

# Aberrant Expression and Potency as a Cancer Immunotherapy Target of Inhibitor of Apoptosis Protein Family, Livin/ML-IAP in Lung Cancer

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## ABSTRACT

**CD8<sup>+</sup> CTLs have an essential role in immune response against tumor. Although an increasing number of tumor-associated antigens that can be recognized by CTLs have been identified from human tumors, a limited number of tumor-associated antigens is known in lung cancer. In addition, because some of them are expressed in noncancerous tissues, there exist limitations in their application to tumor immunotherapy. Livin/ML-IAP is one of recently identified inhibitor of apoptosis protein (IAP) family, which is overexpressed in melanoma cells. In this report, we show that Livin/ML-IAP is aberrantly expressed in many lung cancer cell lines and primary lung cancer tissues, whereas it is not detectable in normal tissues, including lung by reverse transcription-PCR methods. To identify HLA-A24-restricted T-cell epitopes of Livin/ML-IAP, eight peptides were selected from the amino acid sequence of this protein and screened for their binding affinity to HLA-A24. It was revealed that Livin7 peptide (amino acid sequence, KWFPSCQFLL) had the highest affinity to HLA-A24. By stimulating peripheral blood lymphocytes of HLA-A24-positive lung cancer patients with Livin7 peptide *in vitro*, the peptide-specific CTLs were successfully induced from four of five patients with Livin/ML-IAP-positive lung cancer but not from any of four patients without Livin/ML-IAP expression in their cancer tissues. Furthermore, the CTLs induced by Livin7 peptide showed cytotoxicity against Livin/ML-IAP<sup>+</sup> lung cancer cell**

**lines in an HLA-A24-restricted manner. Our data suggest that Livin/ML-IAP may be an excellent target antigen in immunotherapy for lung cancer and Livin7 peptide may serve as a potent peptide vaccine for HLA-A\*2402<sup>+</sup>/Livin<sup>+</sup> lung cancer patients.**

## INTRODUCTION

Lung cancer is one of the most frequent malignancies in many countries. Despite recent progress in chemotherapeutic, radiotherapeutic, and surgical treatment, the 5-year survival rate of lung cancer patients is still low, especially in advanced cases. Hence, a new treatment modality is awaited, and recent progress in the understanding of tumor immunology and immunotherapy has raised expectations that cancer immunotherapy may be within a distinct possibility.

In the past decade, to achieve a successful tumor-specific immunotherapy, large numbers of tumor-associated antigens that can be recognized by CTLs have been identified (1, 2). Of these antigens, antigenic peptide derived from tumor-specific antigens like *MAGE* gene family and melanoma differentiation antigens like pmel17/gp100 were applied for pioneering studies of tumor immunotherapy in cases of advanced melanoma patients (3, 4). Although most reports were still in phase I study, it is noteworthy that tumor regression could be observed in some cases, suggesting that tumor immunotherapy with such antigenic peptides might become a new treatment modality for melanoma patients. In contrast to melanoma, nonmelanocytic tumors generally have lower antigenicity, and the difficulty in establishing CD8<sup>+</sup> CTLs from patients' peripheral blood mononuclear cells (PBMCs) or tumor-infiltrating lymphocytes has delayed identification of tumor-associated antigens expressed in such tumors.

Livin, also known as melanoma inhibitor of apoptosis protein (ML-IAP) and kidney inhibitor of apoptosis protein, has been identified as a new member of the IAP family proteins with one baculovirus IAP repeat domain and COOH-terminal RING finger domain (5–7). Like other IAP family proteins, Livin/ML-IAP interacts with downstream caspases, such as caspase-3, caspase-7, and caspase-9, leading to their inactivation and degradation. Its overexpression can protect cells from several proapoptotic stimuli (i.e., tumor necrosis factor, Fas, DR4, and DR5 stimuli). Very importantly, treatment of cancer cells with Livin/ML-IAP antisense oligo DNA causes apoptotic cell death, indicating that Livin/ML-IAP expression may be essential for survival of certain cancer cells.

Of the IAP family members, cIAP-1, cIAP-2, XIAP, and NAIP are expressed in normal adult tissues, whereas Survivin expression is limited to tumor tissues. It has been reported that Livin/ML-IAP was expressed in some tumor cells (5, 6, 8) and several fetal tissues but not in normal adult tissues. Hence, its expression profiles seem to be very similar to those of Survivin, a cancer-specific IAP family protein (9).

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In this report, we focused on the characteristics of Livin/ML-IAP as a novel lung cancer antigen. We show that Livin/ML-IAP expression was detected in certain lung cancer cell lines and primary lung cancer tissues by reverse transcription-PCR (RT-PCR) and immunohistochemical staining. However, its expression could not be detected in normal adult tissues by the most sensitive RT-PCR method. We identified several 9-mer or 10-mer peptides with HLA-A24-binding motif derived from Livin/ML-IAP protein, and one of the peptides termed Livin7 (KWFPSQFLL) had the highest binding affinity to HLA-A24 molecule. By stimulating PBMCs from *HLA-A\*2402*<sup>+</sup>/Livin<sup>+</sup> lung cancer patients with Livin7 peptide, CTLs specific for the peptide could be successfully induced. CTLs could not be induced from PBMCs of *HLA-A2402*<sup>+</sup> lung cancer patients with immunohistochemically Livin/ML-IAP-negative lung cancer. In addition, CTLs induced by Livin7 peptide were capable of exerting cytotoxicity on Livin/ML-IAP<sup>+</sup> lung cancer cell line in an HLA-A24-restricted manner. These data highlight Livin/ML-IAP as a potent target for immunotherapy of lung cancer, and raise the possibility that Livin7 peptide may be suitable for the peptide-based vaccine for *HLA-A\*2402*<sup>+</sup> lung cancer patients.

## MATERIALS AND METHODS

**Patients and Samples.** The surgically resected tissue specimens and PBMC used in this study were obtained from *HLA-A\*2402*<sup>+</sup> lung cancer patients who were hospitalized at Sapporo Medical University Hospital or Sapporo Hospital of Hokkaido Railway Company (Sapporo, Japan) after obtaining their informed consent.

