immune attack.[37,38] The present study is the first to demonstrate that SPARC can be a target antigen for cancer immunotherapy. Figures 2B, 3A and 4D show that the effect of SPARC as the target antigen was comparable with the previously known melanoma-associated antigen, TRP2, or gp100.

CTL specific to each TAA were sensitized by the in vivo transfer of DC transfected with *GPC3*, *SPARC*, *TRP2*, or *hgp100* gene. However, the anticancer effects of ES-DC expressing single TAA in vivo were insufficient (Fig. 4D). These results are quite similar to the situation of recent T cell-targeted cancer immunotherapy. Using DC expressing multiple TAA for cancer immunotherapy makes sense and several studies have been reported.[39–41] However, there are very few reports that directly demonstrate the advantage of multiple as compared with single TAA-targeted immunotherapy.[42] As shown in Figures 3B, C and 4D, preimmunization with the mixture of 3 independent TAA transfectant ES-DC protected the mice more efficiently than the ES-DC expressing single TAA. The enhancement of antitumor immunity by the transfer of a mixture of 3 kinds of TAA-transfectant DC could be owing to an increase of number of CTL attacking tumor cells and a low frequency of immune escape.

In the past decade, α-GalCer has been attracting attention as a novel immunostimulatory reagent for cancer immunotherapy. CD1d is monomorphic and thus CD1d–α-GalCer–complex on DC can stimulate the NKT cells of any recipients. On the basis of the promising results of preclinical studies demonstrating the anticancer effects of α-GalCer–loaded DC,[20] several phase 1 clinical studies have been conducted. Although the activation and expansion of NKT cells by the administration of α-GalCer–loaded DC has been observed, the results seemed to be unsatisfactory from the viewpoint of the clinical effects.[43–46] The present study evaluated the effects of loading α-GalCer onto ES-DC–expressing endogenous TAA to induce anticancer immunity. Upon loading with α-GalCer, ES-DC had a capacity to activate NKT cells (Fig. 5). Figure 5C showed that the killing activity induced by the in vivo administration of α-GalCer–loaded ES-DC through the activation of NKT cells was sustained 1 week, and after 2 weeks the effect decreased to the background level. Despite this transient NKT cell activating capacity of α-GalCer–loaded ES-DC, anticancer effects induced by a mixture of 3 kinds of TAA-transfectant ES-DC loaded with α-GalCer showed a potent effect on inhibiting the growth of B16-BL6/Luc at 2 or 5 weeks after the administration as shown in Figures 6

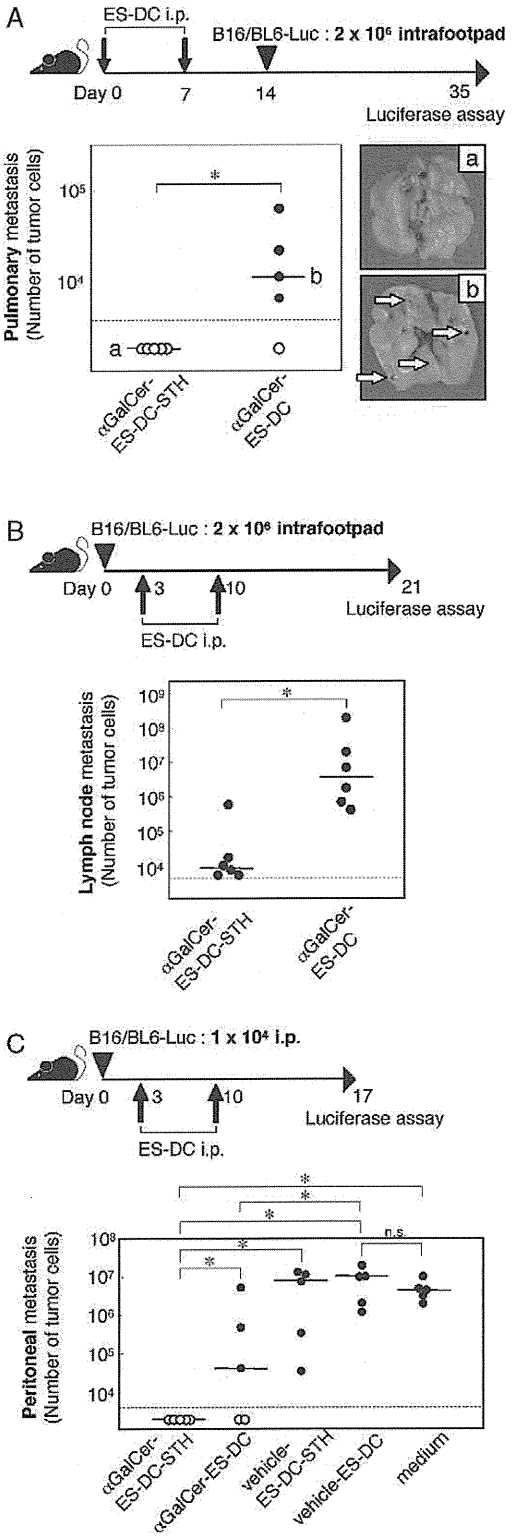

FIGURE 7. Potent anticancer effects of multiple tumor-associated antigen-targeted vaccinations with α-GalCer-loaded ES-DC. A, About 3×10^5 of α-GalCer–ES-DC–STH or α-GalCer–ES-DC were transferred IP into mice twice on days 0 and 7. On day 14, 2×10^6 B16-BL6/Luc cells were inoculated into the footpad of mice. On day 35, the mice were euthanized, the lungs were excised, and luciferase activities were measured. Typical examples of pulmonary metastasis of B16-BL6/Luc cells in mice are shown: above; A, α-GalCer–ES-DC–STH; below; B, α-GalCer–ES-DC. B, On day 0, 2×10^6 B16-BL6/Luc cells were inoculated into the footpad of mice. On days 3 and 10, each ES-DC was transferred IP. On day 21, the mice were euthanized and the luciferase activities of the inguinal lymph nodes were measured. C, On day 0, 1×10^4 B16-BL6/Luc cells were inoculated IP into mice. On days 3 and 10, each ES-DC was transferred IP. On day 17, the mice were euthanized and the luciferase activities of the greater omentum and pancreas were measured. Dotted line indicates the detection limit (A and C, n = 5; B, n = 6; *P<0.05). The data are each representative of 2 independent experiments with similar results. ES-DC indicates embryonic stem cell-derived dendritic cells; IP, intraperitoneal; α-GalCer, α-galactosylceramide.

and 7. In contrast, α-GalCer–loaded ES-DC without TAA showed an insufficient effect under the same conditions. This suggests that α-GalCer–loaded ES-DC have potent, antigen nonspecific effect in the early phase after administration, but sustained anticancer effect requires activation of TAA-specific CTL induced by ES-DC expressing TAA. Interestingly, the others reported that NKT cells activation induced by α-GalCer–loaded mature DC helped to boost adaptive immunity in vivo.[45] We performed an IFN-γ enzyme-linked immunosorbent spot assay to investigate whether the loading of α-GalCer to TAA-expressing DC would enhance the TAA-specific immunoresponse. However, no significant enhancement was observed when the mice were immunized with α-GalCer–loaded TAA-expressing DC in comparison with the vehicle-loaded TAA-expressing DC (unpublished observation). The preventive effects of the immunization with α-GalCer–loaded TAA-expressing DC (compared with vehicle-loaded TAA-expressing DC) were almost totally abrogated when the $CD4^+$ T cells, $CD8^+$ T cells, or NK cells were depleted (Fig. 6C). Collectively, we considered that the enhanced antitumor effects induced by α-GalCer–loaded TAA-expressing DC came from the cooperative work of $CD4^+$ T cells, $CD8^+$ T cells, and NK cells.

Anticancer immunotherapy with DC loaded with human leukocyte antigen (HLA)-binding peptides derived from TAA has been tested clinically in many institutions. In most cases, the DC are generated by culture of monocytes obtained from peripheral blood of the patients. To generate a sufficient number of DC for treatment, apheresis, a procedure that is sometimes invasive for patients with advanced stages of cancer, is necessary. In addition, the culture to generate DC should be carried out separately for each patient and treatment, and thus the procedure used at present may be too labor-intensive and expensive to be applied broadly in a practical setting. Alternately, the source of ES-DC, ES cells, has the capacity to propagate infinitely and multiple gene-transfectant ES-DC can be generated by the sequential transfection of ES cells with vectors bearing different selection markers.[18,24,25] Generation and genetic modification of ES-DC from human ES cells is achieved by the currently established method.[47] It may, therefore, be possible to generate multiple gene-transfectant human ES-DC expressing TAA plus immunostimulating molecules, which could thus more potently stimulate anticancer immunity than monocyte-derived DC do.

