4 O'Reilly MS, Boehm T, Shing Y *et al.* Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* 1997; **88**: 277–85.
5 Fong TA, Shawver LK, Sun L *et al.* SU5416 is a potentand selective inhibitor of vascular endothelial growth factor receptor (Flk-1/KDR) that inhibits tyrosine kinase catalysis, tumor vascularization, and growth of multiple tumor types. *Cancer Res* 1999; **59**: 99–106.
6 Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med* 2003; **9**: 669–76.
7 Hurwitz H, Feherenbacher L, Novotny W *et al.* Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* 2004; **350**: 2335–42.
8 Gerber HP, Ferrara N. Pharmacology and pharmacodynamics of bevacizumab as monotherapy or in combination with cytotoxic therapy in preclinicals studies. *Cancer Res* 2005; **65**: 671–80.
9 Ferrara N, Kerbel RS. Angiogenesis as a therapeutic target. *Nature* 2005; **438**: 967–74.
10 Eklund L, Olsen BR. Tie receptors and their angiopoietin ligands are context-depedent regulators of vascular remodeling. *Exp Cell Res* 2006; **312**: 630–41.
11 Lin P, Polverini P, Dewhirst M, Shan S, Roao PS, Peters KG. Inhibition of tumor angiogenesis using a soluble receptor establishes a role for Tie-2 in pathologic vascular growth. *J Clin Invest* 1997; **100**: 2072–8.
12 Lin P, Buxton JA, Acheson A *et al.* Anti-angiogenic gene therapy targeting the endothelium-specific receptor tyrosine kinase Tie2. *Proc Natl Acad Sci USA* 1998; **95**: 8829–34.
13 Siemeister G, Schirner M, Weindel K *et al.* Two independent mechanisms essential for tumor angiogenesis: inhibition of human melanoma xenograft growth by interfering with either the vascular endothelial growth factor receptor pathway or the Tie-2 pathway. *Cancer Res* 1999; **59**: 3185–91.
14 Stratmann A, Acker T, Burger AM, Amann K, Risau W, Plate KH. Differential inhibition of tumor angiogenesis by Tie2 and vascular endothelial growth factor receptor-2 dominant-negative receptor mutants. *Int J Cancer* 2001; **91**: 273–82.
15 Hayes AJ, Huang WQ, Mallah J, Yang D, Lippman ME, Li LY. Angiopoietin-1 and its receptor Tie-2 participate in the regulation of capillary-like tubule formation and survival of endothelial cells. *Microvasc Res* 1999; **58**: 224–37.
16 White RR, Shan S, Rusconi CP *et al.* Inhibition of rat corneal angiogenesis by a nuclease-resistant RNA aptamer specific for angiopoietin. *Proc Natl Acad Sci USA* 2003; **100**: 5028–33.
17 Niu Q, Perruzzi C, Voskas D, Lawler J, Dumont DJ, Benjamin LE. Inhibition of Tie-2 signaling induces endothelial cell apoptosis, decreases Akt signaling, and induces endothelial cell expression of the endogenous anti-angiogenic molecule, thrombospondin-1. *Cancer Biol Ther* 2004; **3**: 402–5.
18 Tournaire R, Simon M-P, le Noble F, Eichmann A, England P, Pouyssegur J. A short synthetic peptide inhibits signal transduction, migration and angiogenesis mediated by Tie2 receptor. *EMBO Rep* 2004; **5**: 1–6.
19 Sahara H, Ishikawa M, Takahashi N *et al. In vivo* anti-tumour effect of 3′-sulphonoquinovosyl 1′-monoacylglyceride isolated from sea urchin (*Strongylocentrotus intermedius*) intestine. *Br J Cancer* 1997; **75**: 324–32.
20 Sahara H, Hanashima S, Yamazaki T *et al.* Anti-tumor effect of chemically synthesized sulfolipids based on sea urchin's natural sulfonoquinovosylmonoacylglycerols. *Jpn J Cancer Res* 2002; **93**: 85–92.
21 Sakimoto I, Ohta K, Yamazaki T *et al.* α-Sulfoquinovosylmonoacylglycerol is a novel potent radiosensitizer targeting tumor angiogenesis. *Cancer Res* 2006; **66**: 2287–95.
22 Solway J, Seltzer J, Samaha FF *et al.* Structure and expression of a smooth muscle cell-specific gene, SM22α. *J Biol Chem* 1995; **270**: 13 460–9.
23 Zhang L, Yang N, Park J *et al.* Tumor-derived vascular endothelial growth factor up-regulates angiopoietin-2 in host endothelium and destabilizes host vasculature, supporting angiogenesis in ovarian cancer. *Cancer Res* 2003; **63**: 3403–12.
24 Yamori T, Matsunaga A, Sato S *et al.* Potent antitumor activity of MS-247, a novel DNA minor grove binder, evaluated by an *in vitro* and *in vivo* human cancer cell line panel. *Cancer Res* 1999; **59**: 4042–9.
25 Dan S, Tsunoda T, Kitahara O *et al.* An integrated database of chemosensitivity to 55 anticancer drugs and gene expression profiles of 39 human cancer cell lines. *Cancer Res* 2002; **62**: 1139–47.
26 Dumont DJ, Yamaguchi TP, Conlon RA, Rossant J, Breitman ML. *Tek*, a novel tyrosine kinase gene located on mouse chromosome 4, is expressed in the endothelial cells and their presumptive precursors. *Oncogene* 1992; **7**: 1471–80.
27 Dumont DJ, Gradwohl GJ, Fong GH, Auerbach R, Breitman ML. The endothelial-specific receptor tyrosine kinase, *tek*, is a member of a new subfamily of receptors. *Oncogene* 1993; **8**: 1293–301.
28 Dumont DJ, Gradwohl G, Fong GH *et al.* Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, *tek*, reveal a critical role in vasculogenesis of the embryo. *Genes Dev* 1994; **8**: 1897–909.
29 Sato TN, Tozawa Y, Deutsch U *et al.* Distinct roles of the receptor tyrosine kinases Tie1 and Tie-2 in blood vessel formation. *Nature* 1995; **376**: 70–4.
30 Maisonpierre PC, Suri C, Jones PF *et al.* Angiopoietin-2, a natural antagonist for Tie2 that disrupts *in vivo* angiogenesis. *Science* 1997; **277**: 55–60.
31 Hanahan D. Signaling vascular morphogenesis and maintenance. *Science* 1997; **277**: 48–50.
32 Davis S, Yancopoulos GD. The angiopoietins: ying and yang in angiogenesis. *Curr Top Microbiol Immunol* 1999; **237**: 173–85.
33 Hamaguchi I, Morisada T, Azuma M *et al.* Loss of Tie2 receptor compromises embryonic stem cell-derived endothelial but not hematopoitic cell survival. *Blood* 2006; **107**: 1207–13.
34 Wong AL, Haroon ZA, Werner S, Dewhirst MW, Greenberg CS, Peters KG. Tie2 expression and phosphorylation in angiogenic and quiescent adult tissues. *Circ Res* 1997; **81**: 567–74.
35 Witzenbichler B, Maisonpierre PC, Jones P, Yancopoulos GD, Isner JM. Chemotactic properties of angiopoietin-1 and -2, ligands for the endothelial-specific receptor tyrosine kinase Tie2. *J Biol Chem* 1998; **273**: 18 514–21.
36 Koblizek TI, Weiss C, Yancopoulos GD, Deutsch U, Risau W. Angiopoietin-1 induces sprouting angiogenesis *in vitro*. *Curr Biol* 1998; **8**: 529–32.
37 Kim I, Kim HG, Moon SO *et al.* Angiopoietin-1 induces endothelial cell sprouting through the activation of focal adhesion kinase and plasmin secretion. *Circ Res* 2000; **86**: 952–9.
38 Kim I, Kim HG, So JN, Kim JH, Kwak HJ, Koh GY. Angiopoietin-1 regulates endothelial cell survival through the phosphatidylinositol 3V-kinase/Akt signal transduction pathway. *Circ Res* 2000; **86**: 24–9.
39 Daly C, Wong V, Burova E *et al.* Angiopoietin-1 modulates endothelial cell function and gene expression via the transcription factor FKHR (FOXO1). *Genes Dev* 2004; **18**: 1060–71.
40 Davis S, Aldrich TH, Jones PF *et al.* Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. *Cell* 1996; **87**: 1161–9.
41 Hawighorst T, Skobe M, Streit M *et al.* Activation of the tie2 receptor by angiopoietin-1 enhances tumor vessel maturation and impairs squamous cell carcinoma growth. *Am J Pathol* 2002; **160**: 1381–92.
42 Stoeltzing O, Ahmad SA, Liu W *et al.* Angiopoietin-1 inhibits vascular permeability, angiogenesis, and growth of hepatic colon cancer tumors. *Cancer Res* 2003; **63**: 3370–7.

