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

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(Received March 2, 2009/Revised April 8, 2009/Accepted April 8, 2009/Online publication May 19, 2009)

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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doi: 10.1111/j.1349-7006.2009.01187.x

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 (1×10^4 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

doi: 10.1111/j.1349-7006.2009.01187.x

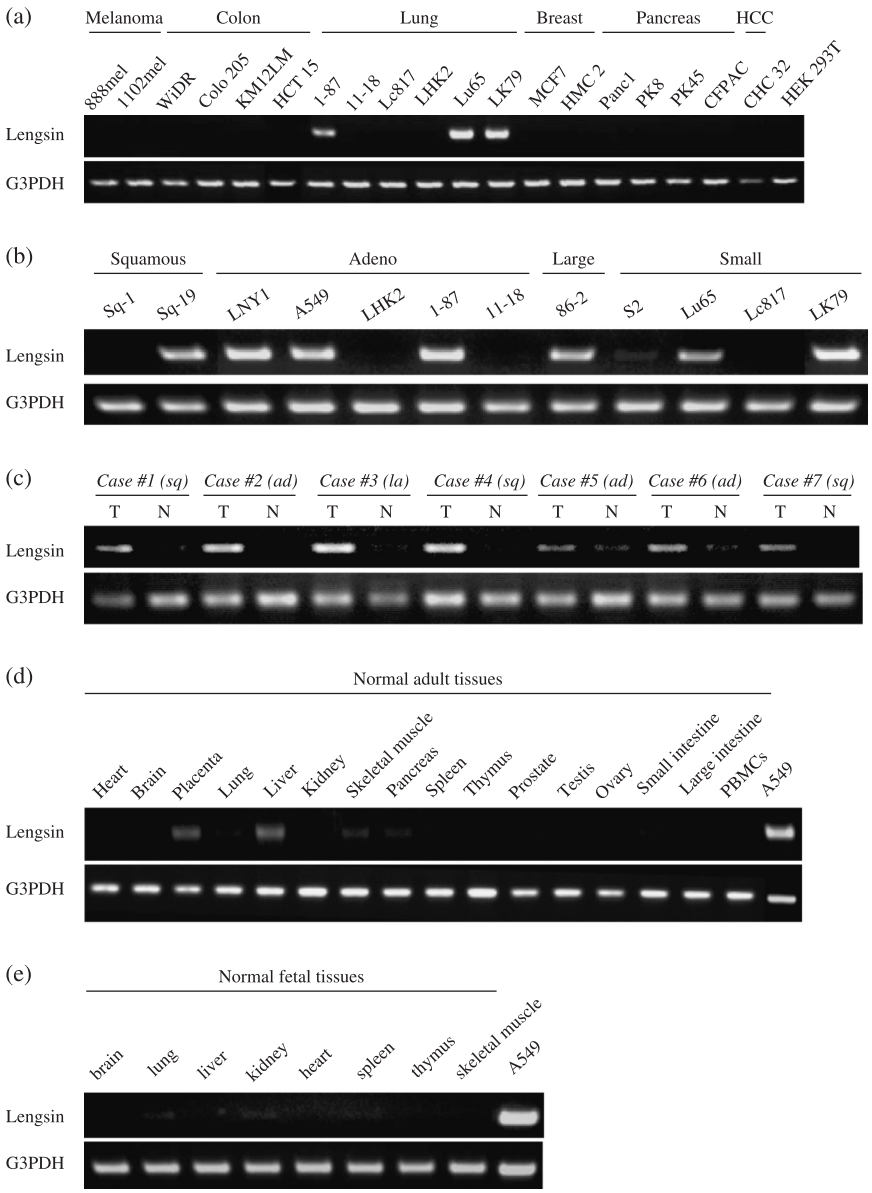

Fig. 1. Expression profiles of Lengsin as assessed by RT-PCR with Lengsin primer pair I in various cancer cell lines, lung cancer cell lines, human primary lung cancer tissues, normal adult tissues and fetal tissues. (a) Expression of Lengsin in various cancer cell lines. Cells include two melanoma cell 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