Considering the future clinical application of ES-DC, allogenicity (ie, differences in the genetic background) between patients to be treated and ES cells as a source for DC may cause problems. However, it is expected that human ES cells sharing some HLA alleles with the patients will be available for most cases. Mouse ES-DC administered into semiallogenic recipients, sharing 1 major histocompatibility complex (MHC) haplotype with the ES-DC, effectively primed antigen-specific CTL, thus suggesting that ES-DC can survive for a sufficient period to stimulate antigen-specific CTL restricted by the shared MHC class I.[48] However, in the same semiallogenic setting, the 5 times injection of nonantigen-loaded ES-DC significantly reduced the efficiency of priming of antigen-specific CTL induced by the subsequent injection of antigen-loaded ES-DC (unpublished observation). Therefore, repetitive stimulation with ES-DC expressing allogenic MHC may result in activation and expansion of allogenic MHC class I-reactive CTL, and in such recipients subsequently transferred ES-DC may be rapidly eliminated. Repeated immunization may be required in clinical applications to induce strong anticancer immunity. Therefore, the problem of the histoincompatibility between ES cell lines and recipients should be resolved. The methods for targeted gene modification of human ES cells and for targeted chromosome elimination of mouse ES cells have been developed. To overcome the problem of histoincompatibility, genetic modification to inhibit expression of endogenous HLA class I in ES-DC may be effective. A disruption of the genes for the molecules necessary for the cell surface expression of HLA class I molecules, such as transporter associated with antigen processing or β2-microglobulin (β2M), is presumably feasible. Along this line, we recently reported that the efficient activation of antigen-specific CTL was induced by TAP1 or β2M disrupted and recipient-matched MHC class I introduced mouse ES-DC.[19] We are now preparing to introduce expression vector encoding for β2M-linked form of recipient-matched HLA class I heavy chain into TAP1-deficient or β2M-deficient human ES cells.

Previous studies on ES-DC were performed by using well-established TT2 ES cells.[15,18,28] The present study confirmed that ES-DC could be generated from B6 ES cells with the same method previously established by using TT2 ES cells.[17] ES-DC generated from B6 ES cells were comparable with TT2 ES cells in differentiation, proliferation, surface phenotype, and antigen presentation. In addition, ES-DC could be generated from other ES cell lines (unpublished observation), thus suggesting that the method to generate ES-DC can be applied to various types of mouse ES cells.

Other studies have reported the generation of induced pluripotent stem (iPS) cells from adult human dermal fibroblasts with the defined 4 factors: Oct3/4, Sox2, Klf4, and c-Myc.[49] Human iPS cells are similar to human ES cells in morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, and telomerase activity. DC can be generated from mouse iPS cells (unpublished observation) and testing is underway to determine whether DC could be generated from human iPS cells. Tailor-made medicine may, therefore, someday be possible if "iPS-DC" can be generated in the future.

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Gene silencing of glypican-3 in clear cell carcinoma of the ovary renders it more sensitive to the apoptotic agent paclitaxel *in vitro* and *in vivo*

Tomokazu Umezu,[1] Kiyosumi Shibata,[1,3] Maiko Shimaoka,[1] Hiroaki Kajiyama,[1] Eiko Yamamoto,[1] Kazuhiko Ino,[1] Akihiro Nawa,[1] Takeshi Senga[2] and Fumitaka Kikkawa[1]

[1]Department of Obstetrics and Gynecology, Nagoya University Graduate School of Medicine, Showa-ku, Nagoya; [2]Division of Cancer Biology, Nagoya University Graduate School of Medicine, Showa-ku, Nagoya, Japan

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Glypican-3 (GPC3) is a heparan sulfate proteoglycan that is bound to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor, and glypicans can regulate the activity of a wide variety of growth and survival factors. We report here that GPC3 was expressed in clear cell carcinoma of the ovary, and not in other carcinomas. To evaluate the phenotype and potential preclinical relevance, we generated an ovarian cancer cell line stably transfected with plasmids encompassing shRNA targeting GPC3. We show that the clear cell carcinoma cell line with silenced GPC3 expression (GPC3 [–]) was more sensitive to paclitaxel than GPC3 (+) cells. In addition, the GPC3 silencing induced sensitization to paclitaxel was associated with the activation of an apoptosis pathway, as shown by flow cytometry. Moreover, we investigated the effect of GPC3 on peritoneal metastases using nude mice. Peritoneal metastases caused by GPC3 (–) were more sensitive to paclitaxel than those caused by GPC3 (+) cells. These results indicate that increased GPC3 expression in a clear cell carcinoma cell line may play a protective role against apoptosis, and so the downregulation of GPC3 may be a potential target to increase sensitivity to paclitaxel-induced apoptosis in clear cell carcinoma. (*Cancer Sci* 2010; 101: 143–148)

Over the last two decades, aggressive cytoreductive surgery and chemotherapy have been used in an attempt to improve the survival rate in patients with EOC.[1–3] However, the long-term survival rate remains poor as a result of recurrence and the emergence of drug resistance. Based on morphological criteria, there are four major types of primary epithelial adenocarcinoma, and the chemosensitivity and biologic behaviors differ among these histologic types. Although ovarian carcinoma cells are generally sensitive to anticancer drugs, CCC is resistant to most of these agents. Thus, the prognosis for CCC patients is poor compared to other types of EOC such as serous adenocarcinoma.[4,5] CCC comprises more than 15% of EOC cases in Japan, although it represents 8–10% of all EOC cases in the USA.[5,6] Therefore, it is important to establish new treatment strategies to improve the prognosis of CCC patients.

Glypicans are a family of heparan sulfate proteoglycans that are linked to the exocytoplasmic surface of the plasma membrane through a glycosylphosphatidylinositol anchor. Six glypicans have been identified in mammals (GPC1–GPC6), and two in *Drosophila*.[7] The physiological function of glypicans is still not well understood. However, it was shown that the GPC3-encoding gene is mutated in patients with Simpson–Golabi–Behmel syndrome, an X-linked disorder characterized by pre- and postnatal overgrowth and a varying range of dysmorphisms.[7] GPC3 regulates cell growth either positively or negatively depending on the cell type. Genetic and functional studies showed that glypicans regulate the signaling activity of various morphogens, including Wnts, Hedgehogs, bone morphogenic proteins, and fibroblast growth factors.[8–12] Previous studies showed that GPC3 was overexpressed in Wilms' tumor, hepatocellular carcinoma, and hepatoblastoma.[13,14] In ovarian carcinoma, GPC3 was overexpressed in yolk sac tumor and CCC.[15–17] However, GPC3 function in CCC was unclear.

In the present study, we examined the phenotype and preclinical relevance of the downregulation of GPC3 *in vitro* and *in vivo* by using an RNA interference system comprising a stably integrated cassette for the expression of shRNA targeting GPC3.

Furthermore, we investigated the role of this molecule in the sensitivity of CCC to paclitaxel, which is a key drug for ovarian cancer, using shRNA targeting GPC3.

Materials and Methods

Cell culture. We used eight human ovarian carcinoma cell lines (SKOV-3, HEY, ES-2, OVCAR-3, HRA, RMG-I, RMG-II, and KOC-7c) in this study. SKOV-3, HRA, RMG-I, and RMG-II cells were cultured and maintained as described previously.[18,19] ES-2, HEY, and KOC-7c cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The cell lines were maintained in RPMI-1640 supplemented with 10% FCS and penicillin–streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

Sensitivity to paclitaxel. The sensitivity of the cells to paclitaxel was determined using a modified tetrazolium salt MTS assay using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA). Briefly, cells were trypsinized and plated out at a density of 5000 cells per well into 96-well plates. The cells were cultured overnight then given fresh medium containing various concentrations of paclitaxel. After incubation for 72 h, cell viability was assayed, and the cytotoxity was expressed as the IC_{50} for each of the cell lines; that is, the concentration of the drug that caused a 50% reduction in absorbance at 490 nm relative to untreated cells.