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Identification of an Immunogenic CTL Epitope of HIFPH3 for Immunotherapy of Renal Cell Carcinoma

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Abstract

Purpose: $CD8^+$ CTLs have an essential role in immune response against tumor. Although tumor-associated antigens have been identified in renal cell carcinoma (RCC), few of these are commonly shared and investigated as therapeutic targets in the clinical medicine. In this report, we show that HIFPH3, a member of prolyl hydroxylases that function as oxygen sensor, is a novel tumor antigen and HIFPH3-specific CTLs are induced from peripheral blood lymphocytes of RCC patients.

Experimental Design: Expression of HIFPH3 was examined by reverse transcription-PCR and immunostaining with anti-HIFPH3 antibody. To identify HLA-A24-restricted T-cell epitopes of HIFPH3, eight peptides were selected from the amino acid sequence of this protein and screened for their binding affinity to HLA-A24. Peptide-specific CTLs were induced by stimulating peripheral blood lymphocytes of HLA-A24-positive RCC patients with these peptides *in vitro*. HLA-A24-restricted cytotoxicity of the CTLs against $HIFPH3^+$ RCC lines was assessed by chromium release assay.

Results: HIFPH3 was overexpressed in many RCC cell lines and primary RCC tissues, whereas it was not detectable in normal adult tissues by reverse transcription-PCR. Of the eight peptides that contained HLA-A24-binding motif, HIFPH3-8 peptide (amino acid sequence, RYAMTVWYF) could induce the peptide-specific CTLs from 3 of 6 patients with HIFPH3-positive RCC. Furthermore, HIFPH3-8 peptide-specific CTLs showed cytotoxicity against $HIFPH3^+$ RCC cell lines in a HLA-A24-restricted manner.

Conclusions: HIFPH3 may be a target antigen in immunotherapy for RCC and HIFPH3-8 peptide could be used as a peptide vaccine for HLA-A*2402$^+$/$HIFPH3^+$ RCC patients.

Surgery is the only known effective therapy for localized renal cell carcinoma (RCC); however, ~20% of all patients surgically treated with curative intent will ultimately experience disease recurrence (1) and ~30% of patients will present metastatic disease. Although systemic therapy with radiation and/or chemotherapeutic drugs is applied for locally advanced or metastatic RCC, its efficacy is limited due to the resistance to the therapy. Nonspecific immunotherapy with IFN-α and/or interleukin (IL)-2 has been also established as the primary therapy for metastatic RCC. However, neither agent provides substantial clinical benefit in the majority of patients. The number of durable responses is limited, and the use of these agents is complicated due to the significant safety and tolerability issues (2, 3). Hence, there is great need for new strategies of target-specific immunotherapy for the treatment of RCC, and recent progress in understanding of tumor immunology has raised expectations that specific immunotherapy may become a new modality of cancer therapy. Since the establishment of methods to isolate genes encoding tumor antigens that were recognized by CTLs, numerous tumor-associated antigens have been identified in melanoma and various other types of cancer (4, 5). Although tumor-associated antigens have been also identified in RCC, few of these are commonly shared and can be studied for clinical applications (6–9). There are no RCC-associated antigens currently being investigated as immunotherapeutic targets of RCC in clinical trials.

Tumor progression is highly regulated by hypoxia, a low level of oxygen, which occurs after excessive tumor cell proliferation that distances cells from oxygen-rich blood vessels. A consequence of increased cell number within a tumor is a corresponding increase in oxygen consumption. The hypoxia-inducible factor-1 (HIF-1), a transcriptional complex composed of an oxygen-sensitive α-subunit and a β-subunit, is the most

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Translational Relevance

Although systemic therapy with radiation and/or chemotherapeutic drugs is applied for locally advanced or metastatic RCC, its efficacy is limited due to the resistance to the therapy. Nonspecific immunotherapy with IFN-α and/or interleukin-2 has been also established as the primary therapy for metastatic RCC. However, neither agent provides substantial clinical benefit in the majority of patients. Hence, there is great need for new strategies of target-specific immunotherapy for the treatment of RCC, and recent progress in understanding of tumor immunology has raised expectations that specific immunotherapy may become a new modality of cancer therapy. In the present article, we showed that HIFPH3 was one of potent immunogenic antigens of RCC and that HIFPH3-8 peptide might serve as a tumor vaccine for HLA-A*2402$^+$ RCC patients. It is expected that HIFPH3 targeting immunotherapy might become a rational modality in therapy for RCC.

important factor involved in the cellular response to hypoxia. Overexpression of the HIF-1 subunit, resulting from intratumoral hypoxia and genetic alterations, has been shown in common human cancers and is correlated with tumor angiogenesis and patient mortality. Under normoxia, HIF-1 is continuously expressed in the cell but immediately degraded via the proteasomal pathway after ubiquitination (10). The von Hippel-Lindau (VHL) protein acts as a particle recognition protein of the responsible E3 ubiquitin-ligase complex if two distinct prolyl residues within a region, referred to as the oxygen-dependent degradation domain of HIF-1, Pro^{402}, and/or Pro^{564}, are hydroxylated (11–15). The site-specific hydroxylation of HIF prolyl residues is catalyzed by a conserved class of 2-oxoglutarate-dependent and Fe (II)-dependent dioxygenases, designated HIF prolyl hydroxylases (HIFPH; refs. 15–18). Three different HIFPHs, HIFPH1, HIFPH2, and HIFPH3, have been identified, but the difference among their *in vivo* roles remain unclear. Some studies have pointed out that HIFPH3 is strikingly expressed by hypoxia, displays high substrate specificity, and has been identified in other signaling pathways. HIFPH3 may therefore hydroxylate divergent substrates and/or connect divergent cellular responses with HIF (19).

In this report, we focused on the characteristics of HIFPH3 as a novel tumor antigen. We show that HIFPH3 expression was detected in certain RCC cell lines and primary RCC 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- or 10-mer peptides with HLA-A24-binding motif derived from HIFPH3 protein, and some of the peptides had relatively high binding affinity to HLA-A24 molecule. By stimulating peripheral blood mononuclear cells (PBMC) from *HLA-A*2402*$^+$/HIFPH3$^+$ RCC patients with HIFPH3 peptides, CTLs specific for HIFPH3-8 peptide could be successfully induced. In addition, CTLs induced by HIFPH3-8 peptide were capable of exerting cytotoxicity on HIFPH3$^+$ RCC cell line in a HLA-A24-restricted manner. These data highlight HIFPH3 as a potent target for immunotherapy of RCC and raise the possibility that HIFPH3-8 peptide may be suitable for the peptide-based vaccine for *HLA-A*2402*$^+$ RCC patients.

Materials and Methods

Patients and samples. The surgically resected tissue specimens and PBMC used in this study were obtained from *HLA-A*2402*$^+$ RCC patients who were hospitalized at Sapporo Medical University Hospital after obtaining their informed consent. PBMCs of RCC patients were obtained just before the nephrectomy and prepared for the CTL induction freshly without cryopreservation.

Cell lines and culture media. RCC cell lines SMKT R-1 (*HLA-A*2402*$^+$), SMKT R-2 (*HLA-A*2402*$^+$), SMKT R-3 (*HLA-A*2402*$^-$), and SMKT R-4 (*HLA-A*2402*$^-$), lung cancer line LHK2, gastric cancer line SSTW, and pancreatic cancer line PUN were established in our laboratory. RCC cell lines Caki-1 (*HLA-A*2402*$^+$), ACHN (*HLA-A*2402*$^-$), melanoma lines 888MEL and LG2MEL, colon cancer line SW450, lung cancer lines LNY-1, A549, 1-87, and LK79, pancreas cancer line HS776T, hepatic cancer line CHC20, and erythroleukemia cell line K562 were purchased from the American Type Culture Collection. All these cell lines were cultured in RPMI 1640 (Sigma) or DMEM (Sigma) supplemented with 10% fetal bovine serum (Filtron). SMKT R-4*2402, a stable transfectant of *HLA-A*2402* cDNA of SMKT R-4 cells was cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 500 ng/mL puromycin (Sigma). 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), was cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 800 μg/mL G418 (Invitrogen Life Technologies).