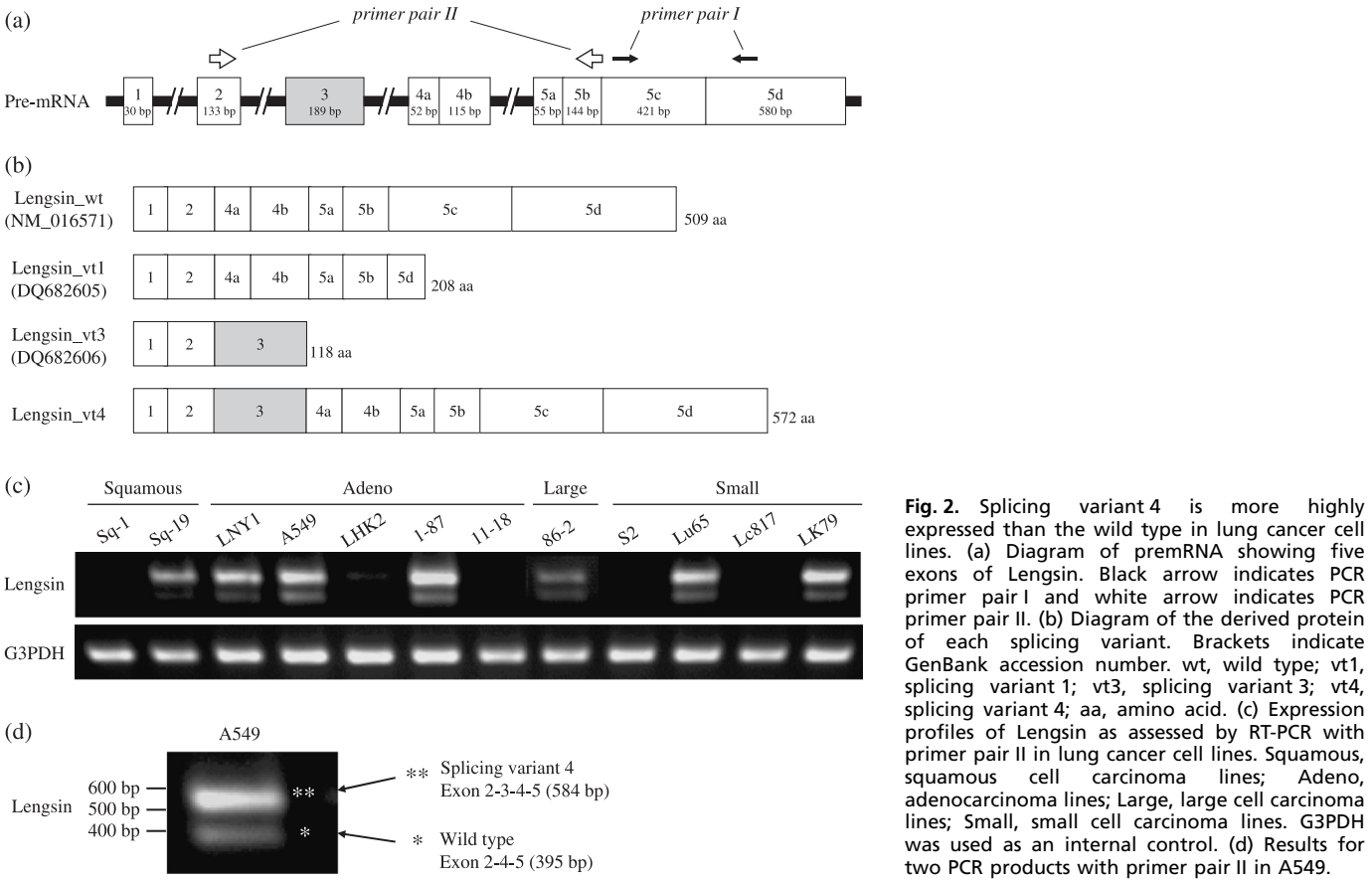

Fig. 2. Splicing variant 4 is more highly expressed than the wild type in lung cancer cell lines. (a) Diagram of premRNA showing five exons of Lengsin. Black arrow indicates PCR primer pair I and white arrow indicates PCR primer pair II. (b) Diagram of the derived protein of each splicing variant. Brackets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