Glypican-3 shRNA and its transfection. Short hairpin RNA was designed by us and synthesized by Qiagen-Xeragon (Germantown, MD, USA) to target GPC3: CCAATGCCATGTTCAAGAATTCAAGAGATTCTTGAACATGGCATTGGTTTTT and its antisense oligonucleotide. The scrambled shRNA (Qiagen-Xeragon) was used as a control. The oligonucleotides were annealed and inserted into RNAi-Ready pSIREN-RetroQ-TetP vector (Takara Bio, Tokyo, Japan). To establish a stable cell line, the vector plasmid was transfected into parental KOC-7c

[3]To whom correspondence should be addressed.
E-mail: shiba@med.nagoya-u.ac.jp

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cells using Lipofectamine 2000 reagent (Invitrogen, San Diego, CA, USA) and selected by adding puromycin.

Western blot analysis. Cell lysates were electrophoresed in SDS polyacrylamide gels under reducing conditions. After electrophoresis, the proteins were transferred electrophoretically to an Immobilon membrane (Millipore, Bedford, MA, USA). After blocking, the membrane was incubated for 1 h with the relevant antihuman primary antibody at the recommended dilution, GPC3 (BioMosaics, Burlington, VT, USA), cleaved-PRAP (Cell Signaling Technology, Danvers, MA, USA), and β-actin (Abcam, Cambridge, MA, USA). The membrane was washed three times with Tween/PBS for 15 min, then incubated with the appropriate secondary antibody for 1 h. After washing with Tween/PBS, the membrane was treated with ECL-Western blotting detecting reagent (Amersham Biosciences KK, Tokyo, Japan).

Proliferation assay. To study the effect of silencing GPC3 on KOC-7c cell growth, 5×10^3 KOC-7c cells per well were seeded in 96-well plates in culture medium supplemented with 10% FCS, and transfected with GPC3 shRNA or control shRNA. Following overnight incubation, the culture medium was replaced with fresh complete medium containing 10% FCS. After 24, 48, and 72 h, cell proliferation was assessed as previously described.[22]

***In vitro* cell migration assay.** The cell migratory potential was evaluated using 24-well Transwell chambers with 8.0 μm pore membranes (Corning, Corning, NY, USA). Cells were suspended in the upper chamber at a density of 5×10^4 in 200 μL serum-free medium. The lower chamber contained 800 μL medium supplemented with 10% FCS as a chemoattractant. After incubation for 16 h, the remaining cells on the upper surface of the filters were removed by wiping with cotton swabs, and migrating cells on the lower surface were stained using May-Grunwald Giemsa. The number of cells on the lower surface of the filters was counted under a microscope. Data were obtained from three individual experiments in triplicate.

Assay for apoptosis. To quantify the apoptosis of KOC-7c cells, annexin V and PI staining was carried out, followed by flow cytometry. Cells were briefly plated at a density of 1×10^6 per well into 10 cm dishes and treated with paclitaxel for 24 h. After treatment, both floating and attached cells were collected by brief trypsinization and washed with PBS twice, then subjected to annexin V and PI staining using a MEBCYTO apoptosis kit (MBL International, Woburn, MA, USA). After staining, quantitative analysis for apoptosis was carried out by flow cytometry.

TUNEL assay. The TUNEL assay (Apoptosis *in situ* Detection Kit; Wako Pure Chemical, Osaka, Japan) was used to detect apoptotic cell death by the enzymatic labeling of DNA strand breaks with fluorescein-deoxyuridine triphosphate and terminal deoxynucleotidyl transferase.

***In vivo* studies.** Female nude mice (BALB/c) at 5 weeks of age were obtained from Japan SLC (Nagoya, Japan). The treatment protocol followed the guidelines for animal experimentation adopted by Nagoya University (Nagoya, Japan). Control and sh GPC3 cells (1×10^7 cells per 0.5 mL of medium/mouse) were injected i.p. to generate peritoneal metastasis of ovarian carcinoma in the mouse model. The i.p. injection of paclitaxel (20 mg/kg body weight) was initiated 48 h after tumor inoculation, and was repeated twice.

Statistical analysis. For data from *in vitro* experiments, statistical comparisons among groups were carried out using Student's *t*-test. Differences between groups were considered significant at $P < 0.05$.

Results

Glypican-3 expression and the IC_{50} value of paclitaxel in EOC cell lines. We evaluated GPC3 expression in EOC cell lines by Western blot (Fig. 1A). Only KOC-7c cells were strongly positive for GPC3 expression. In contrast, no serous carcinoma cell lines showed the expression of GPC3. We evaluated the IC_{50} value of paclitaxel in EOC cell lines. KOC-7c was the most resistant to paclitaxel in CCC cell lines. However, some serous adenocarcinoma cell lines were more resistant to paclitaxel than KOC-7c (Table 1).

Short hairpin RNA-mediated inhibition of GPC3 expression. Previously, we used siRNAs targeting GPC3 to knock down endogenous gene expression. However, the major disadvantage of this strategy is the short duration of the silencing effect. In order to study the function of GPC3 in long-term growth *in vitro* and *in vivo*, we used an shRNA technique targeting GPC3. Control shRNA was also prepared. Each construct produced shRNA composed of two 21-nucleotides with an inverted orientation. GPC3 shRNA and control shRNA were used to transfect KOC-7c cells. The cells were further cultured in selective medium. After 6 weeks, the cell clones were selected and seeded for further expansion in selective medium for Western blot analysis. The protein level of GPC3 shRNA cells was almost zero, compared to that of GPC3 in control shRNA and parent cells. In contrast to GPC3, β-actin was not affected (Fig. 1B).

Effect of GPC3 knockdown on *in vitro* cell proliferation and migration. There was no significant difference in cell proliferation between control and sh GPC3 cells (Fig. 2A). Similarly, the silencing of GPC3 did not affect the migratory potential (Fig. 2B).

Effect of GPC3 knockdown on *in vitro* paclitaxel sensitivity. Previous studies suggested that GPC3 might be involved in chemoresistance to anticancer agents in gastric carcinoma cell lines.[20] Thus, we subsequently evaluated chemosensitivity on the GPC3 silencing of KOC-7c cells *in vitro*. We observed that

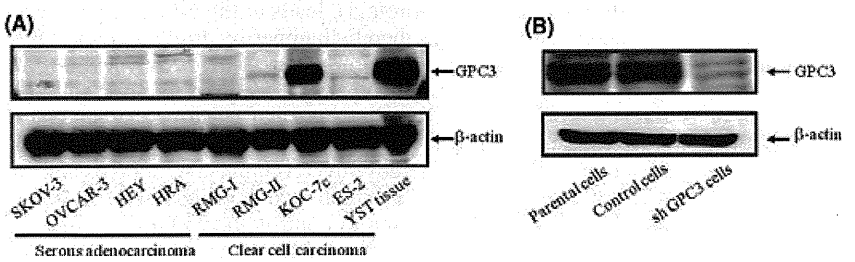

Fig. 1. (A) Glypican-3 (GPC3) expression in various epithelial ovarian cancer cell lines. YST, yolk sac tumor. (B) Effect of the suppression of GPC3 expression by sh RNA in KOC-7c ovarian cancer cells.

Table 1. IC_{50} values of various epithelial ovarian carcinoma cell lines

SKOV-3	OVCAR-3	HEY	HRA	RMG-I	RMG-II	KOC-7c	ES-2
16 ± 3.4	6.8 ± 1.8	7.4 ± 1.0	69.6 ± 10.2	4.8 ± 1.2	4.3 ± 1.1	15.4 ± 2.6	11.3 ± 2.2

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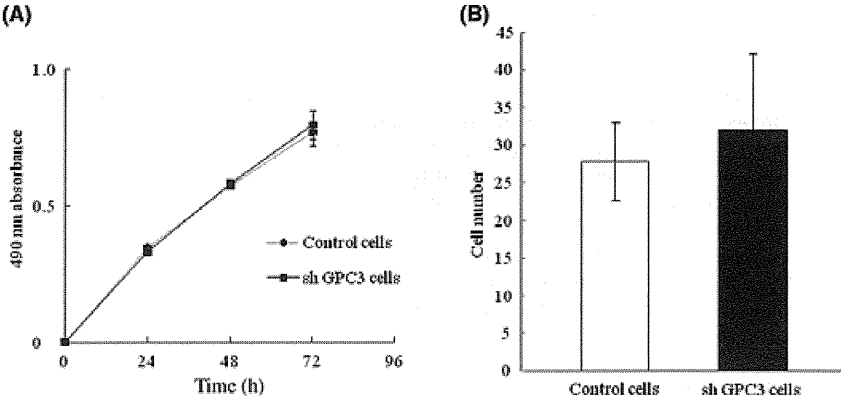

Fig. 2. (A) *In vitro* proliferation assay to assess the effect of silencing glypican-3 (sh GPC3) on growth of KOC-7c ovarian cancer cells. (B) *In vitro* cell migration assay to evaluate the potential of KOC-7c cells to migrate into Transwell chambers. The silencing of GPC3 did not affect the cells' migratory potential.

sh GPC3 cells were more sensitive to paclitaxel than control cells. The IC_{50} value for paclitaxel of sh GPC3 cells was 4.5 ± 1.6 ng/mL. In contrast, that in control cells was 9.5 ± 1.5 ng/mL (Table 2). Cell death with morphological changes associated with apoptosis was more frequently observed in sh GPC3 cells (Fig. 3A–D).