Development of monoclonal anti-HIFPH3 antibody. Monoclonal antibody against HIFPH3 was generated by immunizing mice eight times every week with recombinant His-tag HIFPH3 protein, which was produced and purified by Ni-NTA agarose column (Qiagen) as described previously (20). Spleen cells were fused with NS-1 myeloma cells by using polyethylene glycol 4000 (Kanto Kagaku) and plated into 96-well plates. Hybridoma supernatants were initially screened using an ELISA with recombinant His-HIFPH3 protein and then screened by Western blotting. The third screening of the supernatants was done by immunostaining of formalin-fixed, paraffin-embedded human tissue sections. The resulting hybridoma EMR-PHD3 was cloned by limiting dilution and finally its subclone EMR-PHD3-7 that produced monoclonal anti-HIFPH3 antibody with IgG1 subclass and κ chain was established.

Immunohistochemical staining of tissue sections. Immunohistochemical staining was done with formalin-fixed, paraffin-embedded sections of surgically resected tumor specimens of RCCs. Sections (4-5 μm thick) were deparaffinized in xylene and rehydrated in graded alcohols. Antigen retrieval was done by boiling sections for 20 min in a microwave oven in preheated 0.01 mol/L sodium citrate (pH 6.0). Endogenous peroxidase activity was blocked by 3% hydrogen peroxide in ethanol for 10 min. After blocking with 1% nonfat dry milk in PBS (pH 7.4), the sections were reacted with monoclonal anti-HIFPH3 antibody EMR-PHD3-7 for 1 h followed by incubation with biotinylated anti-mouse IgG (Nichirei) for 30 min. 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) 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). cDNA mixture was synthesized from 1 μg total RNA by reverse transcription using SuperScript II and oligo(dT) primer (Invitrogen Life Technologies) according to the manufacturer's protocol. PCR amplification was done in 50 μL PCR mixture containing 1 μL cDNA mixture, 1 μL KOD Plus DNA polymerase (Toyobo), and

15 pmol 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 64°C for 30 s, and extension at 72°C for 30 s. For specific detection of HIFPH3, the primer pairs 5′-CATCCCTGTCTTGTGTGTGG-3′ and 5′-CCAACAGCCCTGGATTAAGA-3′ were employed as forward and reverse primers, respectively. The expected size of PCR product for HIFPH3 is 420 bp. For an internal control, glyceraldehyde 3-phosphate dehydrogenase expression was detected by using forward primer 5′-ACCACAGTCCATGCCATCAC-3′ and reverse primer 5′-TCCACCAC-CCTGTTGCTGTA-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).

Peptides and cytokines. HIFPH3-derived peptides carrying HLA-A24-binding motif HIFPH3-1 (IMRLDLEKI), HIFPH3-2 (NWDAKLHGGI), HIFPH3-3 (IFPEGKSFI), HIFPH3-4 (SFIADVEPI), HIFPH3-5 (GFCYLDNFL), HIFPH3-6 (SFLLSLIDRL), HIFPH3-7 (YYVKERSKAM), HIFPH3-8 (RYAMTVWYF), EBV LMP2-derived HLA-A24-binding peptide (TYGPVFMSL; ref. 21), and HIV env-derived HLA-A24-binding peptide (RYLRDQQLLGI; ref. 22) were purchased from Greiner Bio-One. The peptides were dissolved in DMSO at the concentration of 5 mg/mL and stored at -80°C. Human recombinant IL-2, IL-4, and granulocyte-macrophage colony-stimulating factor were kind gifts from Takeda Pharmaceutical, Ono Pharmaceutical, and Novartis Pharmaceutical, 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 (22) based on the findings that MHC class I molecules could be stabilized on the cell surface in the presence of binding peptides. T2-A*2402 cells are the peptide transporter-negative B/T hybrid cell line 174 CEM.T2 (referred to as T2) transfected with a plasmid expressing HLA-A*2402. After incubation of T2-A*2402 cells in culture medium at 26°C for 18 h, 2×10^5 cells were washed with PBS and suspended with 1 mL Opti-MEM (Life Technologies) with or without 100 μg peptide followed by incubation at 26°C for 3 h and then at 37°C for 2.5 h. After washing with PBS, the cells were incubated with anti-HLA-A24 monoclonal antibody at 4°C for 1 h followed by incubation with FITC-conjugated rabbit anti-mouse IgG at 4°C for 30 min. The cells were then suspended with 1 mL PBS containing 1% formaldehyde and analyzed by FACScan (Becton Dickinson). Binding affinity was evaluated by comparing mean fluorescence intensity (MFI) of HLA-A24 expression in the presence of peptide pulsation.

Peptide-specific CTL induction with immature dendritic cells and phytohemagglutinin blasts. CTLs were induced from PBMCs of cancer patients by using autologous dendritic cells and phytohemagglutinin (PHA) blasts as antigen-presenting cells (APC; refs. 23, 24). Briefly, PBMCs (1×10^7-1×10^8) were isolated from blood of cancer patients by using Lymphoprep (Nycomed) and then separated into $CD14^+$ and $CD14^-$ cells by using MACS separation system (Miltenyi Biotech) and anti-CD14 monoclonal antibody coupled with magnetic microbeads according to the manufacturer's instruction. Autologous immature dendritic cells were generated from $CD14^+$ cells in the plastic flask by culturing in AIM-V medium supplemented with 10% human serum, 10 mmol/L HEPES, 50 μmol/L 2-mercaptoethanol, 100 ng/mL granulocyte-macrophage colony-stimulating factor, and 1,000 units/mL IL-4 for 7 days. $CD8^+$ cells were isolated from $CD14^-$ cells by using MACS separation system and anti-CD8 monoclonal antibody coupled with magnetic microbeads according to the manufacturer's instruction. PHA blasts were derived from $CD14^-CD8^-$ cells by culturing in AIM-V medium supplemented with 10 mmol/L HEPES, 50 μmol/L 2-mercaptoethanol, 100 units/mL IL-2, and 1 μg/mL PHA for 2 days followed by culture in AIM-V medium without PHA for 5 days. APCs (dendritic cells and PHA blasts) were cultured in AIM-V medium supplemented with 50 μmol/L peptide at room temperature for 2 h followed by washing with AIM-V medium once and then irradiated (100 Gy) and used for stimulation of CTL. CTL induction procedure was initiated by stimulating $CD8^+$ cells with peptide-pulsed autologous dendritic cells at a 20:1 effecter/APC ratio in AIM-V medium supplemented with HEPES, 2-mercaptoethanol, and 10 ng/mL IL-7 for 7 days at 37°C. The following stimulation was done with peptide-pulsed PHA blasts at a 10:1 effector/APC ratio. On the next day of the second stimulation, IL-2 was added to the culture at a concentration of 10 units/mL. The same CTL stimulation cycle with PHA blasts was then done twice more over the period of 2 weeks. One week after