doi: 10.1111/j.1349-7006.2009.01187.x

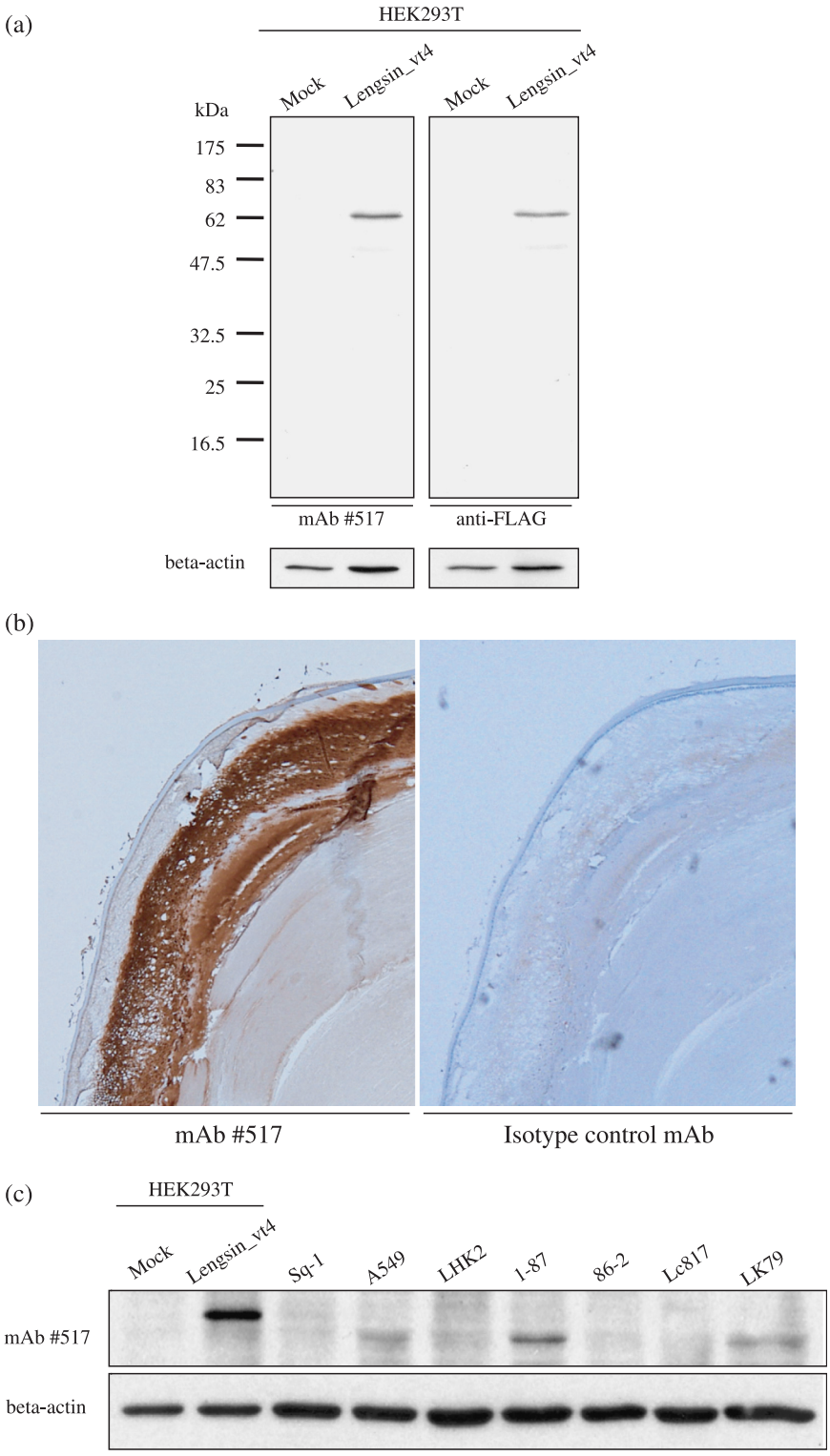

Fig. 3. Detection of Lengsin protein as assessed by Western blot analysis and immunohistochemical staining with anti-Lengsin mAb #517. (a) Specific detection of Lengsin protein in HEK293T cells transfected with expression vectors of FLAG-epitope-tagged Lengsin_vt4 as assessed by Western blot analysis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