**Cell Lines and Culture Media.** Lung adenocarcinoma cell lines LHK-2 (*HLA-A\*0207*, *A\*2402*, *B\*4601*, *B\*4801*, and *Cw\*0102*) and LNY-1 (*HLA-A26*, *A31*, *B51*, *B54*, *C4*, and *C10*) were established in our laboratory. Lung squamous cell carcinoma cell lines LK79 and Sq-1, adenocarcinoma cell line 1-87, and small cell carcinoma cell line Lu65 were kindly provided by Dr. S. Kobayashi (Department of Surgery, Institute of Developmental Aging and Cancer, Tohoku University, Sendai, Japan). Melanoma cell line LG-2 mel was kindly provided by Dr. B.J. Van den Eynde (Ludwig Institute for Cancer Research, Brussels, Belgium). Melanoma cell line 888 mel (*HLA-A1*, *A24*) and 1353 mel (*HLA-A26*, *A31*) was kindly provided by Dr. F.M. Marincola (Department of Transfusion Medicine, Clinical Center, NIH, Bethesda, MA). Lung adenocarcinoma cell line A549 and erythroleukemia cell line K562 were purchased from American Type Culture Collection (Manassas, VA). Lung small cell carcinoma cell line SBC-2 was purchased from Japanese Cancer Research Resources Bank. All these cell lines were cultured in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10 % fetal bovine serum (Filtron, Brooklyn, Australia). C1R-A\*2402, a stable transfectant of *HLA-A\*2402* cDNA of C1R cells (a kind gift from Dr. M. Takiguchi, Kumamoto, Japan) was cultured in RPMI 1640 supplemented with 10 % fetal bovine serum and 500 µg/mL of hygromycin B (WAKO chemicals, Osaka, Japan). T2-A\*2402, a stable transfectant of *HLA-A\*2402* cDNA of T2 cells (a kind gift from Dr. K. Kuzushima, Aichi Cancer Research Institute, Nagoya, Japan)

was cultured in RPMI 1640 supplemented with 10 % fetal bovine serum and 800 µg/mL of G418 (Invitrogen Life Technologies Co., Carlsbad, CA). LNY-1-A\*2402 and SBC-2-A\*2402, a stable transfectant of each cells with *HLA-A\*2402* cDNA was cultured in RPMI 1640 supplemented with 10 % fetal bovine serum and 500 ng/mL of puromycin (Sigma).

**Production and Purification of Anti-Livin/ML-IAP Antibody.** Polyclonal antibody against Livin/ML-IAP was generated by immunizing rabbits eight times every week with recombinant His-tag Livin/ML-IAP protein, which was produced and purified by Ni-NTA agarose column (Qiagen, Valencia, CA) as previously described (10). The serum immunoglobulin was precipitated with ammonium sulfate and resolved into PBS, followed by dialysis overnight in a membrane (Spectrum Laboratories, Inc., Ft. Lauderdale, FL) to remove ammonium sulfate.

**Immunohistochemical Staining of Tissue Sections.** Immunohistochemical staining was done with formalin-fixed paraffin-embedded sections of surgically resected tumor specimens of clear cell sarcoma (malignant melanoma of soft tissue), and biopsy specimens of lung cancers. Four- to 5-µm-thick sections were deparaffinized in xylene and rehydrated in graded alcohols. Antigen retrieval was done by boiling sections for 20 minutes in a microwave oven in preheated 0.01 mol/L sodium citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by 3 % hydrogen peroxide in ethanol for 10 minutes. After blocking with 1% nonfat dry milk in PBS (pH 7.4), the sections were reacted with rabbit polyclonal anti-Livin/ML-IAP antibody at 25 µg/mL or preimmune sera for 1 hour, followed by incubation with biotinylated goat anti-rabbit IgG (Nichirei, Tokyo, Japan) for 30 minutes. Subsequently, the sections were stained with streptavidin-biotin complex (Nichirei), followed by incubation with 3,3-diaminobenzidine used as the chromogen and counterstaining with hematoxylin.

**RT-PCR Analysis.** Multiple Tissue cDNA Panels (BD Biosciences Clontech, Palo Alto, CA) were used as a template of normal tissue cDNA. Total RNA was isolated from cultured cells and tumor tissues by using ISOGEN reagent (Nippon Gene, Tokyo, Japan). cDNA mixture was synthesized from 1 µg of total RNA by reverse transcription (RT) using Superscript II and oligo (dT) primer (Invitrogen Life Technologies) according to the manufacture's protocol. PCR amplification was done in 25 µL of PCR mixture containing 0.25 µL of the cDNA mixture, 0.5 µL of KOD Plus DNA polymerase (TOYOBO, Osaka, Japan), and 15 pmol of primers. The PCR mixture was initially incubated at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds and extension at 68°C for 1 minute. For specific detection of Livin/ML-IAP, the primer pairs 5'-CTCCTTCTAT-GACTGGC-3' and 5'-ACACTCAGCACAGACC-3' were employed as a forward and a reverse primer, respectively. The expected size of PCR product for Livin/ML-IAP is 496 bp. For an internal control, G3PDH expression was detected by using forward primer 5'-ACCACAGTCCATGCCATCAC-3' and reverse primer 5'-TCCACCACCCTGTTGCTGTA-3' with

an expected PCR product of 452 bp. The PCR products were visualized with ethidium bromide staining under UV light following electrophoresis on 1.0% agarose gel. The nucleotide sequence of the PCR products was confirmed by direct sequencing using an ABI Genetic analyzer PRISM 310 and an AmpliCycle sequencing kit (Perkin-Elmer, Foster City, CA).

**Peptides and Cytokines.** Livin/ML-IAP-derived peptides carrying HLA-A24 binding motif Livin1 (AWDHVGQI), Livin2 (AWDHVGQIL), Livin3 (AFPGMGSEEL), Livin4 (GMGSEELRL), Livin5 (PWTEHAKWF), Livin6 (KWFPSCQFL), Livin7 (KWFPSCQFLL), Livin8 (WFPCQFLL), EBV LMP2-derived HLA-A24 binding peptide (TYGPFVMSL; ref. 11), HIV env-derived HLA-A24 binding peptide (RYLRDQQLGI; ref. 12), HLA-A31 binding peptide F4.2 (YSWMDISCWI; ref. 13) and synovial sarcoma chromosomal translocation product SYT-SSX-derived HLA-A24 binding peptide (PYGYDQIMP; ref. 14) were purchased from Sigma Genosis (Ishikari, Japan). The peptides were dissolved in DMSO at the concentration of 5 mg/mL and stored at  $-80^{\circ}\text{C}$ . Human recombinant interleukin (IL)-2, IL-4, and granulocyte macrophage colony-stimulating factor were a kind gift from Takeda Pharmaceutical Co. (Osaka, Japan), Ono Pharmaceutical Co. (Osaka, Japan), and Novartis Pharmaceutical (Basel, Switzerland), respectively. Human recombinant IL-7 was purchased from Invitrogen Life Technologies.