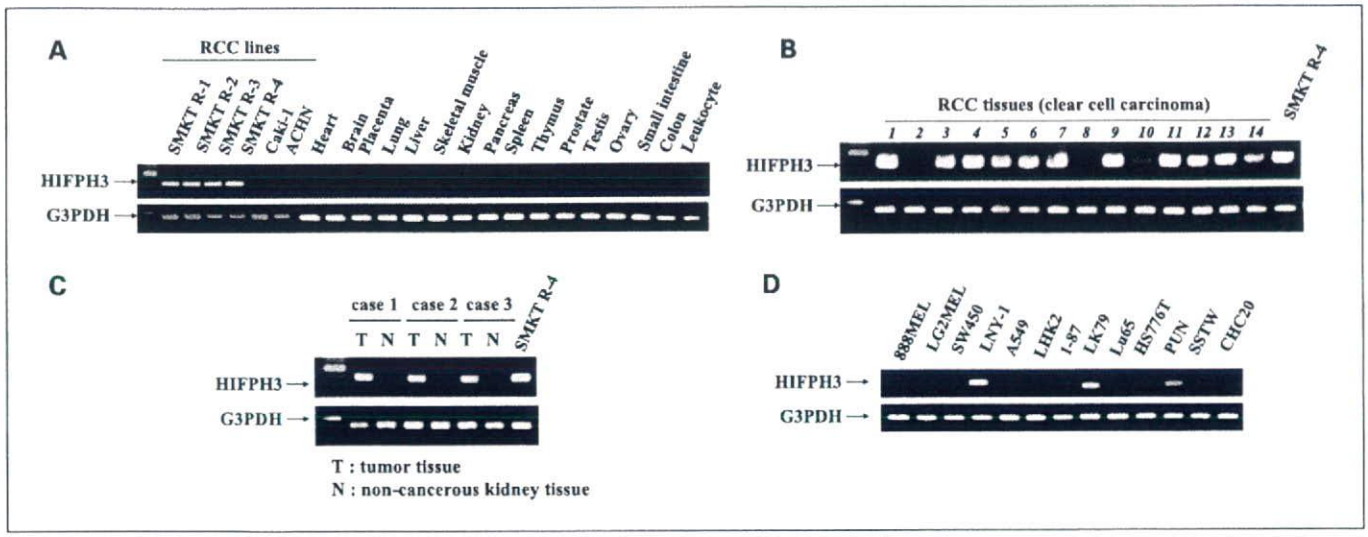

Fig. 1. Expression profiles of HIFPH3 as assessed by RT-PCR in normal adult tissues, RCC cell lines, and primary RCC tissues. *A*, expression of HIFPH3 in RCC cell lines and normal tissues including heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and leukocyte. Glyceraldehyde 3-phosphate dehydrogenase expression was detected as an internal control. *B*, expression of HIFPH3 in primary RCC tissues. *C*, expression of HIFPH3 in RCC tumor tissue (*T*) and noncancerous tissue (*N*) of 3 RCC cases. *D*, expression of HIFPH3 in various tumor cells including melanoma lines 888MEL and LG2MEL, colon cancer line SW450, lung cancer lines LNY-1, A549, LHK2, 1-87, LK79, and Lu65, pancreatic cancer lines HS776T and PUN, gastric cancer line SSTW, and hepatic cell cancer line CHC20.

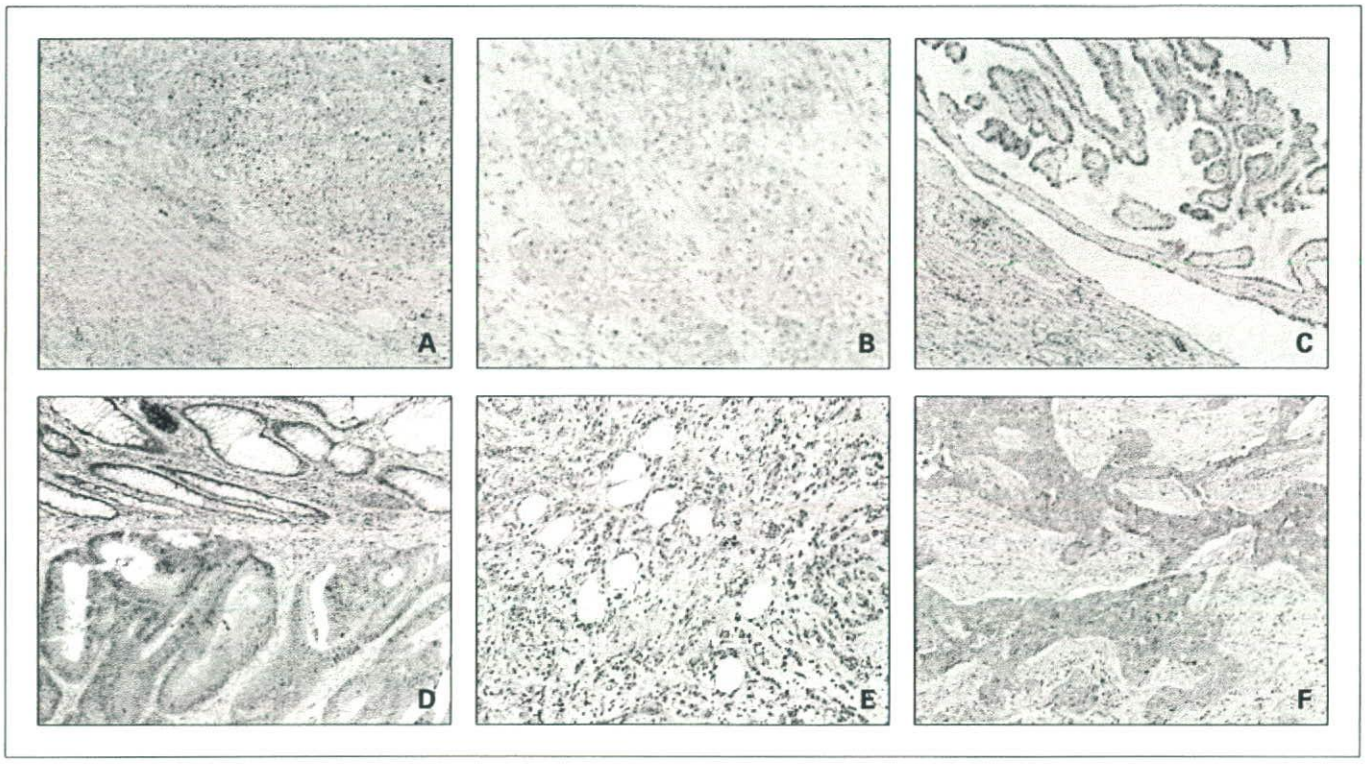

Fig. 2. Immunohistochemical staining of HIFPH3 in RCC and other types of tumors. *A*, low magnified view (×40) of clear cell RCC. *B*, high magnified view (×100) of clear cell RCC. *C*, low magnified view (×40) of papillary RCC. *D*, low magnified view (×40) of colon cancer tissue. *E*, low magnified view (×40) of breast cancer tissue. *F*, low magnified view (×40) of lung cancer tissue.

the fourth stimulation, cytotoxic activity of the CTL was measured by ^{51}Cr release assay.

Cytotoxicity assay. The cytotoxic activities of CTLs were measured by ^{51}Cr release assay as described previously (25). Briefly, target cells were labeled with 100 μCi ^{51}Cr for 1 h at 37°C and washed with RPMI 1640 thrice. Then, 2×10^3 ^{51}Cr-labeled target cells were incubated with effecter cells at various E:T ratios at 37°C for 6 h in V-bottomed 96-well microtiter plates. Then, supernatants were collected and the radioactivity was measured with a γ-counter. % Specific lysis was calculated as follows: % specific lysis = (test sample release - spontaneous release) × 100 / (maximum release - spontaneous release). For preparation of peptide-pulsed target cells, target cells were incubated with 100 μg/mL peptide at room temperature for 1 h before the assay.

Results

HIFPH3 is expressed in RCC cell lines and primary RCC tissues but not in normal adult tissues. In this report, HIFPH3 expression profiles in normal adult tissues and RCC cell lines and tissues were analyzed by RT-PCR method. We first studied HIFPH3 expression in RCC cell lines and normal adult tissues including heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and leukocyte. As shown in Fig. 1A, HIFPH3 mRNA was detected in 4 of 6 RCC cell lines (SMKT R-1, R-2, R-3, and R-4). Nucleic acid sequence of the HIFPH3-specific band was confirmed by DNA sequence analysis (data not shown). In contrast, no overt expression of HIFPH3 mRNA was observed in these normal adult tissues on the condition of detecting the expression of glyceraldehyde 3-phosphate dehydrogenase mRNA. These data indicate that HIFPH3 is aberrantly expressed in certain RCC cell lines. We then analyzed the HIFPH3 expression in primary RCC tissue specimens. As shown in Fig. 1B, the expression of HIFPH3 was detected in certain RCC tissues in 13 of 15 (87%) cases. These data indicate that HIFPH3 was expressed in primary RCC tissues as well as in RCC cell lines.

We then examined the expression of HIFPH3 in cancerous tissue and noncancerous tissue of 3 RCC cases (Fig. 1C). HIFPH3 was selectively expressed in cancerous tissue but not in noncancerous tissue.