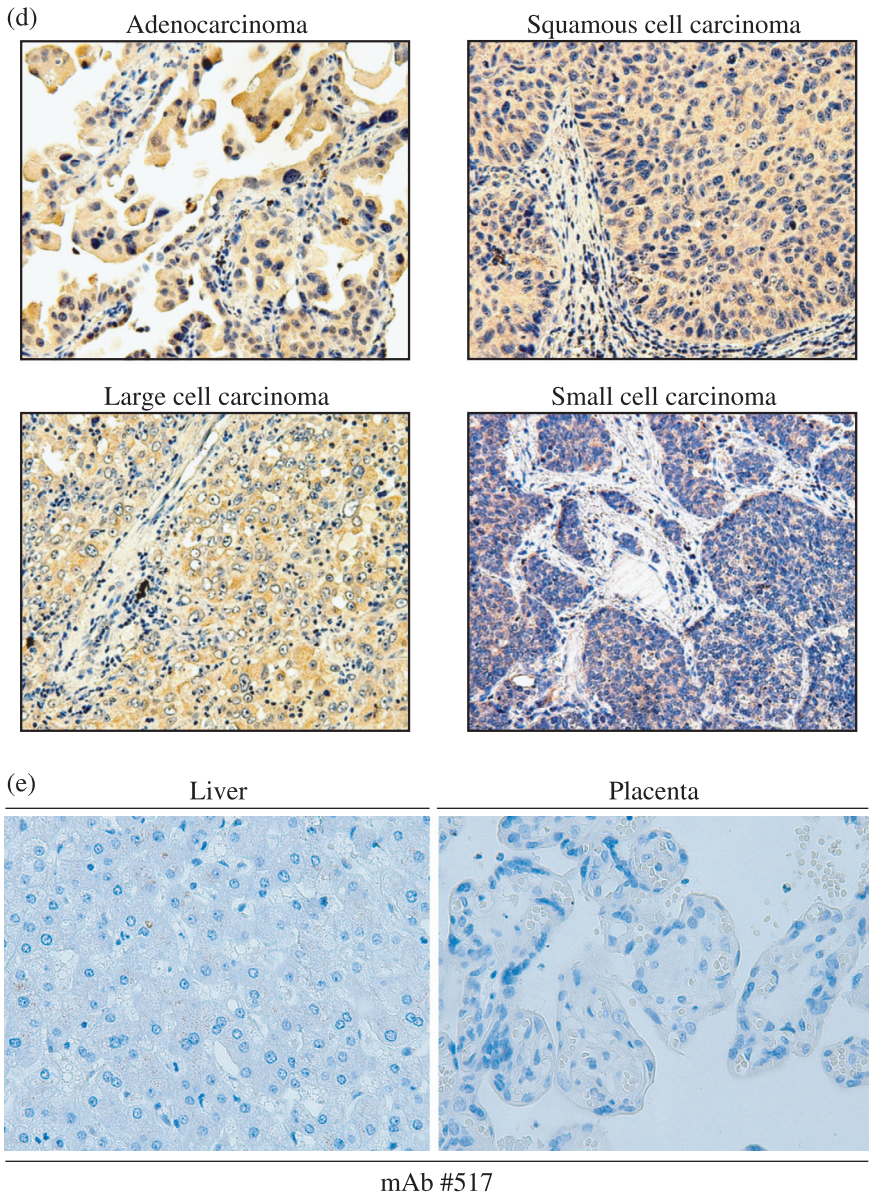

Fig. 3. *Continued.*

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.