**Peptide Binding Assay.** Peptide binding affinity to HLA-A24 molecule was assessed by HLA-A24 stabilization assay as described previously (12), based on the findings that MHC class I molecules could be stabilized on the cell surface in the presence of binding peptides. RMA-S-A\*2402/Kb cells are TAP-deficient mouse RMA-S cells transfected with a chimeric MHC class I cDNA consisting of  $\alpha 1$  and  $\alpha 2$  domains derived from human HLA-A\*2402 molecule and  $\alpha 3$ , transmembrane, and intracellular domains derived from mouse H-2Kb molecule (kindly provided from Dr. H. Takasu, Sumitomo Pharmaceutical, Osaka, Japan). After incubation of cells in culture medium at  $26^{\circ}\text{C}$  for 18 hours, cells ( $2 \times 10^5$ ) were washed with PBS and suspended with 1 mL of Opti-MEM (Life Technologies) containing 3  $\mu\text{g}/\text{mL}$  of  $\beta 2$ -microglobulin with or without 100  $\mu\text{g}$  of peptide, followed by incubation at  $26^{\circ}\text{C}$  for 3 hours and then at  $37^{\circ}\text{C}$  for 3 hours. After washing with PBS, the cells were incubated with anti-HLA-A24 monoclonal antibody at  $4^{\circ}\text{C}$  for 30 minutes, followed by incubation with FITC-conjugated rabbit anti-mouse IgG at  $4^{\circ}\text{C}$  for 30 minutes. The cells were then suspended with 1 mL of PBS containing 1% formaldehyde, and analyzed by FACScan (Becton Dickinson, Mountain View, CA). Binding affinity was evaluated by comparing mean fluorescence intensity (MFI) of HLA-A24 expression in the presence of peptide pulsation to MFI in the absence of the peptide.

**Peptide-Specific CTL Induction with Immature Dendritic Cell and Phytohemagglutinin Blast.** CTLs were induced from PBMCs of cancer patients by using autologous dendritic cells (DC) and phytohemagglutinin (PHA) blasts as antigen-presenting cells (APC; refs. 14, 15). Briefly, PBMCs were isolated from blood of cancer patients by using Lymphoprep (Nycomed, Oslo, Norway) and cultured in AIM-V medium (Life

Technologies) at  $37^{\circ}\text{C}$  for 1 hour, then separated into adherent cells and nonadherent cells. Autologous immature DC were generated from adherent cells in the plastic flask by culturing in AIM-V medium supplemented with HEPES (10 mmol/L), 2-mercaptoethanol (50  $\mu\text{mol}/\text{L}$ ), granulocyte macrophage colony-stimulating factor (1,000 units/mL), and IL-4 (1,000 units/mL) for 7 days.  $\text{CD8}^{+}$  cells were isolated from nonadherent cells in the plastic flask by using MACS separation system (Miltenyi Biotech, Bergish Blabach, Germany) and anti-CD8 monoclonal antibody coupled with magnetic microbeads according to the manufacturer's instruction. PHA blasts were derived from  $\text{CD8}^{-}$  cells by culturing in AIM-V medium supplemented with HEPES (10 mmol/L), 2-ME (50  $\mu\text{mol}/\text{L}$ ), IL-2 (100 units/mL), and PHA (1  $\mu\text{g}/\text{mL}$ ) for 3 days followed by culture in AIM-V medium without PHA for 4 days. APCs (DC and PHA blasts) were cultured in AIM-V medium supplemented with 50  $\mu\text{mol}/\text{L}$  of peptide at room temperature for 1 hour, followed by washing with AIM-V thrice, then irradiated (100 Gy) and used for stimulation of CTL. CTL induction procedure was initiated by stimulating  $2 \times 10^6$   $\text{CD8}^{+}$  cells with peptide-pulsed autologous DC at a 20:1 effector/APC ratio in AIM-V supplemented with HEPES, 2-ME, and IL-7 (10 ng/mL) for 7 days at  $37^{\circ}\text{C}$ . The following stimulation was done with peptide-pulsed PHA blasts at a 5:1 effector/APC ratio. On the next day of the 2nd stimulation, IL-2 was added to the culture at a concentration of 50 units/mL. The same CTL stimulation cycle with PHA blasts was then done twice more over the period of 2 weeks. One week after the 4th stimulation, cytotoxic activity of the CTL was measured by  $^{51}\text{Cr}$  release assay.

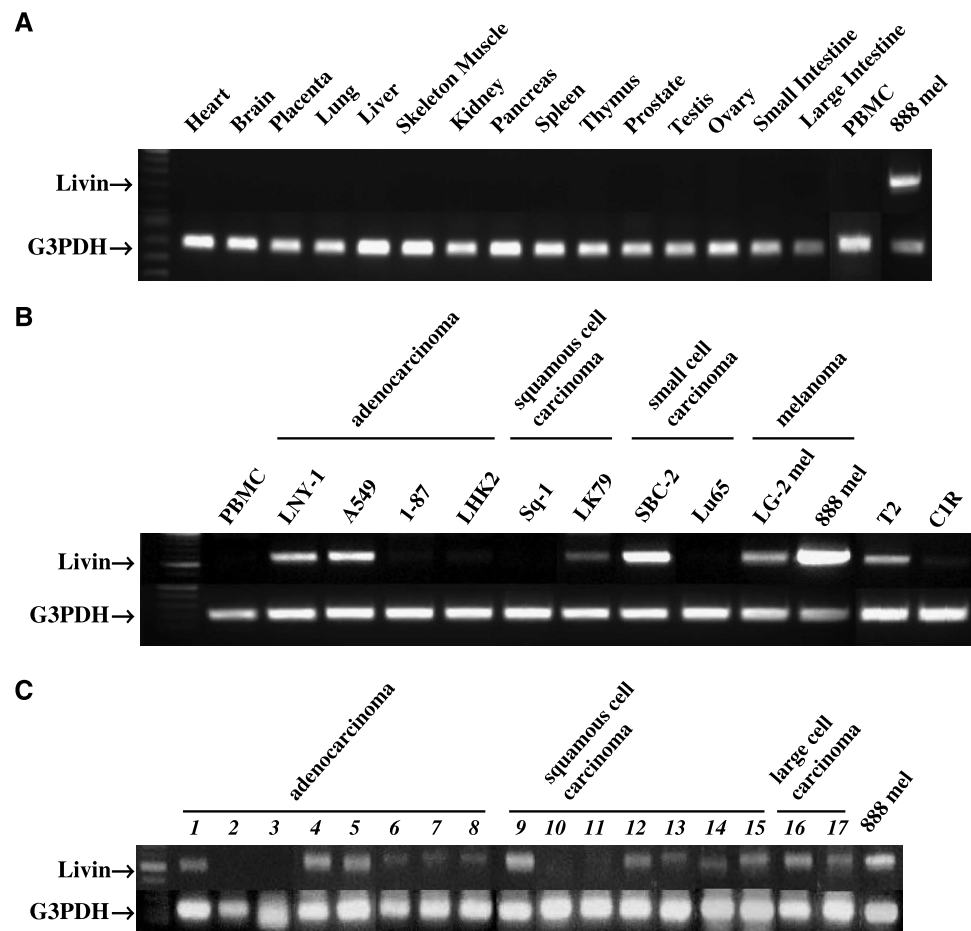
**Cytotoxicity Assay.** The cytotoxic activities of CTLs were measured by  $^{51}\text{Cr}$  release assay as described previously (16). Briefly, target cells were labeled with 100  $\mu\text{Ci}$  of  $^{51}\text{Cr}$  for 1 hour at  $37^{\circ}\text{C}$  and washed with RPMI 1640 thrice. Then,  $2 \times 10^3$   $^{51}\text{Cr}$ -labeled target cells were incubated with effector cells at various effector/target ratios at  $37^{\circ}\text{C}$  for 6 hours in V-bottomed 96-well microtiter plates. Then supernatants were collected and the radioactivity was measured with a gamma-counter. % Specific lysis was calculated as follows: % specific lysis = (test sample release – spontaneous release)  $\times$  100/(maximum release – spontaneous release). For preparation of peptide-pulsed target cells, target cells were incubated with 1  $\mu\text{g}/\text{mL}$  of peptide at room temperature for 1 hour before the assay.