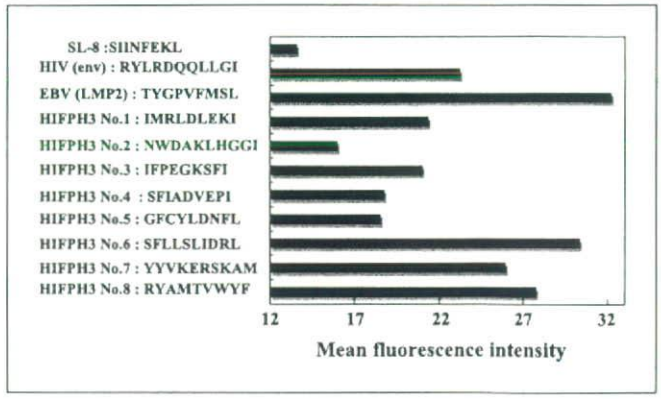

Fig. 3. Amino acid sequences of HIFPH3-derived peptides with HLA-A24-binding motif and their binding assay to HLA-A24 molecule. Eight peptides carrying HLA-A24-binding motif (HIFPH3-1-HIFPH3-8) were synthesized. Binding affinity of HIFPH3-derived peptides to HLA-A24 molecule was evaluated by MFI of cell surface HLA-A24 molecule onT2-A*2402 cells that were pulsed with each peptide. HLA-A24-bound EBV LMP2-derived peptide (*TYGPVFMSL*) and HIV env-derived peptide (*RYLRDQQLLGI*) were used as positive controls. SL-8 peptide (*SIINFEKL*) was used as a negative control. Histograms of MFI were displayed for each peptide.

Table 1. Summary of clinicopathologic characteristics and peptide-reactive CTL induction from PBMCs of HIFPH3$^+$ clear cell carcinoma patients

Case no.	Age	Sex	Stage*	Peptides	HLA-A*2402	CTL induction†
1	56	M	$T_{1a}N_0M_0$	HIFPH3-8	+	+
2	54	F	$T_{1b}N_0M_0$	HIFPH3-8	+	+
3	72	M	$T_{1a}N_0M_0$	HIFPH3-8	+	+
4	68	F	$T_{1b}N_0M_0$	HIFPH3-8	+	-
5	50	M	$T_{1a}N_0M_0$	HIFPH3-8	+	-
6	56	M	$T_{1a}N_0M_0$	HIFPH3-8	+	-
7	61	F	$T_{1a}N_0M_0$	HIFPH3-1 to HIFPH3-4	+	-
8	65	M	$T_{1a}N_0M_0$	HIFPH3-1 to HIFPH3-4	+	-
9	53	M	$T_{1a}N_0M_0$	HIFPH3-5 to HIFPH3-8	+	-
10	75	M	$T_{1a}N_0M_0$	HIFPH3-5 to HIFPH3-8	+	+
11	68	F	$T_{1b}N_0M_0$	HIFPH3-8	-	-
12	82	M	$T_{1a}N_0M_0$	HIFPH3-8	-	-

*Tumor-node-metastasis classification.
†<20% specific lytic activity against HIFPH3 peptide-pulsed T2-A*2402 target cells was indicated as +.

To know if HIFPH3 is expressed in non-RCC tumor cells, various tumor cells were examined by RT-PCR, including melanoma lines, lung cancer lines, colon cancer line, pancreatic cancer line, gastric cancer line, and hepatic cell cancer line. Of these tumor cells, two of lung cancer lines and one of pancreatic cancer lines had expression of HIFPH3 (Fig. 1D).

Immunohistochemical staining of HIFPH3. To detect the HIFPH3 protein expressed in RCC tissues, we generated HIFPH3-specific monoclonal antibody suitable for immunohistochemical staining. HIFPH3-specific reactivity of the antibody was confirmed by Western blotting (data not shown). Representative pictures of RCC tissue staining are shown in Fig. 2A to C, indicating cytoplasmic staining of HIFPH3 in RCC cells. Of 18 cases of clear cell RCC, 13 (72%) cases were HIFPH3 positive by immunostaining (Fig. 2A and B). In contrast, only 3 of 9 (33%) cases of non-clear cell RCC were

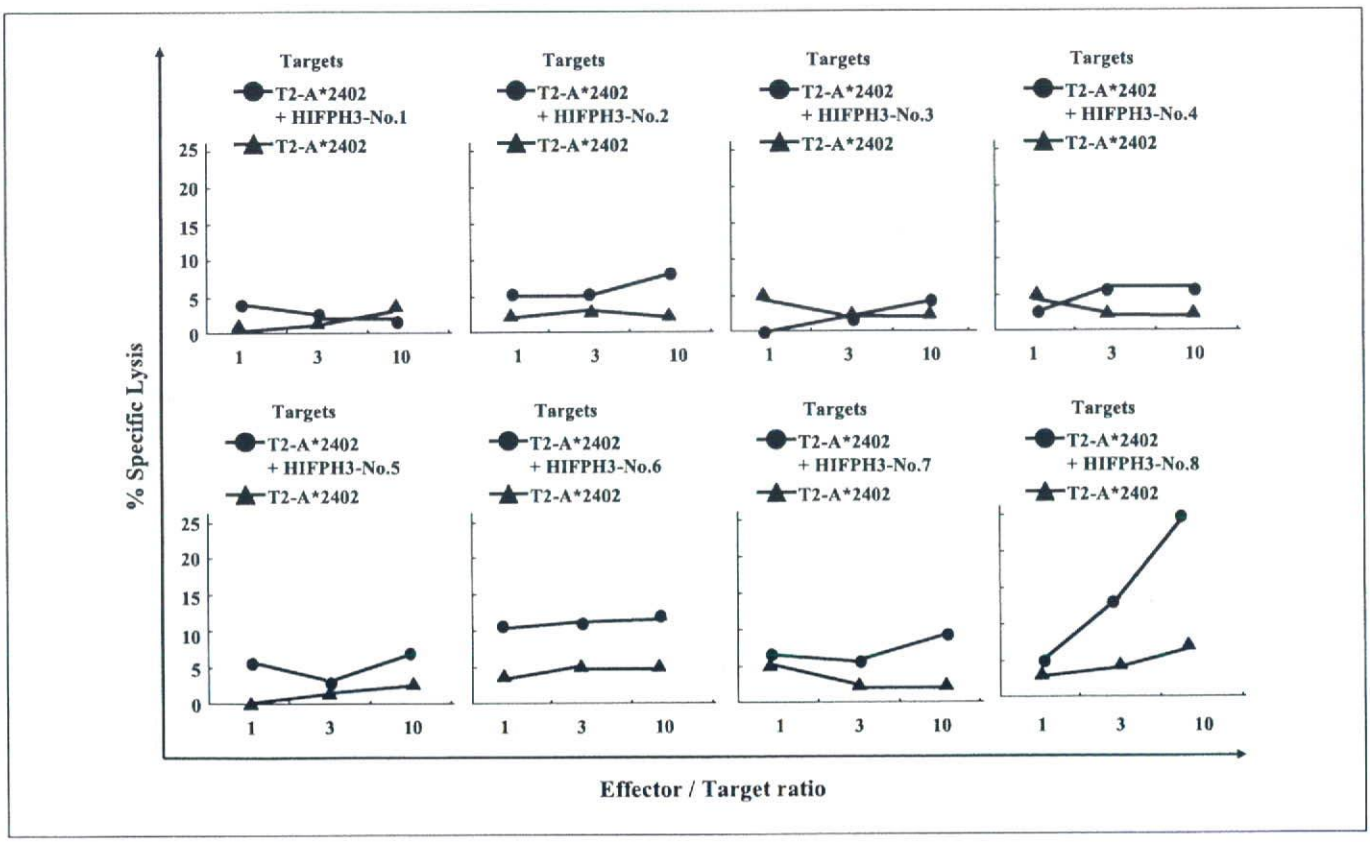

Fig. 4. Induction of HIFPH3 peptide-specific CTLs and their peptide-specific cytotoxicity. CTLs were induced from PBMCs of a HLA-A*2402$^+$ RCC patient by stimulating with group A (HIFPH3-1-HIFPH3-4) peptide-pulsed APCs (*top four graphs*) or group B (HIFPH3-5-HIFPH3-8) peptide-pulsed APCs (*bottom four graphs*). After four times of stimulation, CTLs were subjected to standard ^{51}Cr release assay at the indicated E:Tratio. Peptide-pulsed T2-A*2402 cells and nonpulsed cells were used as target cells.