doi: 10.1111/j.1349-7006.2009.01187.x

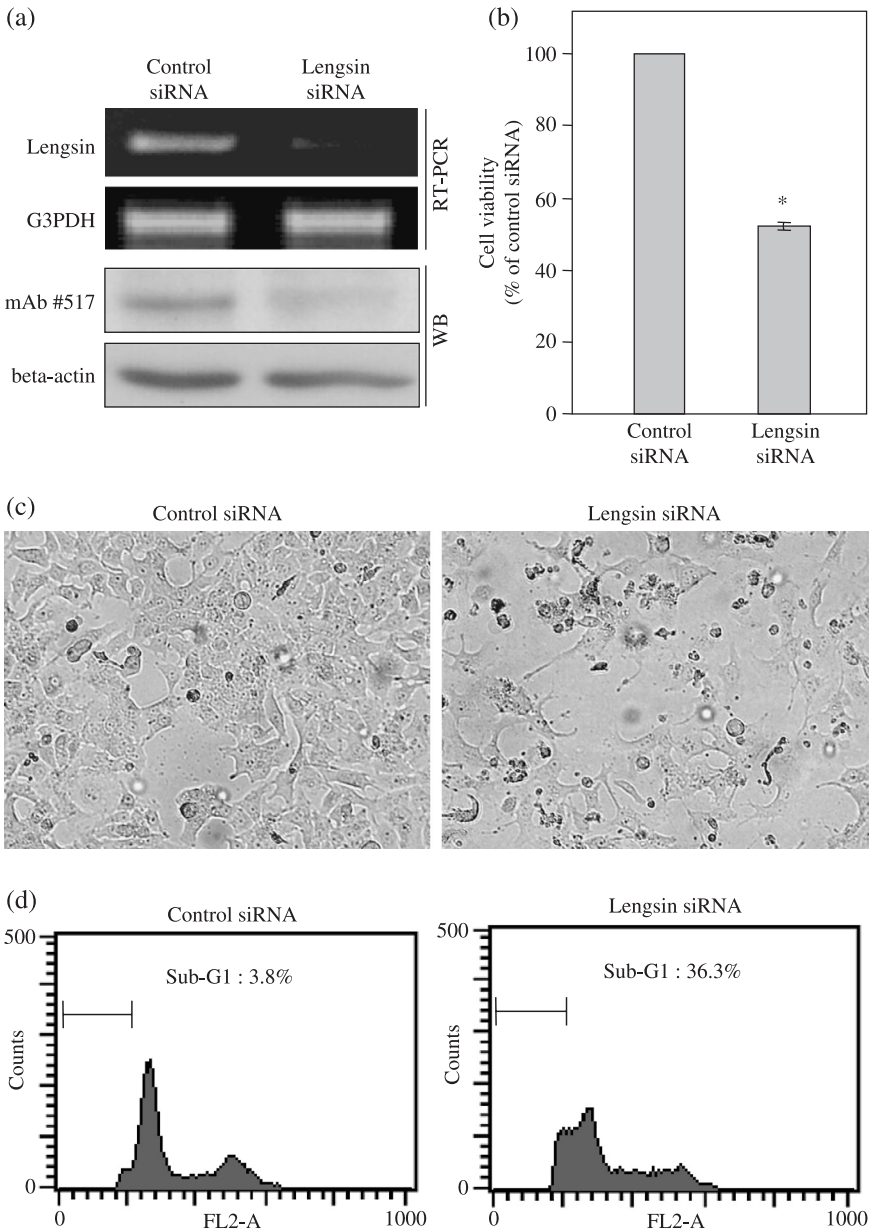

Fig. 4. Effect of Lengsin on cell viability in lung cancer cell line 1-87. (a) Gene silencing was performed using Lengsin siRNA. RT-PCR analysis was done using Lengsin primer pair I. G3PDH was used as an internal control. (b) WST-1 assay shows a decrease in the numbers of viable cells after knockdown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

Acknowledgments

Thanks to Drs K Imai, K Itoh, BJ van den Eynde, PG Coulie and K Kuzushima for kindly providing cell lines and S Ottonello for kindly providing the plasmid of Lengsin. This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology in Japan.

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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