## RESULTS

### Livin/ML-IAP Is Expressed in Lung Cancer Cell Lines and Primary Tissues, but not in Normal Adult Tissues.

Livin/ML-IAP has been primarily reported to be overexpressed in malignant melanoma cells. In this report, Livin/ML-IAP expression profiles in normal adult tissues and lung cancers were analyzed by RT-PCR method. We first studied Livin/ML-IAP expression in normal adult tissues including heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, large intestine, and PBMC. As shown in Fig. 1A, no overt expression of Livin/ML-IAP mRNA was observed in these normal adult tissues on the condition of detecting the expression of G3PDH mRNA. To test

**Fig. 1** Expression profiles of Livin/ML-IAP as assessed by RT-PCR in normal tissues, lung cancer cell lines, and primary lung cancer tissues. **A**, expression of Livin/ML-IAP in normal tissues including heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, large intestine, and PBMC. 888 mel melanoma cell line was used as a positive control for Livin/ML-IAP expression. G3PDH expression was detected as an internal control. **B**, expression of Livin/ML-IAP in lung cancer cell lines. PBMCs from a healthy volunteer were used as a negative control. **C**, expression of Livin/ML-IAP in primary lung cancer tissues, including adenocarcinoma, squamous cell carcinoma, and large cell carcinoma.



the expression of Livin/ML-IAP in lung cancers, several lung cancer cell lines were examined by RT-PCR. As shown in Fig. 1B, Livin/ML-IAP mRNA was expressed in two of four lung adenocarcinoma cell lines (LNY-1, A549), one of two squamous cell carcinoma cell lines (LK-79), and one of two small cell carcinoma cell lines (SBC-2). Nucleic acid sequences of the Livin/ML-IAP-specific band was confirmed by DNA sequence analysis (data not shown). These data indicate that Livin/ML-IAP is aberrantly expressed in certain lung cancer cell lines. We then analyzed the Livin/ML-IAP expression in primary lung cancer tissue specimens. As shown in Fig. 1C, the expression of Livin/ML-IAP was detected in certain lung cancer tissues in six of eight (75%) adenocarcinoma (lanes 1-8), five of seven (70%) squamous cell carcinoma (lanes 9-15) and two of two (100%) large cell carcinoma specimens (lanes 16 and 17). These data indicate that Livin/ML-IAP was expressed in primary lung cancer tissues as well as lung cancer cell lines with considerable frequency independently of their histologic types.

**Immunohistochemical Staining of Livin/ML-IAP.** To detect the Livin/ML-IAP protein expressed in lung cancer tissues, we generated Livin/ML-IAP-specific polyclonal antibody suitable for immunohistochemical staining. Livin/ML-IAP-specific reactivity of the polyclonal antibody was

confirmed by Western blotting (data not shown). We used clear cell sarcoma (malignant melanoma of soft tissue) as a positive control since this tumor expressed high levels of Livin/ML-IAP protein and lacked melanin pigment, thereby being suitable for immunohistochemical staining. As shown in Fig. 2A and B, Livin/ML-IAP was stained clearly in both cytoplasm and nucleus of this sarcoma. In Fig. 2C and D, representative staining of a lung adenocarcinoma tissue section is shown, indicating cytoplasmic and nuclear expression of Livin/ML-IAP in lung cancer cells.

**Binding Analysis of Livin/ML-IAP-Derived Peptides to HLA-A24 Molecules.** Because Livin/ML-IAP is expressed in lung cancer cells but not in normal tissues, we hypothesized that it might be a suitable target for tumor immunotherapy. Immune tolerance toward Livin/ML-IAP is considered to be very weak because anti-Livin/ML-IAP autoantibody is produced in many cancer patients (10, 17). Thus, it was reasoned that antigenic peptides derived from Livin/ML-IAP might be presented by MHC class I molecules as well as MHC class II and recognized by CD8<sup>+</sup> T cells. To evaluate if Livin/ML-IAP might become a target of CTLs, we focused on HLA-HLA-A\*2402 allele because of its high frequency worldwide. The total amino acid sequence of Livin/ML-IAP was searched for peptides that have HLA-A24 binding motif as 9- or 10-mer peptide with Y, F, M, or W at 2nd