Effect of GPC3 knockdown on paclitaxel-induced apoptotic death of KOC-7c cells. To further investigate the mechanism of GPC3 silencing-induced chemosensitivity, we also assessed the paclitaxel-induced apoptosis of control and sh GPC3 cells using flow cytometry. Cells were treated with paclitaxel (10 ng/mL) for 8 h, then subjected to annexin V/PI staining and flow cytometric analysis. As shown in Figure 3(E,F), sh GPC3 cells treated with paclitaxel showed a stronger apoptotic effect than control cells. The quantitative data showed that, compared to control cells treated with paclitaxel (10.8 ± 1.6% apoptotic cells), paclitaxel-treated sh GPC3 cells showed a significant increase in apoptosis (37.0 ± 3.9% apoptotic cells) (Fig. 3G). We measured cleaved PARP in control and sh GPC3 cells after exposure to paclitaxel by Western blot analysis. As shown in Figure 3(H), cleaved PARP expression in sh GPC3 cells showed an increase at 3 h after treatment with 10 ng/mL paclitaxel.

Effect of GPC3 knockdown on peritoneal progression in nude mice. To determine the role of GPC3 in peritoneal progression, the peritoneal progression of control and sh GPC3 cells was evaluated in a nude mouse model. Carcinomatous peritonitis was observed for 3 weeks after the inoculation of mice with 1×10^7 control and sh GPC3 cells. As shown in Figure 4A,B, the progression rate of peritoneal dissemination in sh GPC3 cells-inoculated was not significantly different from that in control cell-inoculated. The total cell-inoculated mice tumor volume was not different between control and sh GPC3 cells ($P = 0.67$) (Fig. 4C). Histologic findings confirmed that xenografted tumors of sh GPC3 cells showed attenuated GPC3 expression (Fig. 4D,E).

Effect of GPC3 knockdown on *in vivo* paclitaxel sensitivity. We tested whether GPC3 influenced CCC sensitivity to paclitaxel in a model using nude mice. Two days after inoculation of the mouse peritoneum with 1×10^7 control and sh GPC3 cells, 20 mg/kg body weight of paclitaxel was given once a week, a total of three times. Twenty-one days after the final treatment with paclitaxel, mice were dissected. The control cell group showed more disseminated tumors and a larger amount of bloody ascites compared with the sh GPC3 cell group (Fig. 5A,B). The total tumor volume in the mouse peritoneum in the presence of control cells was relatively large, compared to that in the presence of sh GPC3 cells ($P = 0.09$) (Fig. 5C). Finally, we used the TUNEL method to visualize DNA fragmentation at the single-cell level (Fig. 5D,E). After paclitaxel treatment, more apoptotic cells were observed in the sh GPC3 cell xenograft group than in the control cell group after paclitaxel treatment, although this was not significant ($P = 0.42$) (Fig. 5F).

Table 2. Effect of shRNA on the sensitivity of KOC-7c ovarian cancer cells to paclitaxel

	Mean IC_{50} ± SEM Paclitaxel (ng/mL)
Control shRNA	9.5 ± 1.5
Glypican-3 shRNA	4.5 ± 1.6

Discussion

Clear cell carcinoma has been classified as a subgroup of EOC and reported to be an interesting histologic type with unique clinical features. CCC showed a poorer prognosis compared to serous adenocarcinoma because it tended to be resistant to antineoplastic agents, including paclitaxel.[4,5] Paclitaxel is a first-line chemotherapeutic agent that is effective for the treatment of ovarian carcinoma. Paclitaxel exerts its effect through the stabilization of microtubules, induction of cell cycle arrest in G_2-M, and activation of pro-apoptotic signaling. However, in spite of the comparatively high sensitivity of ovarian carcinoma to paclitaxel, the prognosis of CCC patients remains poor as most deaths are the result of metastasis that is refractory to conventional chemotherapy. To overcome the paclitaxel resistance of CCC, a variety of additional molecular targeting therapies combined with paclitaxel have been investigated. The mechanisms of paclitaxel resistance have previously been explained by *MDR-1*/P-glycoprotein overexpression, and the selective expression of β-tubulin isotypes. Overexpression of the *MDR-1* gene, encoding an efflux pump (P-glycoprotein), leads to the efflux of paclitaxel and other cationic drugs, thereby hampering drug retention.[21]

Such a mechanism is easily observable in *in vitro*-cultured cancer cells. However, in the present analysis, there was no difference in *MDR-1* expression between control and sh GPC3 cells (data not shown). Class III β-tubulin seems to enhance dynamic instability, thereby overcoming the suppression of microtubule dynamicity by paclitaxel.[22] Thus, we evaluated class III β-tubulin expression in control and sh GPC3 cells. The results showed that there were no differences in class III β-tubulin between control and sh GPC3 cells (data not shown).

A previous report showed that 60% of ovarian CCC cases expressed GPC3. Our data also showed that 40% of CCC cases expressed GPC3. However, the function of GPC3 in CCC remains unclear. One reason for this might be the lack of a stable knockdown of GPC3 in human cancer cell lines available for both *in vitro* and *in vivo* experiments. In this study, we were the