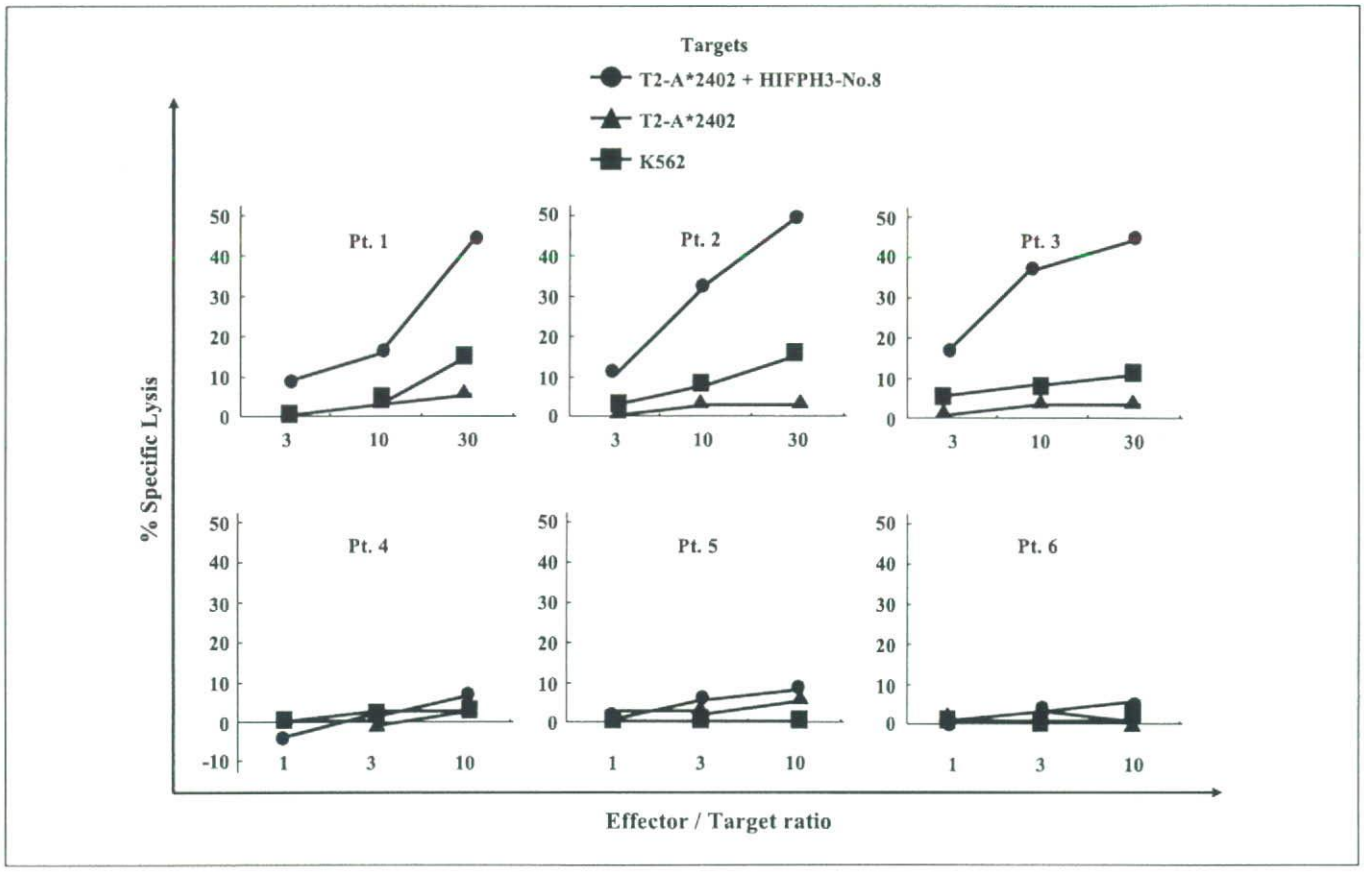

Fig. 5. HIFPH3-8 peptide-specific CTL induction from PBMCs of HLA-A*2402$^+$ RCC patients. CTLs were induced from PBMCs of 6 HLA-A*2402$^+$ RCC patients (patients 1-6) by stimulating with HIFPH3-8 peptide-pulsed APCs. The cytotoxic activity was examined by ^{51}Cr release assay at the indicated E:Tratio. HIFPH3-8 peptide-pulsed T2-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.

HIFPH3 positive. The histology of HIFPH3-positive cases included granular cell carcinoma, papillary RCC (Fig. 2C), and chromophobe cell carcinoma. Immunostaining of non-RCC tumors revealed that HIFPH3 was expressed in some types of tumors besides RCC, including colon cancer (Fig. 2D, 10 of 24 cases), breast cancer (Fig. 2E, 14 of 24 cases), and lung cancer (Fig. 2F, 10 of 24 cases).

Binding analysis of HIFPH3-derived peptides to HLA-A24 molecules. Because HIFPH3 is expressed in RCC cells but not in normal tissues, we hypothesized that it might be a suitable target for tumor immunotherapy. Immune tolerance toward HIFPH3 is considered to be weak because anti-HIFPH3 autoantibody was detected in sera of RCC cancer patients (data not shown). Thus, it was reasoned that antigenic peptides derived from HIFPH3 might be presented by MHC class I molecules and recognized by CD8$^+$ T cells. To evaluate if HIFPH3 might become a target of CTLs, we focused on *HLA-A*2402* allele because of its high frequency worldwide. The total amino acid sequence of HIFPH3 was searched for peptides that have HLA-A24-binding motif as 9- or 10-mer peptide with Y, F, M, or W at the second position and L, I, F, or M at the COOH-terminal position (26). Consequently, we found eight peptides (HIFPH3-1-HIFPH3-8) carrying HLA-A24-binding motif, and to assess their binding ability to HLA-A24 molecule, binding assay using T2-A*2402 cells was done as described previously (22). Two positive control peptides, HLA-A24-restricted EBV epitope and HIV epitope, and negative control peptide, SL-8 peptide, were used in the assay. HLA-A24 level on the cell surface of T2-A*2402 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. 3). Both EBV and HIV peptides increased MFI of HLA-A24 clearly, whereas SL-8 peptide failed, indicating adequate qualification of this assay system. HIFPH3-1 to HIFPH3-5 peptides could just increased the cell surface HLA-A24 level to mild or moderate levels, whereas HIFPH3-6 to HIFPH3-8 peptides were capable of up-regulating the HLA level to almost similar levels to EBV peptide. It was indicated that HIFPH3-6 to HIFPH3-8 might have relatively high binding affinity to HLA-A24 molecule among all the peptides.

CTL induction from PBMCs of HLA-A*2402$^+$ RCC patients. To know which HIFPH3-derived peptides can be recognized by T cells of cancer patients in the context of HLA-A24, we attempted to induce peptide-specific CTL and compare their cytotoxic activity. PBMCs were collected from RCC patients with *HLA-A*2402* (patient profiles in Table 1), and T cells sorted out from the PBMCs were incubated with peptide-pulsed autologous monocyte-derived dendritic cells or autologous PHA blasts (27). Eight peptides were grouped into two peptide mixtures: group A consisting of HIFPH3-1 to HIFPH3-4, and group B consisting of HIFPH3-5 to HIFPH3-8. After four times stimulation with either of the peptide mixtures and APCs,

cytotoxic activity against each peptide-pulsed target cells was examined by ^{51}Cr release assay. As shown in Fig. 4, CTLs induced from PBMCs by *in vitro* stimulation with group B peptides could react specifically to the HIFPH3-8-pulsed T2-A*2402 cells, whereas they could not react to HIFPH3-5, HIFPH3-6, or HIFPH3-7 peptide-pulsed target cells. The other CTLs induced by stimulation with group A peptides failed to exert cytotoxicity against HIFPH3-1, HIFPH3-2, HIFPH3-3, or HIFPH3-4 peptide-pulsed T2-A*2402 cells. Therefore, we determined that HIFPH3-8 peptide could be the best candidate for the CTL epitope presented by HLA-A*2402.

CTL induction efficiency was examined by using HIFPH3-8 peptide-pulsed autologous APCs from PBMCs of 6 RCC patients. As shown in Fig. 5, CTLs reacting specifically to HIFPH3-8 peptide-pulsed T2-A*2402 cells were successfully induced from 3 of 6 patients (patients 1-3). These data indicate that HIFPH3-8 peptide-specific CTLs could be efficiently induced from PBMCs of *HLA-A*2402*$^+$ RCC patients.

The results of peptide-specific CTL induction from PBMCs of RCC patients were summarized in Table 1. CTLs were not induced by pulsation with group A peptides. No. 8 peptide-specific CTLs could not be induced from two of HLA-A*2402-negative RCC patients.