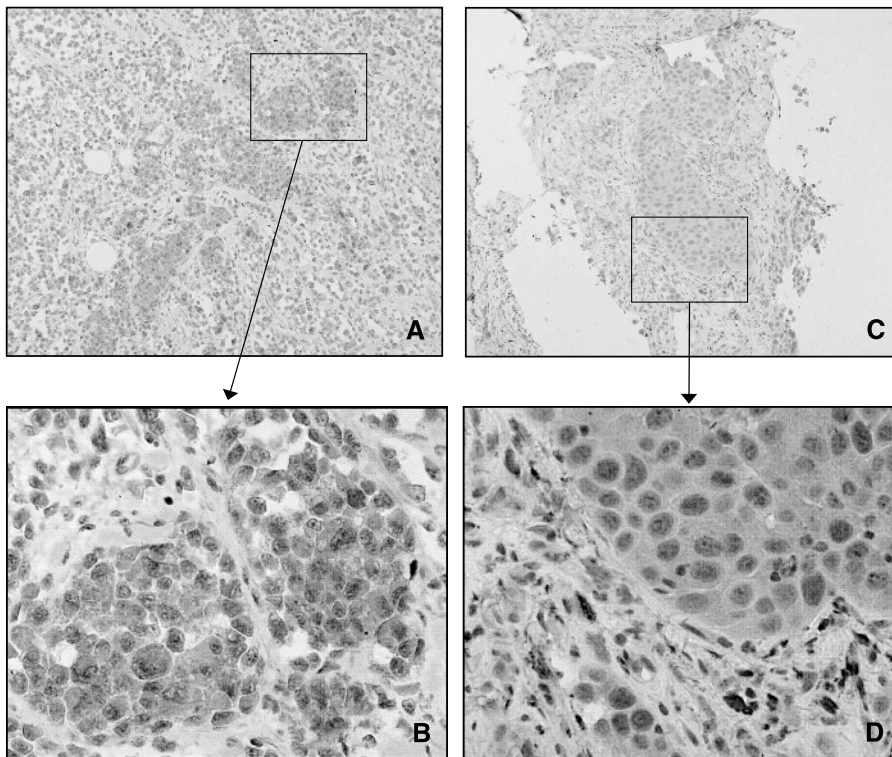


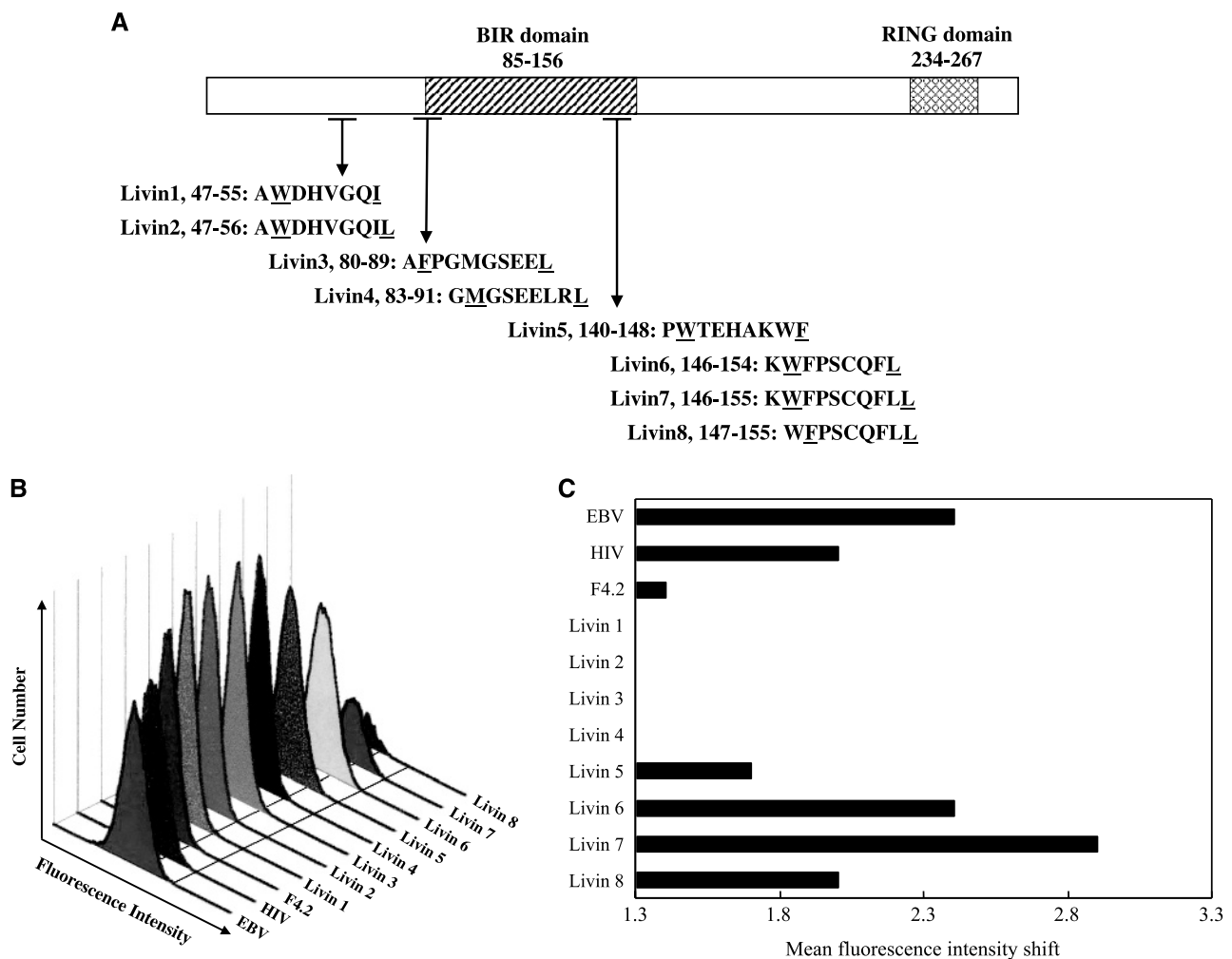
Fig. 2 Immunohistochemical staining of Livin/ML-IAP in clear cell sarcoma (malignant melanoma of soft tissue, A and B) and lung adenocarcinoma (C and D). Bottom, magnified views of top.

position and L, I, F, or M at COOH-terminal position (18). Consequently, we found eight peptides (Livin1-8) carrying HLA-A24 binding motif (Fig. 3A), and to assess their binding ability to HLA-A24 molecule, binding assay using transporters associated with antigen processing (TAP) deficient and HLA-A\*2402-transfected cell line, RMA-S-A\*2402/Kb, was done as described previously (12). Two positive control peptides, HLA-A24-restricted EBV epitope and HIV epitope, and negative control peptide, HLA-A31-bound F4.2 peptide, were used in the assay. HLA-A24 level on the cell surface of RMA-S-A\*2402/Kb cells is up-regulated in the presence of HLA-A24-binding peptides. Up-regulation of MFI of cell surface HLA-A24 was detected by flow cytometer (Fig. 3B and C). Both EBV peptide and HIV peptide increased MFI of HLA-A24 clearly (EBV = 2.4 and HIV = 2.0), whereas F4.2 peptide failed (F4.2 = 1.4), indicating adequate qualification of this assay system. Livin1, Livin2, Livin3, and Livin4 peptides could not increase cell surface HLA-A24 level (Livin1, Livin2, Livin3, Livin4 = 1.3), whereas Livin5, Livin6, Livin7, and Livin8 peptides were capable of up-regulating the HLA level to a various extent (Livin5 = 1.7, Livin6 = 2.4, Livin7 = 2.9, and Livin8 = 2.0). It was noted that all those peptides that could bind to HLA-A24 shared overlapping sequences as shown in Fig. 3A. It was indicated that Livin7 might have the highest binding affinity to HLA-A24 molecule among all the peptides.

**CTL Induction from PBMCs of HLA-A\*2402+ Lung Cancer Patients.** To know which Livin/ML-IAP-derived peptides can be most recognized by T cells of cancer patients in the context of HLA-A24, we attempted to induce simultaneously each peptide-specific CTL and compare their

cytotoxic activity. Because Livin6, Livin7, and Livin8 shared the same amino acid sequences, we chose Livin7 peptide whose MFI is the highest of them and Livin 5 as another candidate. PBMCs were collected from one lung cancer patients with HLA-A\*2402, and T cells sorted out from the PBMCs were incubated with peptide-pulsed autologous monocyte-derived DC or autologous PHA blasts (19). After four times stimulation, cytotoxic activity against peptide-pulsed target cells was examined by  $^{51}\text{Cr}$  release assay. As shown in Fig. 4A, CTLs induced from PBMCs by *in vitro* stimulation with either Livin5 peptide or Livin7 peptide could react specifically to the peptide-pulsed C1R-A\*2402 cells. However, the Livin5 peptide-specific CTLs failed to recognize HLA-A24<sup>+</sup>/Livin/ML-IAP<sup>+</sup> melanoma cell lines (Fig. 4B). Therefore, we determined that Livin7 peptide could be the best candidate for the CTL epitope presented by HLA-A\*2402.