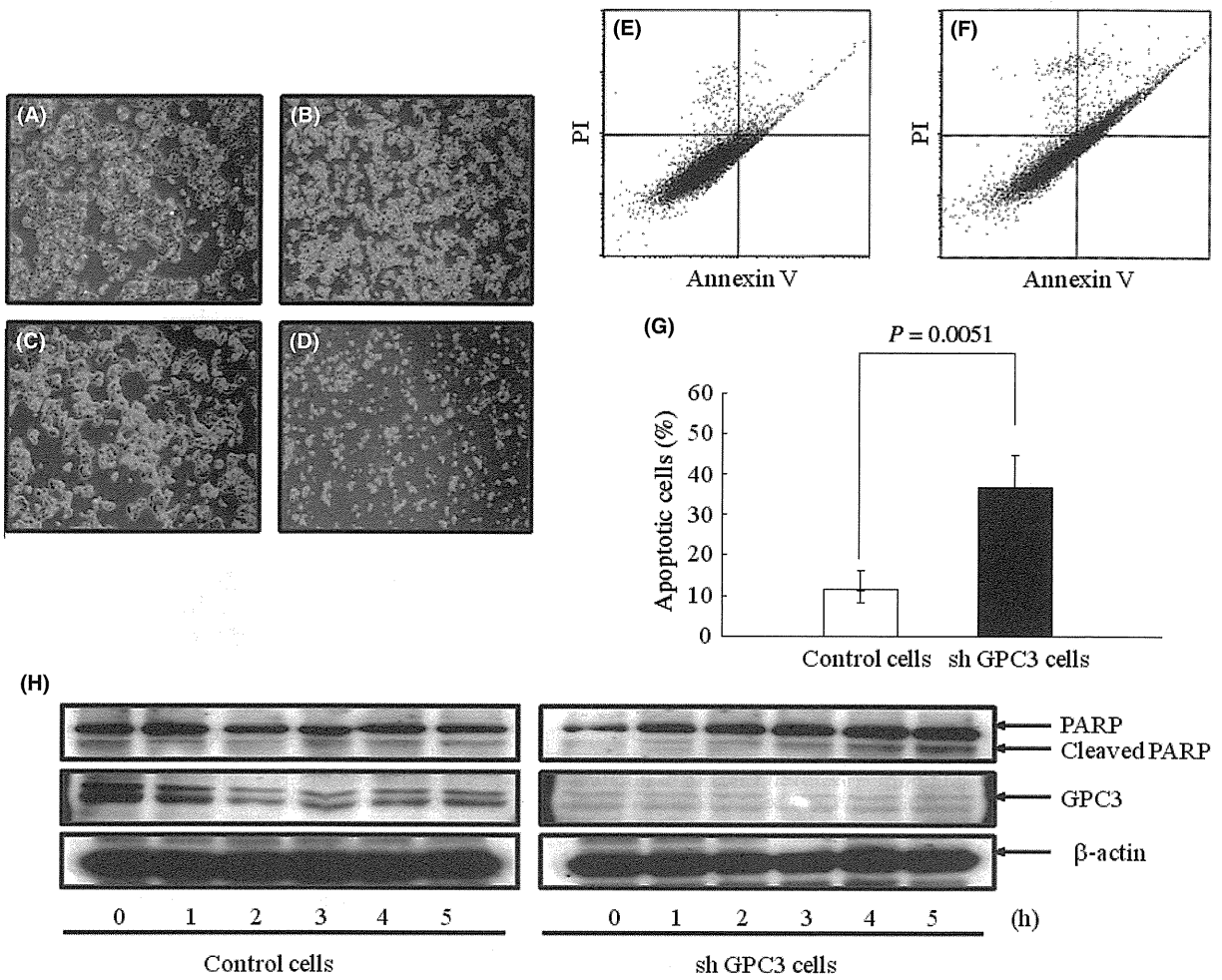

Fig. 3. (A–D) Morphological changes in control cells and clear cell carcinoma cells with silenced glypican-3 expression (sh GPC3) incubated with paclitaxel (10 ng/mL). (A) Control cells treated with paclitaxel for 24 h. (B) sh GPC3 cells treated with paclitaxel for 24 h. (C) Control cells treated with paclitaxel for 48 h. (D) sh GPC3 cells treated with paclitaxel for 48 h. (E–H) Effect of paclitaxel on the apoptosis of control and sh GPC3 cells. Cells were incubated with paclitaxel (10 ng/mL) for 8 h. The cells were then harvested, stained with annexin V/propidium iodide (PI), and analyzed by flow cytometry. (E) Control cells. (F) sh GPC3 cells. (G) Percentage of apoptotic cells. (H) Expression level of cleaved PARP, GPC3, and β-actin after paclitaxel treatment.

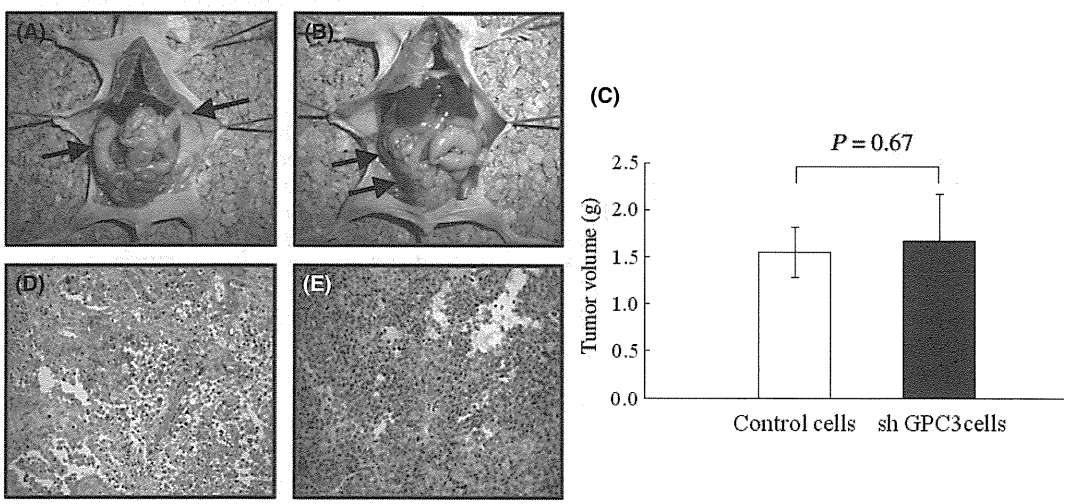

Fig. 4. Peritoneal progression of control cells and clear cell carcinoma cells with silenced glypican-3 expression (sh GPC3) in a nude mouse model. (A,B) Intraperitoneal appearance of mice killed 3 weeks after inoculation with control (A) and sh GPC3 (B) cells. Arrows show peritoneal dissemination. (C) Tumor volume of control and sh GPC3 cells. (D,E) Immunoreactivity of GPC3 in peritoneal dissemination. (D) Control cells. (E) sh GPC3 cells.

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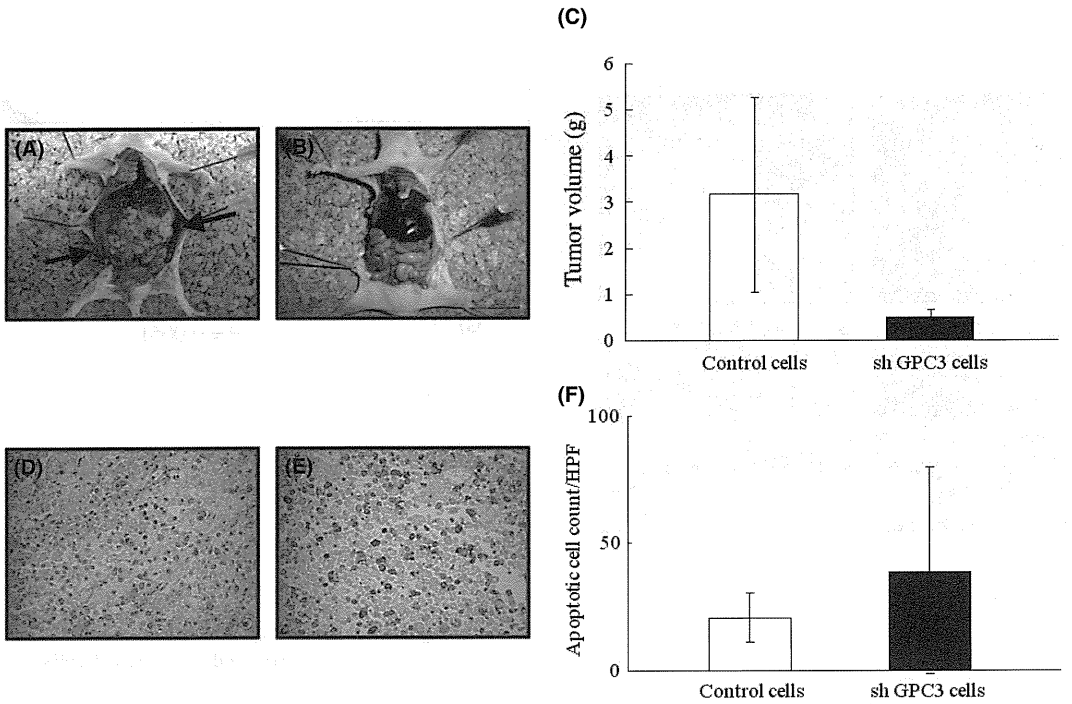

Fig. 5. Effect of paclitaxel treatment on the peritoneal progression of control cells and clear cell carcinoma cells with silenced glypican-3 expression (sh GPC3) in a nude mouse model. (A,B) Intraperitoneal appearance of mice killed 2 weeks after the last paclitaxel treatment. Arrows show peritoneal dissemination. (A) Control cells. (B) sh GPC3 cells. (C) Tumor volume of control and sh GPC3 cells. (D,E) Evaluation of apoptotic cells by TUNEL staining. (D) Control cells. (E) sh GPC3 cells. (F) Apoptotic cell count/high power field (HPF).

first to successfully establish the stable knockdown of a GPC3 expressing human cancer cell line using an sh RNA technique, using the CCC cell line KOC-7c, and revealed the functional implications of GPC3 in the tumor phenotype both *in vitro* and *in vivo*. Although GPC3 was not associated with the proliferation and migration potential of the CCC cell line, the sh RNA-mediated suppression of GPC3 protein expression is associated with a significant restoration of the sensitivity of CCC cells to paclitaxel. KOC-7c was the most resistant to paclitaxel of the CCC cell lines, but some serous carcinoma cell lines were more resistant to paclitaxel than KOC-7c. This suggests that the resistance to paclitaxel of serous carcinoma cell lines is due to other mechanisms, such as class III β tubulin or MDR-1, and not GPC3. The tumor volume in the control mouse peritoneum was significantly larger than in the sh GPC3 mouse. However, the evaluation of apoptosis using the TUNEL method showed no significant differences between the sh GPC3 cell xenograft and control groups after paclitaxel treatment. It may be that the observation period after paclitaxel treatment is so long that paclitaxel-resistant cells survived. Furthermore, individual differences between mice might have been too large. It has been shown that the GPC3 encoding gene is mutated in patients with Simpson–Golabi–Behmel syndrome,[7] a disorder characterized by pre- and postnatal overgrowth and various visceral and skeletal dysmorphisms. Some of these dysmorphisms could be the result of a deficiency in growth inhibition or programmed cell death. Indeed, it has been shown that GPC3 is able to induce apoptosis in a cell-specific manner.[23] GPC3 induced programmed cell death in rat mesothelioma and human breast cancer cells, but not in human colon carcinoma cells or murine NIH3T3 fibroblasts. GPC3 probably maintains the homeostatic balance between cell growth and cell death, which is ultimately the main pharmacological target of cell death-inducing antitumor agents, including paclitaxel.