Cytotoxic activity of HIFPH3-8-specific CTLs against HLA-A24$^+$ and HIFPH3$^+$ RCC cell line. To confirm that CTLs induced with HIFPH3-8 peptide can exert cytotoxicity on HIFPH3-expressing cancer cells in the context of HLA-A*2402, we examined their cytotoxic activity against RCC cell lines: SMKT R-1 that expresses endogenous HIFPH3 and HLA-A*2402 and SMKT R-4-A*2402 that expresses both endogenous HIFPH3 and gene-transfected HLA-A*2402. As shown in Fig. 6, CTLs induced with HIFPH3-8 peptide from PBMCs of RCC patient 1 and RCC patient 3 (Table 1; Fig. 5) exerted significant cytotoxicity against SMKT R-1 and SMKT R-4-A*2402 cells but not against SMKT R-4 lacking HLA-A24 expression or K562 cells. These data implied that HIFPH3-8 peptide-specific CTLs were capable of recognizing endogenously processed HIFPH3-8 peptide in a HLA-A24-restricted manner.

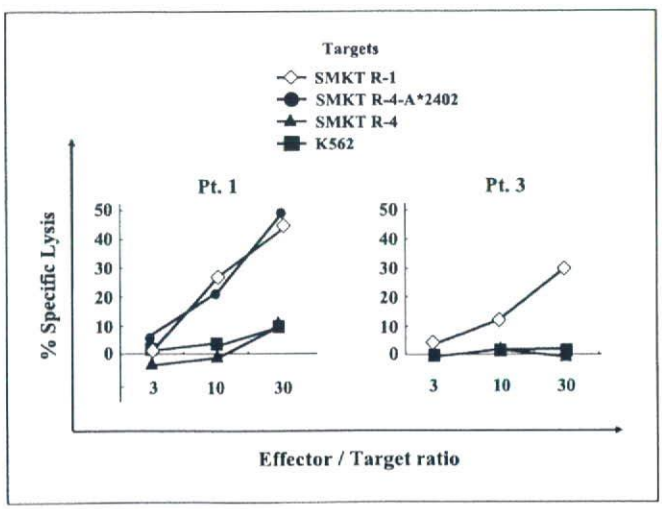

Fig. 6. Cytotoxic activity of HIFPH3-8-specific CTLs against HLA-A24$^+$ and HIFPH3$^+$ RCC cell lines. CTLs were induced from PBMCs of RCC patients 1 and 3 (Table 1; Fig. 5) and their cytotoxic activity against HIFPH3$^+$ HLA-A*2402$^+$ SMKT R-1 cells, HIFPH3$^+$ HLA-A*2402$^-$ SMKT R-4 RCC cells, HLA-A*2402-transfected SMKT R-4-A*2402, and K562 cells was examined by ^{51}Cr release assay at the indicated E:T ratio.

Discussion

The process of tumor progression (proliferation, local invasion, and distant metastasis) is characterized by rapid cellular growth accompanied by alterations of the microenvironment of the tumor cells. To a large extent, the alterations in the cellular microenvironment are due to an inadequate oxygen supply and the resultant hypoxia or even anoxia (28, 29). Among these conditions, changes in the expression of genes for erythropoietin, the angiogenic vascular endothelial growth factor, transferrin receptors, and other proteins allow for the development of a more effective oxygen (and nutrient) supply. Expression of the genes for most of these proteins is regulated by HIF-1α and HIF-2α. This transcription factor was first identified by Semenza et al. as a regulator of hypoxia-induced erythropoietin expression (30–32) and has since been shown to regulate the expression of >30 target genes. These genes also play roles in tumor progression, thereby contributing to tumor aggressiveness.

The activity of the transcriptional complex of HIF is regulated by oxygen-dependent post-translational modifications that are mediated by HIFPH (HIFPH1; refs. 2, 3). HIFPHs hydroxylate two conserved proline residues of HIF-1α and HIF-2α, leading to capture by the corresponding E3 ubiquitin-ligase VHL complex and degradation (11–15). Although all three HIFPHs can hydroxylate HIF-1α and HIF-2α *in vitro*, they exhibit different patterns of expression among tissues and distinct substrate specificity (19, 33). It has been shown that HIFPH1 and HIFPH2 are expressed in various normal adult tissues and predominantly contributes to HIF-1α hydroxylation, leading to setting low steady-state levels of HIF-1α in normoxic condition (34). In contrast, as shown in our results, HIFPH3 was barely detected in normal adult tissues. It is induced in hypoxic condition and retains its activity in mediating HIF-2α hydroxylation in the condition (35), thus serving as a negative feedback loop by limiting physiologic activation of HIF in hypoxia (19, 33, 36). In the present study, we showed for the first time that HIFPH3 was overexpressed in some of RCC cell lines and tissues. Complete VHL gene sequence analysis of RCC lines revealed that 4 HIFPH3-positive RCC lines had VHL mutations and 2 HIFPH3-negative RCC lines had no mutation in VHL genes (data not shown). Therefore, it was indicated that HIFPH3 expression might be associated with VHL mutation in RCC. However, as shown in our immunohistochemical studies, tumors without VHL mutation, such as papillary RCC and non-RCC cancers, had also expression of HIFPH3, indicating that VHL alone probably does not regulate the expression of this gene. Although we have no clear explanation about the molecular mechanism of HIFPH3 overexpression and the roles of HIFPH3 in cancer cells, its expression selectively in tumor cells indicate that it may serve as a cancer-associated antigen applicable to specific immunotherapies. Although the frequency of HIFPH3 expression was highest among various malignant tumors that we tested, our immunohistochemical studies showed that expression of HIFPH3 was not limited to RCC. Therefore, HIFPH3 may be an immunotherapy target for lung, breast, and colon cancer besides RCC.

We identified HLA-A24-restricted CTL epitope of HIFPH3. Eight HIFPH3-derived peptides were shown to bind to HLA-A24 molecule with various affinities, and we succeeded in inducing HIFPH3-8 peptide-specific CTL from PBMCs of RCC patients. Stimulation of PBMCs from *HLA-A24*$^+$/HIFPH3$^+$ RCC patients with HIFPH3-8 peptide could lead to efficient induction of CTLs that exerted cytotoxicity against *HLA-A24*$^+$/HIFPH3$^+$ RCC cell lines. These data indicate that HIFPH3-8 peptide might be one of the naturally processed antigenic peptides derived from HIFPH3 with considerable immunogenicity, thus serving as a potent peptide vaccine in immunotherapy for *HLA-A***2402*$^+$ RCC patients. In addition, we found that anti-HIFPH3 autoantibody was detectable in sera of 10 of 32 RCC patients (data not shown). These observations suggest that HIFPH3 has high antigenic potential *in vivo* in both cellular immunity and humoral immunity. Indeed, in our current study, HIFPH3-specific CTLs were successfully induced from 3 of 6 HIFPH3$^+$ RCC patients' PBMCs. The reason for CTL induction failure in 3 patients remains unknown because HIFPH3 was detected in RCC tissues by immunostaining.

In conclusion, we showed that HIFPH3 was one of potent immunogenic antigens of RCC and HIFPH3-8 peptide might serve as a tumor vaccine for HLA-A*2402$^+$ cancer patients. It is expected that HIFPH3 targeting immunotherapy might become a rational modality in therapy for RCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