CTL induction efficiency was examined by using Livin7 peptide-pulsed autologous APCs from PBMCs of nine lung cancer patients. As shown in Fig. 5, CTLs reacting specifically to Livin7 peptide-pulsed C1R-A\*2402 cells were successfully induced from four patients (patients 1, 2, 3, and 4), but not from the other five patients (patients 5, 6, 7, 8, and 9). The Livin7-specific CTLs had cytotoxicity against Livin7 peptide-pulsed T2-A\*2402 cells as well (data not shown). These data indicate that Livin7 peptide-specific CTLs could be induced from PBMCs of some HLA-A\*2402<sup>+</sup> lung cancer patients. Then, we studied the correlation between the immunohistochemical expression of Livin/ML-IAP in lung tumor tissues and CTL induction efficiency. Successful CTL induction was achieved from four (cases 1-4) out of five cases with Livin/ML-IAP expression in



**Fig. 3** Amino acid sequences of Livin/ML-IAP peptides with HLA-A24 binding motif and their binding affinity to HLA-A24 molecule. **A**, Livin/ML-IAP has one putative BIR domain and COOH-terminal RING domain. Eight peptides carrying HLA-A24 binding motif (Livin1-8) were synthesized. Bold amino acids, residues corresponding to the HLA-A24-binding anchor motif. **B**, binding affinity of Livin/ML-IAP-derived peptides to HLA-A24 molecule was evaluated by MFI of cell surface HLA-A24 molecule on RMA-S-A\*2402/Kb cells that were pulsed with each peptide. HLA-A24-bound EBV LMP2-derived peptide (*TYGPFVMSL*) and HIV env-derived peptide (*RYLRDQQLGI*) were used as positive controls. HLA-A31-bound F4.2 peptide (*YSWMDISCWT*) was used as a negative control. Histograms of MFI were displayed for each peptide. **C**, MFI increases of **B** for each peptide. MFI increase was calculated as MFI increase = (MFI of RMA-S-A\*2402/Kb cells pulsed with the peptide) – (MFI of RMA-S-A\*2402/Kb cells without peptide pulsation).

their primary lung cancer tissues (Table 1). In contrast, CTLs could not be induced from all four cases (cases 5-8), in which Livin/ML-IAP expression was not detected by immunohistochemical staining of primary lung cancer tissues. These observations suggested a positive correlation between Livin/ML-IAP protein expression and CTL induction efficiency both in squamous cell carcinoma and in adenocarcinoma of lung.

To confirm that CTLs induced with Livin7 peptide can react with Livin/ML-IAP-expressing cancer cells in the context of HLA-A\*2402, we examined cytotoxic activity against lung adenocarcinoma LNY-1-A\*2402 cells that express both endogenous Livin/ML-IAP and gene-transfected HLA-A\*2402 (Fig. 1B). As shown in Fig. 6, CTLs induced with Livin7 peptide from lung cancer patient's PBMCs exerted significant cytotoxicity against LNY-1-A\*2402 cells but not against LNY-1

lacking HLA-A24 expression or K562 cells. Similarly, we confirmed that CTLs induced from another *HLA-A\*2402*<sup>+</sup> lung cancer patient's PBMCs were cytotoxic against HLA-A\*2402-transfected small cell carcinoma line SBC-2-A\*2402 and HLA-A\*2402<sup>+</sup> melanoma cell line 888 mel (data not shown), both of which lines have high levels of Livin/ML-IAP expression (Fig. 1B). These data implied that Livin7 peptide-specific CTLs were capable of recognizing endogenously processed Livin7 peptide in an HLA-A24-restricted manner.

## DISCUSSION

This is the first report showing that recently identified IAP family Livin/ML-IAP is overexpressed in certain lung cancer cell lines and primary lung cancers. This protein is considered to be one of the universal lung cancer associated antigens because it