Recently, GPC3 was shown to be involved in atypical multidrug resistance in gastric cancer cells. The suppression of GPC3 expression by an anti-GPC3 ribozyme not only restored sensitivity to mitoxantrone but also attenuated cross-resistance to etoposide.[20] Furthermore, the degree of drug resistance correlated with the level of GPC3 mRNA.

There is increasing evidence that GPC3 is an effective immunotherapeutic target in the treatment of GPC3 expressing cancers. Previous reports indicated that GPC3 was an effective target antigen for immunotherapy in hepatocellular carcinoma, and melanoma, raising the possibility that GPC3 peptides may be applicable to cancer immunotherapy for GPC3 overexpressing tumors.[24–26] Phase I clinical trials of immunotherapy targeting GPC3 in hepatocellular carcinoma patients is ongoing.

In conclusion, we have shown that the suppression of GPC3 in CCC cells restored paclitaxel sensitivity *in vitro*. Furthermore, the present study clearly showed the therapeutic potential of GPC3 inhibition *in vivo*. Taken together, our data could support the use of GPC3-targeted therapies for CCC patients. We suggest that therapy targeting to GPC3 may be a novel treatment strategy that could potentially help to prevent the appearance, progression, and/or recurrence of CCC.

Abbreviations

CCC	clear cell carcinoma
EOC	epithelial ovarian carcinoma
MDR-1	multi-drug resistance gene
PI	propidium iodide
sh	short hairpin

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

Munehide Nakatsugawa,[1,2] Yoshihiko Hirohashi,[1,9] Toshihiko Torigoe,[1,9] Hiroko Asanuma,[1,3] Akari Takahashi,[4] Satoko Inoda,[1] Kenji Kiriyama,[1] Emiri Nakazawa,[4] Kenji Harada,[5] Hideo Takasu,[5] Yasuaki Tamura,[1] Kenjiro Kamiguchi,[1] Noriharu Shijubo,[1,6] Ryoichi Honda,[7] Naohiro Nomura,[8] Tadashi Hasegawa,[3] Hiroki Takahashi[2] and Noriyuki Sato[1]

[1]Department of Pathology, [2]The Third Department of Internal Medicine, [3]Division of Clinical Pathology, Sapporo Medical University School of Medicine, South-1, West-17, Chuo-ku Sapporo 060-8556, [4]Japan Science and Technology Agency, Innovation Plaza Hokkaido, North-19, West-11, Kita-ku, Sapporo, 060-0819, [5]Dainippon Sumitomo Pharma Co., Ltd, Osaka 541-0045, [6]Sapporo Railway Hospital, North-3, East-1, Chuo-ku, Sapporo 060-0033, [7]Sapporo Tokushukai Hospital, Sakaedori 18-chome, Shiroishi-ku, Sapporo 003-0021, [8]Kitahiroshima Hospital, Sakaemachi 1-chome, Kitahiroshima 061-1133, Japan

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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,

[9]To whom correspondence should be addressed. E-mail: hirohash@sapmed.ac.jp or torigoe@sapmed.ac.jp

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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 (1×10^4 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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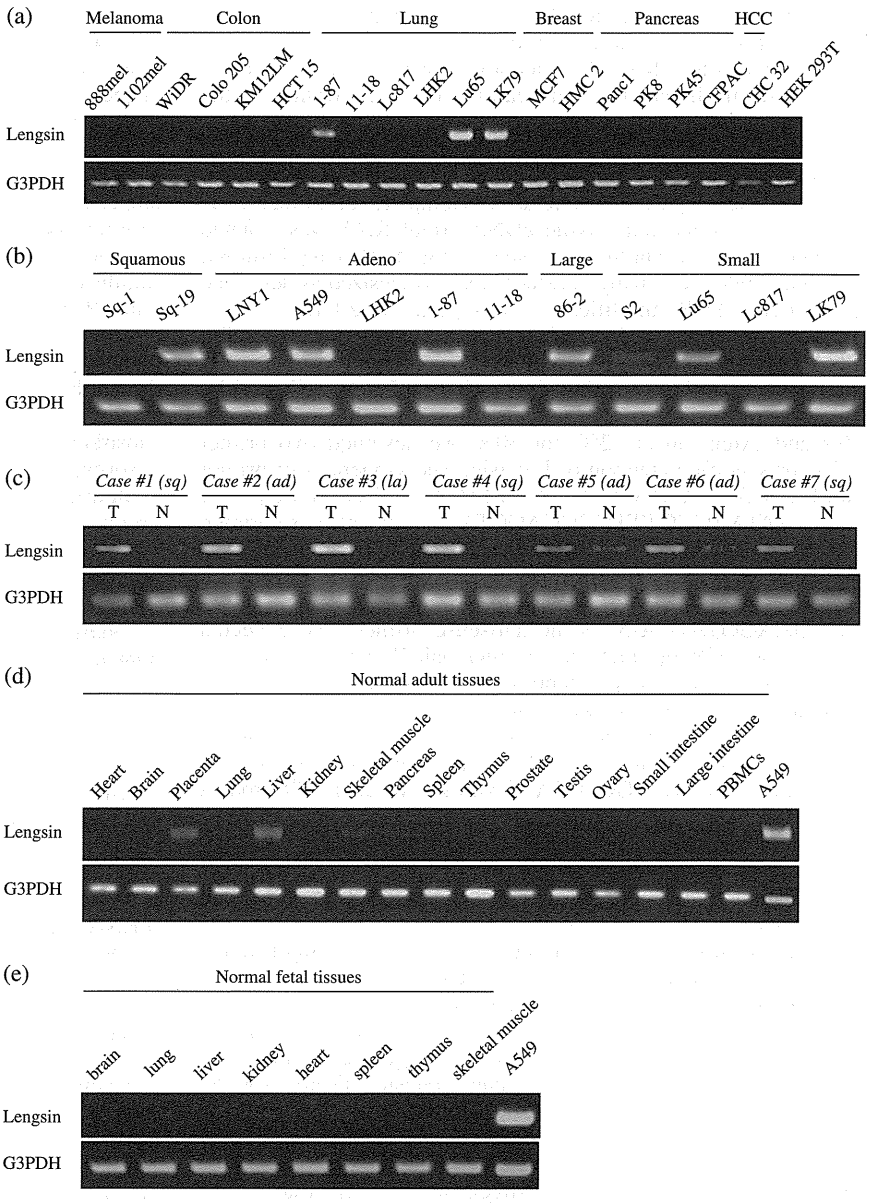

Fig. 1. Expression profiles of Lengsin as assessed by RT-PCR with Lengsin primer pair I in various cancer cell lines, lung cancer cell lines, human primary lung cancer tissues, normal adult tissues and fetal tissues. (a) Expression of Lengsin in various cancer cell lines. Cells include two melanoma cell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

