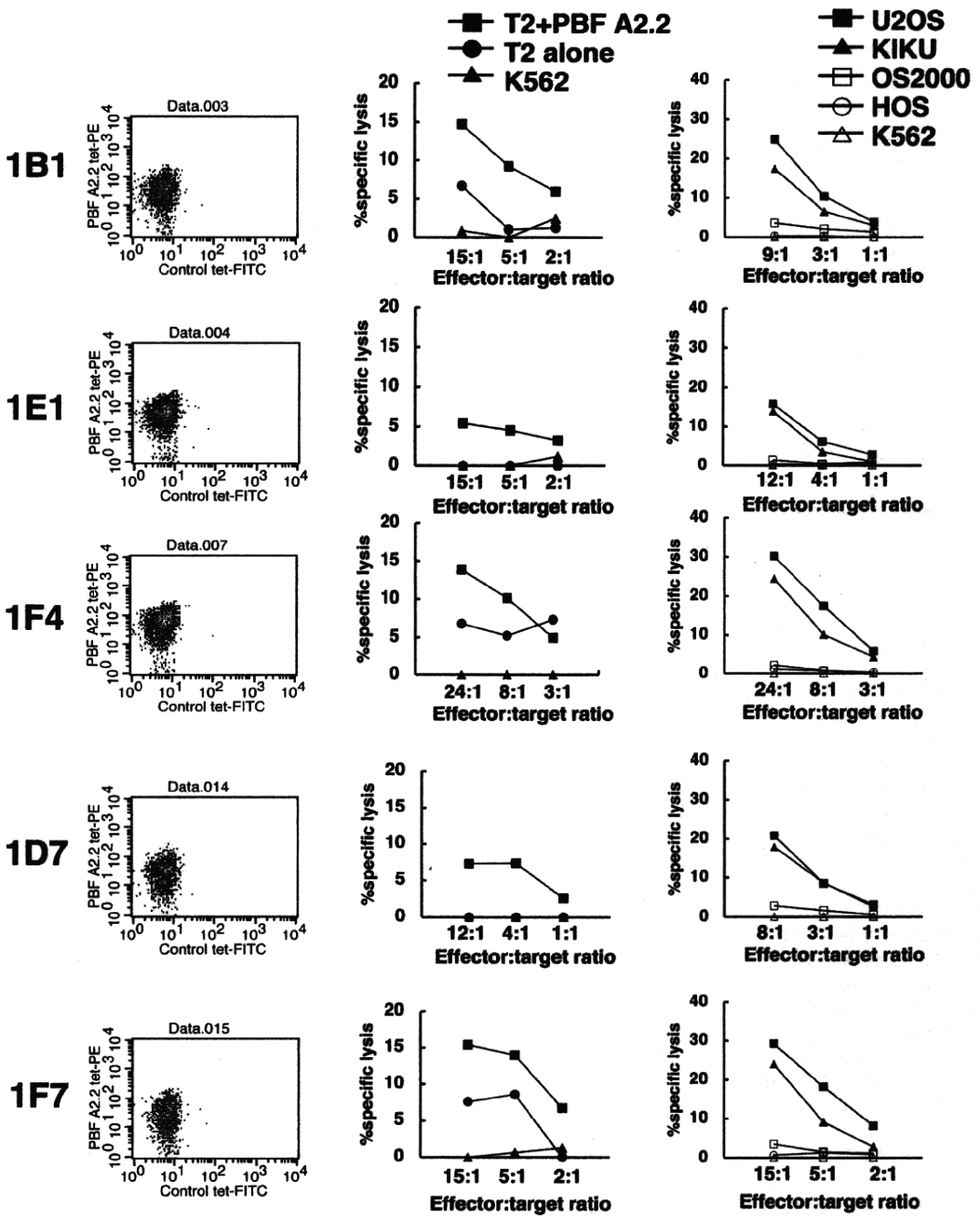


**Figure 5**

**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 $2 \times 10^{-7}$ to $5 \times 10^{-6}$ in patients with osteosarcoma. On the other hand, the frequency of the PBF A2.2-specific CTL precursors in healthy donors ranged from $1 \times 10^{-7}$ and $3 \times 10^{-7}$. In our previous study [17], the frequency of PBF A24.2-specific CTL precursors was between $5 \times 10^{-7}$ and $7 \times 10^{-6}$. 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 μg/ml) was evaluated by a HLA class I stabilization assay.*

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### 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.*

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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

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.

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