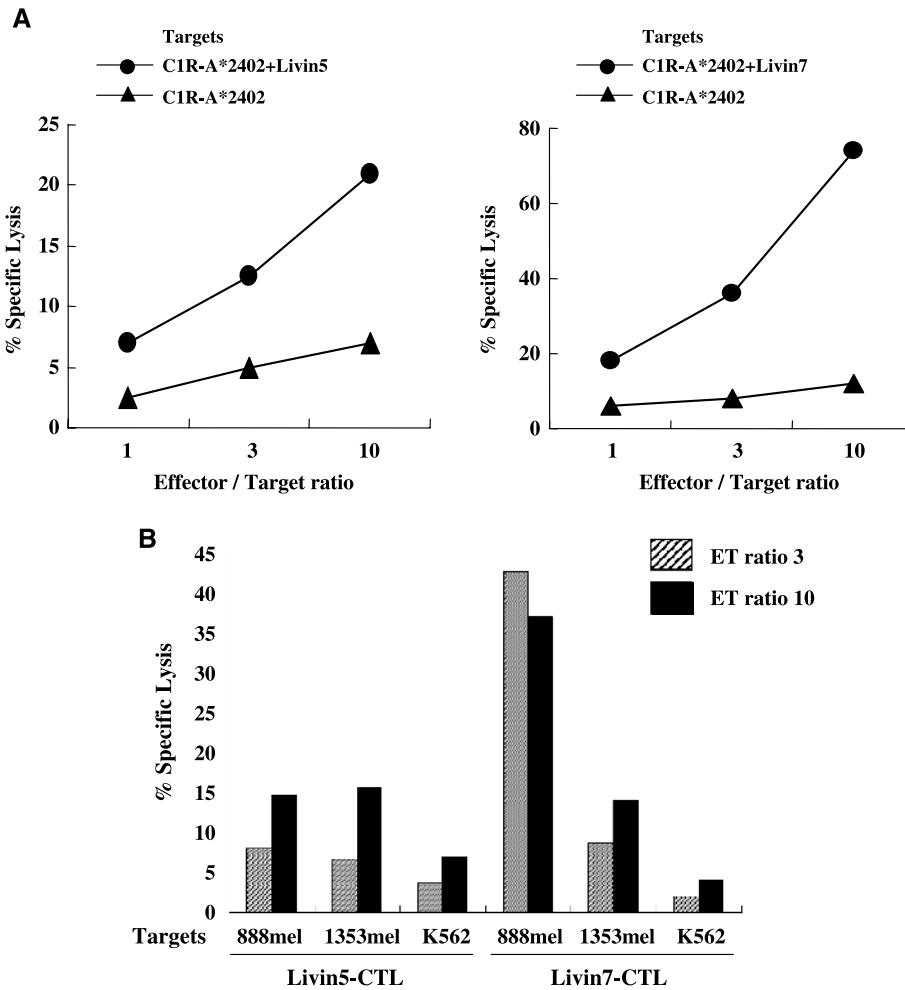


Fig. 4 Induction of Livin5 peptide- or Livin7 peptide-specific CTLs and their cytotoxicity against Livin/ML-IAP-expressing melanoma cell lines. *A*, CTLs were induced from PBMCs of an *HLA-A\*2402*<sup>+</sup> lung cancer patient by stimulating with Livin5 peptide- or Livin7 peptide-pulsed APCs. After four times stimulation, CTLs were subjected to standard <sup>51</sup>Cr release assay at the indicated effector/target ratio. Peptide-pulsed C1R-A\*2402 cells and nonpulsed cells were used as target cells. *B*, CTLs were induced as in *A*, and their cytotoxic activity against Livin/ML-IAP-expressing *HLA-A\*2402*<sup>+</sup> melanoma cell line 888 mel and *HLA-A\*2402*<sup>-</sup> melanoma cell line 1353 mel was examined by <sup>51</sup>Cr release assay at the indicated effector/target ratio. K562 target cells were used for monitoring natural killer activity and lymphokine-activated nonspecific cytotoxicity.

was expressed in small cell carcinoma and non-small cell lung carcinoma. Livin/ML-IAP is known to play an important role in antiapoptotic cell survival by suppression of caspase family proteins. The other antiapoptotic proteins, including IAP family and Bcl-2 family, were also reported to be overexpressed in lung cancer cells. Namely, Survivin, one of IAP family, was expressed in 85.5% of non-small cell lung cancer specimens as assessed by RT-PCR, and patients with Survivin expression had significant unfavorable prognosis (20). We have reported previously that Survivin was one of tumor-associated antigens recognized by both humoral and cellular immunity of cancer patients, and could become a target of CTL (19). Overexpression of Bcl-2 and Bcl-X<sub>L</sub> was also reported in lung cancer cells, and reduction of Bcl-2 or Bcl-X<sub>L</sub> by using antisense oligo DNA could lead to apoptotic death of certain cancer cells. However, expression of Bcl-2 family was not related to the patients' prognosis, indicating that it might be independent of the malignant potential of lung cancer (21). It has been reported that Livin/ML-IAP might be involved in the progression of superficial bladder cancer and used as a marker of early recurrence (8). Because loss of Livin/ML-IAP expression could lead to apoptotic cell death in cervical cancer cells (5), suppression of Livin/ML-IAP should have much advantage in

cancer treatment. From this perspective, Livin/ML-IAP might be a good candidate as a molecular target for the treatment as well as having a prognostic value for lung cancer. It is noted that cancer patients produced anti-Livin/ML-IAP antibody in their sera (10) whereas healthy individuals did not. This raises the possibility that Livin/ML-IAP might be one of immunodominant tumor-associated antigens targeted by cellular immunity as well as humoral immunity. Actually, there has been one report as to the HLA-A2-restricted Livin/ML-IAP-specific CTL induction in melanoma patients (17).

We identified HLA-A24-restricted antigenic peptide of Livin/ML-IAP. Four Livin/ML-IAP-derived peptides were shown to bind to HLA-A24 molecule, and we succeeded in inducing Livin5 peptide-specific CTL from PBMCs of one lung cancer patient as well as Livin7-specific CTL. However, the Livin5 peptide-specific CTLs had not high cytotoxic activity against Livin5 peptide-pulsed C1R-A\*2402 cells and failed to recognize HLA-A24<sup>+</sup>/Livin/ML-IAP<sup>+</sup> lung cancer cell lines, suggesting that Livin5 peptide might not be processed naturally nor presented by HLA-A24 molecule. Livin6, Livin7, and Livin8 share the same amino acid sequences, and among these peptides, Livin7 peptide showed the highest HLA-A24 binding affinity. Stimulation of PBMCs from *HLA-A24*<sup>+</sup>/Livin/ML-IAP<sup>+</sup>