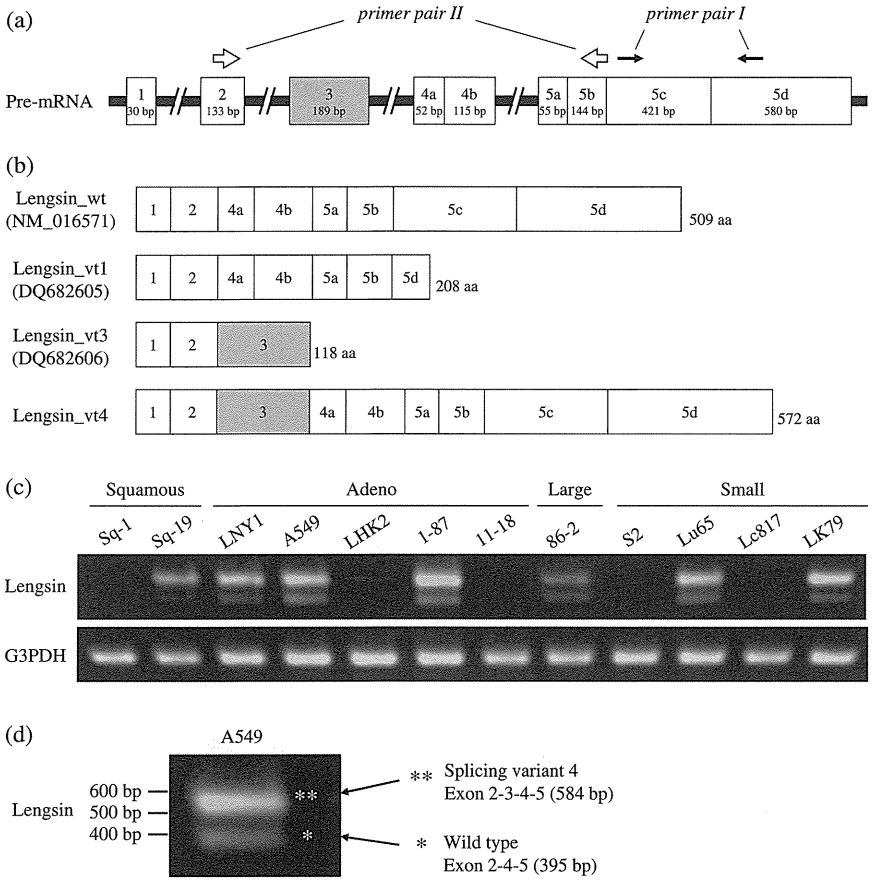

Fig. 2. Splicing variant 4 is more highly expressed than the wild type in lung cancer cell lines. (a) Diagram of premRNA showing five exons of Lengsin. Black arrow indicates PCR primer pair I and white arrow indicates PCR primer pair II. (b) Diagram of the derived protein of each splicing variant. Brackets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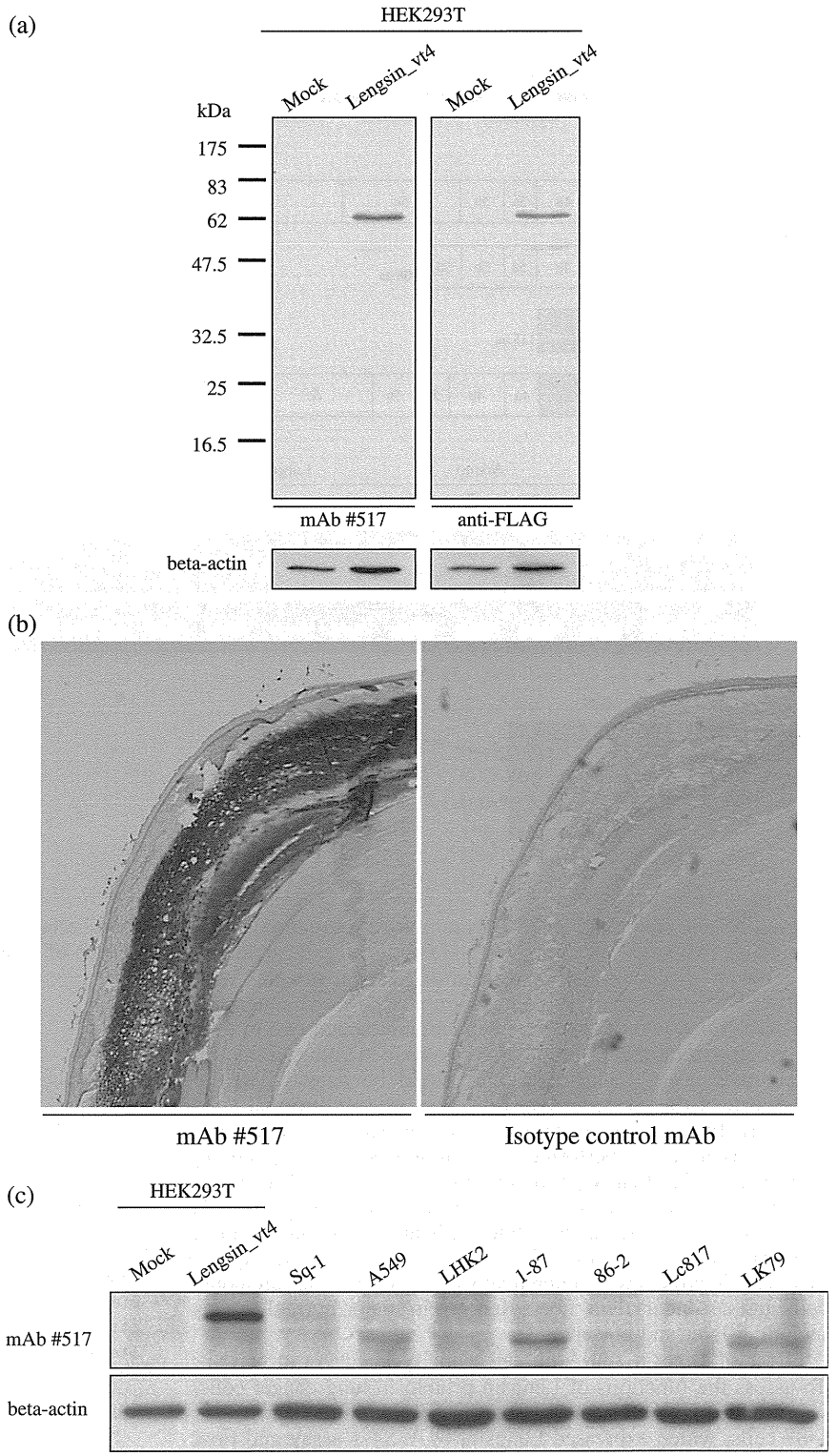

Fig. 3. Detection of Lengsin protein as assessed by Western blot analysis and immunohistochemical staining with anti-Lengsin mAb #517. (a) Specific detection of Lengsin protein in HEK293T cells transfected with expression vectors of FLAG-epitope-tagged Lengsin_vt4 as assessed by Western blot analysis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

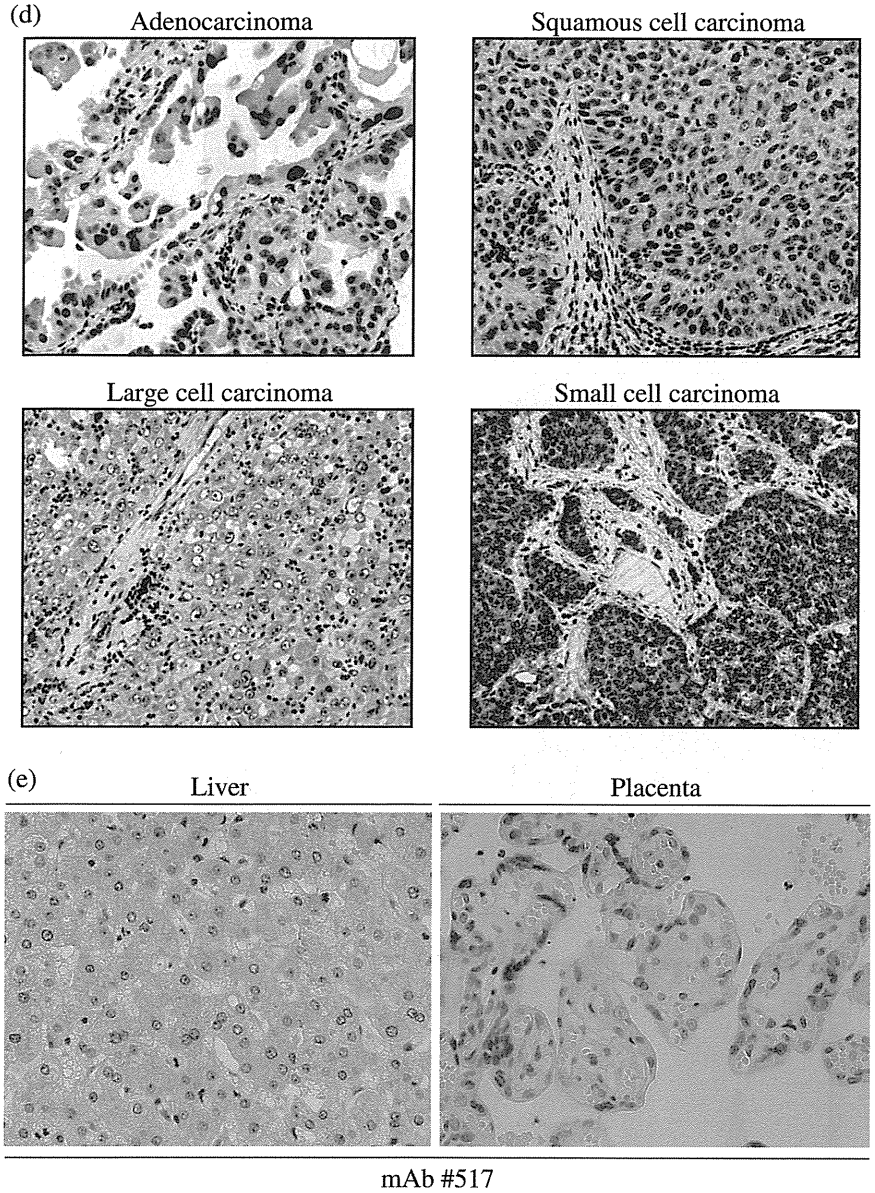

Fig. 3. *Continued.*

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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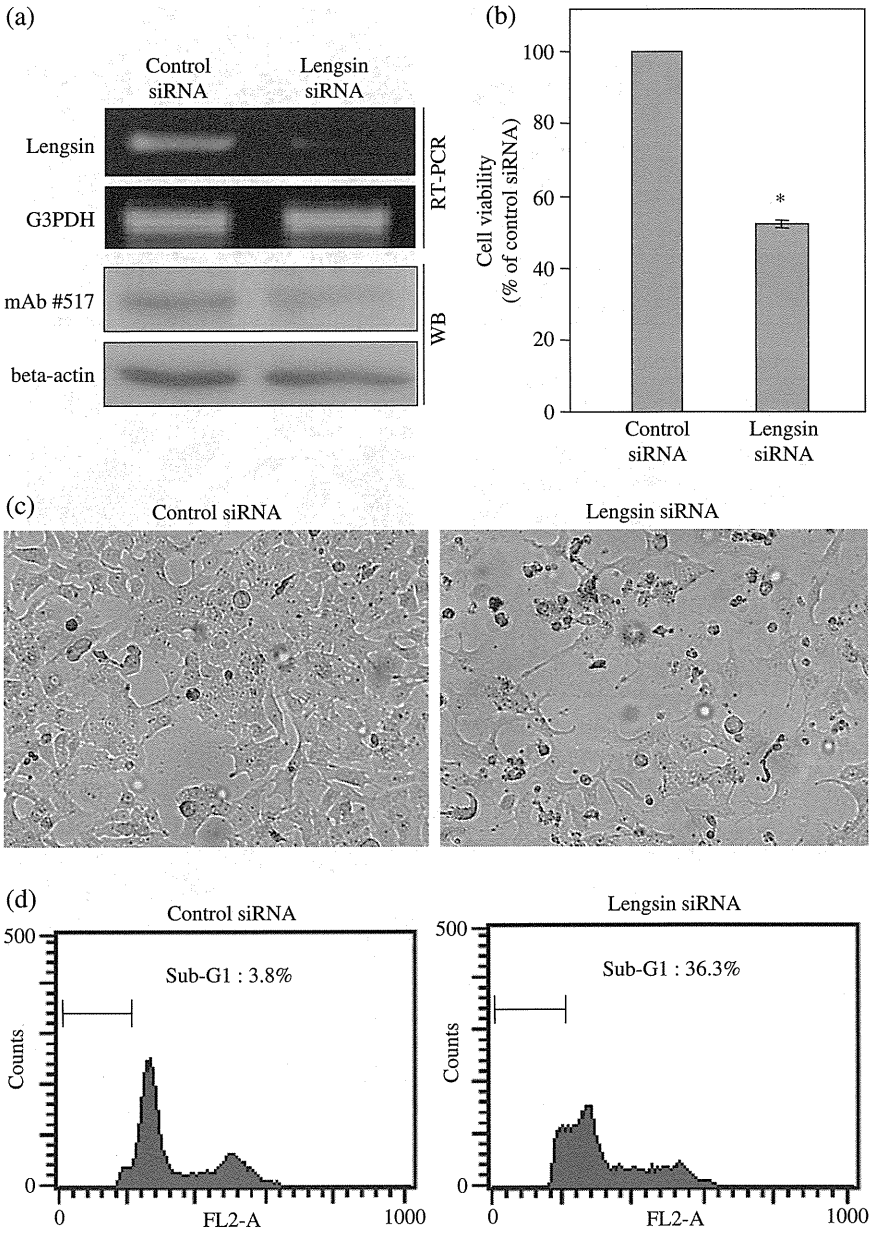

Fig. 4. Effect of Lengsin on cell viability in lung cancer cell line 1-87. (a) Gene silencing was performed using Lengsin siRNA. RT-PCR analysis was done using Lengsin primer pair I. G3PDH was used as an internal control. (b) WST-1 assay shows a decrease in the numbers of viable cells after knockdown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

Phase I clinical study of anti-apoptosis protein survivin-derived peptide vaccination for patients with advanced or recurrent urothelial cancer

Ichiya Honma · Hiroshi Kitamura · Toshihiko Torigoe · Akari Takahashi · Toshiaki Tanaka · Eiji Sato · Yoshihiko Hirohashi · Naoya Masumori · Taiji Tsukamoto · Noriyuki Sato

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Abstract Survivin, a member of the inhibitor of apoptosis protein family, is expressed in many malignant tumors including urothelial cancer but is hardly detectable in normal, differentiated adult tissues. Previously we reported CD8-positive cytotoxic T-lymphocytes (CTLs) were successfully induced by stimulation with survivin-2B80-88 peptide in vitro. We started a phase I clinical study of survivin-2B80-88 peptide vaccination for advanced urothelial cancer patients to assess the safety and efficacy of this vaccination. Nine patients were received vaccination and were evaluated for immunological evaluation, adverse events, and clinical responses. A total of 46 vaccinations were carried out. There was no severe adverse event. HLA-A24/survivin-2B80-88 peptide tetramer analysis revealed a significant increase in the peptide-specific CTL frequency after the vaccination in five patients. Slight reduction of the tumor volume was observed in one patient. Survivin-2B80-88 peptide-based vaccination is safe and should be further considered for potential immune and clinical efficacy in urothelial cancer patients.

I. Honma (✉) · H. Kitamura · T. Tanaka · E. Sato · N. Masumori · T. Tsukamoto
Department of Urology, Sapporo Medical University School of Medicine, Sapporo, Japan
e-mail: ichiya@sapmed.ac.jp

T. Torigoe · Y. Hirohashi · N. Sato
Department of Pathology, Sapporo Medical University, Sapporo, Japan

A. Takahashi
Innovation Plaza Hokkaido, Japan Science and Technology Corporation, Sapporo, Japan

Keywords Immunotherapy · Survivin · Peptide vaccination · Urothelial cancer

Abbreviations

CR	Complete response
CT	Computed tomography
CTL	Cytotoxic T-lymphocyte
DTH	Delayed-type hypersensitivity
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSP	Heat shock protein
IFA	Incomplete Freund's adjuvant
IFN	Interferon
NC	No change
PBMC	Peripheral blood mononuclear cell
PD	Progressive disease
PR	Partial response

Introduction

Increasing numbers of T-lymphocyte epitopes derived from various cancer-associated antigens have been reported, and they have been proved to play significant roles in cytotoxic T-lymphocyte (CTL)-based immunotherapy [1]. Survivin, a member of the inhibitor of apoptosis protein family, is expressed in various malignant tumors but is undetectable in normal and differentiated adult tissues [2–4]. Because of its cancer-specific expression, survivin might be an attractive target for immunotherapy via CTL responses.

We previously reported that survivin and its splicing variant survivin-2B were expressed abundantly in various cancer tissues and cancer cell lines, including urothelial cancer, and were suitable as target antigens for active-specific anticancer immunization [5]. Subsequently, we identified the