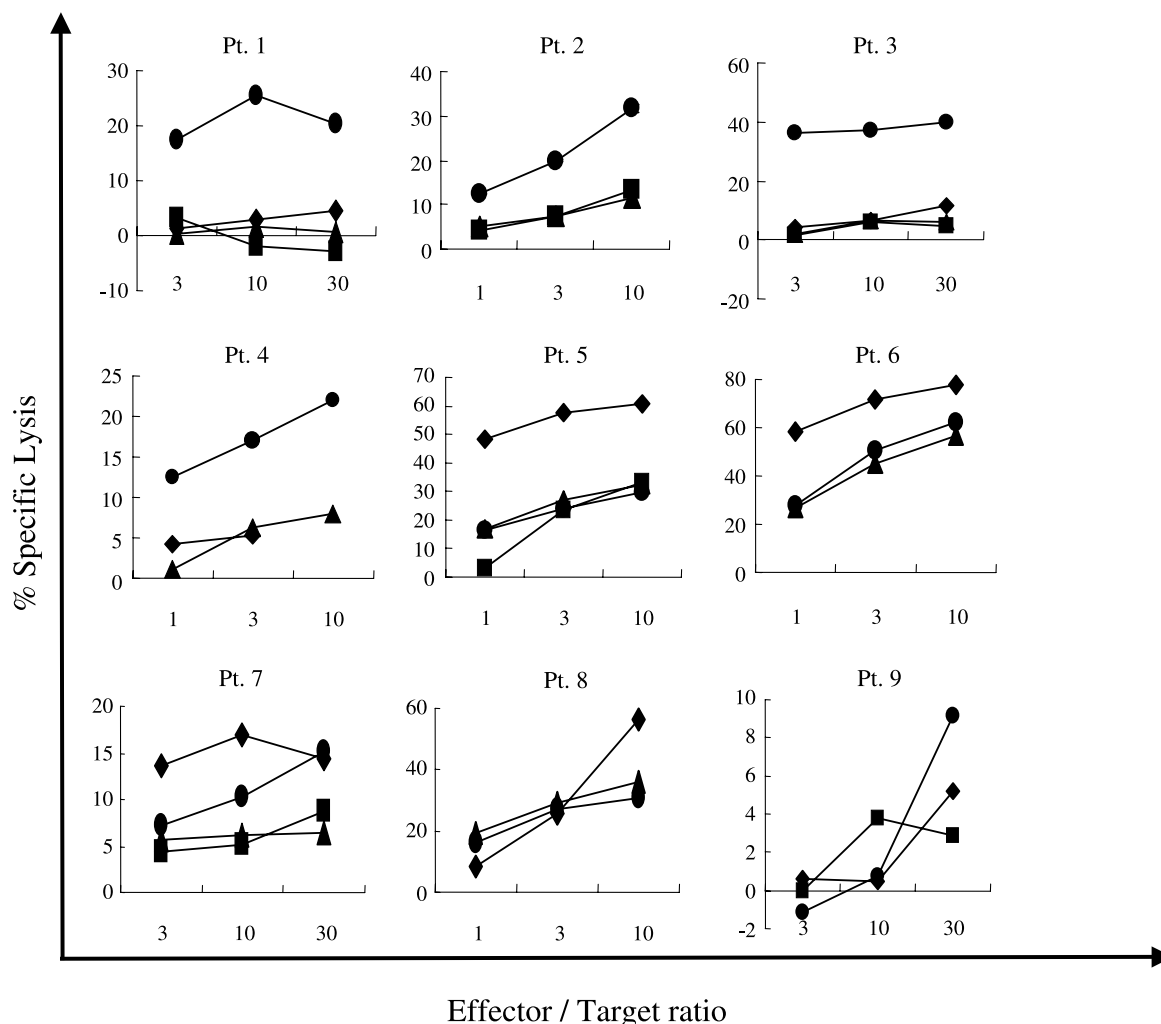


Fig. 5 Livin7 peptide-specific CTL induction from PBMCs of HLA-A\*2402+ lung cancer patients. CTLs were induced from PBMCs of nine HLA-A\*2402+ lung cancer patients by stimulating with Livin7 peptide-pulsed APCs and their cytotoxic activity was examined by <sup>51</sup>Cr release assay at the indicated effector/target ratio. Livin7 peptide- or SYT-SSX peptide-pulsed C1R-A\*2402 cells and nonpulsed cells were used as target cells. K562 target cells were used for monitoring natural killer activity and lymphokine-activated nonspecific cytotoxicity.

lung cancer patients with Livin7 peptide could lead to efficient induction of CTLs that exerted cytotoxicity against HLA-A24+/Livin/ML-IAP+ lung cancer cell lines. These data indicate that

Livin7 peptide might be one of the naturally processed antigenic peptides derived from Livin/ML-IAP with considerable immunogenicity, thus serving as a potent peptide vaccine in

Table 1 Livin/ ML-IAP expression and CTL induction in lung cancer patients

Case no.	Age	Sex	Histology	Stage*	Immunohistochemistry†	CTL induction‡
1	78	M	squamous cell carcinoma	T2N2M1	+	+
2	80	M	squamous cell carcinoma	T4N2M0	+	+
3	59	F	adenocarcinoma	T4N2M0	+	+
4	46	M	adenocarcinoma	T2N2M0	+	+
5	74	M	squamous cell carcinoma	T4N0M0	-	-
6	61	M	squamous cell carcinoma	T3N1M0	-	-
7	77	M	adenocarcinoma	T3N0M0	-	-
8	60	M	adenocarcinoma	T1N2M0	-	-
9	76	M	adenocarcinoma	T4N0M0	+	-

Abbreviations: F, Female; M, male.

\*Tumor-nodes-metastasis classification.

†(-) negative or (+) positive staining of cancer cells as compared with noncancerous normal tissues.

‡≥20% specific lytic activity at effector/target ratio = 10:1 against Livin7 peptide-pulsed C1R-A\*2402 target cells was described as (+) by <sup>51</sup>Cr release assay.



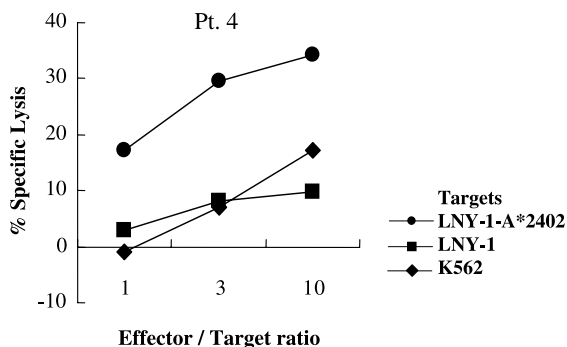


Fig. 6 Cytotoxic activity of Livin7-specific CTLs against HLA-A24<sup>+</sup> and Livin/ML-IAP<sup>+</sup> lung cancer cell line. CTLs were induced as in Fig. 5, and their cytotoxic activity against Livin/ML-IAP-expressing LNY-1 lung adenocarcinoma cells (HLA-A26, A31), HLA-A\*2402-transfected LNY-1 cells and K562 cells was examined by <sup>51</sup>Cr release assay at the indicated effector/target ratio.

immunotherapy for HLA-A\*2402<sup>+</sup> lung cancer patients. Livin/ML-IAP was reported to have two splicing variants with 54 nucleotides difference (Livin  $\alpha$  and Livin  $\beta$ ; ref. 22), which have different antiapoptotic effects in tumor cells. Because both splicing variants share the same amino acid sequence of Livin7, expression of either form should be able to produce Livin7 peptide presented by HLA-A24 molecule.

Schmollinger et al. showed that after several treatments of melanoma patients with lethally irradiated granulocyte macrophage colony-stimulating factor–transfected autologous melanoma cells, high titer anti-Livin/ML-IAP antibody was detected in the patients' sera, and B-cell and T-cell invasion was observed in the metastatic region. Furthermore, CD4<sup>+</sup> T cells and HLA-A2-restricted CD8<sup>+</sup> T cells from the metastatic regions responded against Livin/ML-IAP<sup>+</sup> tumor cells (17). We also have found that anti-Livin/ML-IAP antibody was detectable in 17 of 35 (47%) gastrointestinal cancer patients (10) as well as in lung cancer patients. These observations suggest the high antigenic potential of Livin/ML-IAP *in vivo*. Indeed, in our current study Livin/ML-IAP–specific CTLs were successfully induced from four out of five Livin/ML-IAP<sup>+</sup> lung cancer patients' PBMCs. There was a good correlation between Livin/ML-IAP expression detected by immunohistochemistry and CTL induction efficiency except for case 9. Considering that a chemotherapy had been done just before the examination in the patient of case 9, it is possible that CTL precursor frequency might be too low to respond to the peptide stimulation in this patient.

Chemotherapy resistance or radiotherapy resistance is one of the major issues in treatment of cancers. It has been reported that overexpression of IAP family proteins in cancerous cells and tissues aggravated chemoresistance or radioresistance (23, 24). There was one report that showed a correlation between chemoresistance and expression level of Livin/ML-IAP in melanoma cells (25). We have found also that chemoresistant variant cells had higher levels of Livin/ML-IAP as compared with the wild type lung cancer cells (data not shown). In chemoresistant cases of lung cancer patients, Livin/ML-IAP might become a predictive marker and a suitable target for tumor immunotherapy.

In conclusion, we showed that Livin/ML-IAP was one of potent immunogenic antigens of lung cancer and Livin7 peptide might serve as a tumor vaccine for HLA-A\*2402<sup>+</sup> patients. It is possible that Livin/ML-IAP–targeting therapy might become a rational modality in immunotherapy for lung cancer.

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