1. Kattan MW, Reuter V, Motzer RJ, et al. A postoperative prognostic nomogram for renal cell carcinoma. J Urol 2001;166:63–7.
2. Robert CF, Sydney ES, Brent AB, et al. Nephrectomy followed by interferon alfa-2b compared with interferon alfa-2b alone for metastatic renal-cell cancer. N Engl J Med 2001;345:1655–9.
3. Allan JP, Arie SB, Robert AF. Nephrectomy and interleukin-2 for metastatic renal-cell carcinoma. N Engl J Med 2001;345:1711–2.
4. Boon T, Coulie PG, Van den Eynde B. Tumor antigens recognized by T cells. Immunol Today 1997;18:267–8.
5. Rosenberg SA. A new era for cancer immunotherapy based on the genes that encode cancer antigens. Immunity 1999;10:281–7.
6. Li G, Passebose-Faure K, Lambert C, et al. Flow cytometric analysis of antigen expression in malignant and normal renal cells. Anticancer Res 2000;20:2773–8.
7. Neumann E, Engelsberg A, Decker J, et al. Heterogeneous expression of the tumor-associated antigens RAGE-1, PRAME, glycoprotein 75 in human renal cell carcinoma: candidates for T-cell-based immunotherapies?. Cancer Res 1998;58:4090–5.
8. Hanada K, Perry-Lalley DM, Ohnmacht GA, et al. Identification of fibroblast growth factor-5 as an overexpressed antigen in multiple human adenocarcinomas. Cancer Res 2001;61:5511–6.
9. Takahashi Y, Harashima N, Kajigaya S, et al. Regression of human kidney cancer following allogeneic stem cell transplantation is associated with recognition of an HERV-E antigen by T cells. J Clin Invest 2008;118:1099–109.
10. Huang J, Zhao Q, Mooney SM, et al. Sequence determinants in hypoxia-inducible factor-1α for hydroxylation by the prolyl hydroxylases PHD1, PHD2, and PHD3. J Biol Chem 2002;277:39792–800.
11. Cockman ME, Masson N, Mole DR, et al. Hypoxia inducible factor-α binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. J Biol Chem 2000;275:25733–41.
12. Ivan M, Kondo K, Yang H, et al. HIFα targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. Science 2001;292:464–8.
13. Jaakkola P, Mole DR, Tian YM, et al. Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science 2001;292:468–72.
14. Yu F, White SB, Zhao Q, et al. HIF-1α binding to VHL is regulated by stimulus-sensitive proline hydroxylation. Proc Natl Acad Sci U S A 2001;98:9630–5.
15. Bruick RK, McKnight SL. A conserved family of prolyl-4-hydroxylases that modify HIF. Science 2001;294:1337–40.
16. Ivan M, Haberberger T, Gervasi DC, et al. Biochemical purification and pharmacological inhibition of a mammalian prolyl hydroxylase acting on hypoxia-inducible factor. Proc Natl Acad Sci U S A 2002;99:13459–64.
17. Erez N, Milyavsky M, Goldfinger N, et al. Falkor, a novel cell growth regulator isolated by a functional genetic screen. Oncogene 2002;21:6713–21.
18. Epstein AC, Gleadle JM, McNeill LA, et al. *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell 2001;107:43–54.
19. Appelhoff RJ, Tian YM, Raval RR, et al. Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor. J Biol Chem 2004;279:38458–65.
20. Yagihashi A, Asanuma K, Tsuji N, et al. Detection of anti-livin antibody in gastrointestinal cancer patients. Clin Chem 2003;49:1206–8.
21. Lee SP, Tierney RJ, Thomas WA, et al. Conserved CTL epitopes within EBV latent membrane protein 2: a potential target for CTL-based tumor therapy. J Immunol 1997;158:3325–34.
22. Kuzushima K, Hayashi N, Kimura H, et al. Efficient identification of HLA-A*2402-restricted cytomegalovirus-specific CD8$^+$ T-cell epitopes by a computer algorithm and an enzyme-linked immunospot assay. Blood 2001;98:1872–81.
23. Sato Y, Nabeta Y, Tsukahara T, et al. Detection and induction of CTLs specific for SYT-SSX-derived peptides in HLA-A24(+) patients with synovial sarcoma. J Immunol 2002;169:1611–8.
24. Maeda A, Ohguro H, Nabeta Y, et al. Identification of human antitumor cytotoxic T lymphocytes epitopes of recoverin, a cancer-associated retinopathy antigen, possibly related with a better prognosis in a paraneoplastic syndrome. Eur J Immunol 2001;31:563–72.
25. Sato T, Sato N, Takahashi S, et al. Specific cytotoxicity of a long-term cultured T-cell clone on human autologous mammary cancer cells. Cancer Res 1986;46:4384–9.
26. Kondo A, Sidney J, Southwood S, et al. Prominent roles of secondary anchor residues in peptide binding to HLA-A24 human class I molecules. J Immunol 1995;155:4307–12.
27. Hirohashi Y, Torigoe T, Maeda A, et al. An HLA-A24-restricted cytotoxic T lymphocyte epitope of a tumor-associated protein, survivin. Clin Cancer Res 2002;8:1731–9.
28. Vaupel P, Kallinowski F, Okunieff P. Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. Cancer Res 1989;49:6449–65.
29. Ryan HE, Poloni M, McNulty W, et al. Hypoxia-inducible factor-1α is a positive factor in solid tumor growth. Cancer Res 2000;60:4010–5.
30. Semenza GL, Wang GL. A nuclear factor induced by hypoxia via *de novo* protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol 1992;12:5447–54.
31. Wang GL, Jiang BH, Rue EA, et al. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. Proc Natl Acad Sci U S A 1995;92:5510–4.
32. Wang GL, Semenza GL. Purification and characterization of hypoxia-inducible factor 1. J Biol Chem 1995;270:1230–7.
33. Cioffi CL, Liu XQ, Kosinski PA, et al. Differential regulation of HIF-1α prolyl-4-hydroxylase genes by hypoxia in human cardiovascular cells. Biochem Biophys Res Commun 2003;303:947–53.
34. Berra E, Benizri E, Ginouves A, et al. HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1α in normoxia. EMBO J 2003;22:4082–90.
35. Nakayama K, Frew IJ, Hagensen M, et al. Siah2 regulates stability of prolyl-hydroxylases, controls HIF1α abundance, and modulates physiological responses to hypoxia. Cell 2004;117:941–52.
36. Hirsila M, Koivunen P, Gunzler V, et al. Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor. J Biol Chem 2003;278:30772–80.

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Research

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Clinical and immunological evaluation of anti-apoptosis protein, survivin-derived peptide vaccine in phase I clinical study for patients with advanced or recurrent breast cancer

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Abstract

Background: We previously reported that survivin-2B, a splicing variant of survivin, was expressed in various types of tumors and that survivin-2B peptide might serve as a potent immunogenic cancer vaccine. The objective of this study was to examine the toxicity of and to clinically and immunologically evaluate survivin-2B peptide in a phase I clinical study for patients with advanced or recurrent breast cancer.

Methods: We set up two protocols. In the first protocol, 10 patients were vaccinated with escalating doses (0.1–1.0 mg) of survivin-2B peptide alone 4 times every 2 weeks. In the second protocol, 4 patients were vaccinated with the peptide at a dose of 1.0 mg mixed with IFA 4 times every 2 weeks.

Results: In the first protocol, no adverse events were observed during or after vaccination. In the second protocol, two patients had induration at the injection site. One patient had general malaise (grade 1), and another had general malaise (grade 1) and fever (grade 1). Peptide vaccination was well tolerated in all patients. In the first protocol, tumor marker levels increased in 8 patients, slightly decreased in 1 patient and were within the normal range during this clinical trial in 1 patient.

With regard to tumor size, two patients were considered to have stable disease (SD). Immunologically, in 3 of the 10 patients (30%), an increase of the peptide-specific CTL frequency was detected. In the second protocol, an increase of the peptide-specific CTL frequency was detected in all 4 patients (100%), although there were no significant beneficial clinical responses. ELISPOT assay showed peptide-specific IFN-γ responses in 2 patients in whom the peptide-specific CTL frequency in tetramer staining also was increased in both protocols.

Conclusion: This phase I clinical study revealed that survivin-2B peptide vaccination was well tolerated. The vaccination with survivin-2B peptide mixed with IFA increased the frequency of peptide-specific CTL more effectively than vaccination with the peptide alone, although neither vaccination could induce efficient clinical responses. Considering the above, the addition of another effectual adjuvant such as a cytokine, heat shock protein, etc. to the vaccination with survivin-2B peptide mixed with IFA might induce improved immunological and clinical responses.

Background

The incidence of breast cancer has continuously increased in Japan, similar to European countries and the USA, whereas mortality from breast cancer has declined, indicating improving survival because of the development of early diagnosis [1-3]. However, metastatic recurrence still occurs, and once the cancer has spread beyond the breast and locoregional nodal areas it is felt to be incurable [4]. In the case of metastatic recurrence, the prevailing treatment is systemic chemotherapy, which is fraught with various adverse effects. Thus, we considered the availability of immunotherapy, which is generally reported to be safe, for advanced or recurrent breast cancer.

Tumor cells express antigens that can be recognized by the host's immune system. In the past decade, many antigenic peptides, which can be recognized by CTLs, have been identified [5-9]. As a result, clinical trials of peptide-based immunotherapy for cancer have taken place. Melanoma antigen peptides were the first to be tested in phase I and phase II studies for active immunization of metastatic melanoma patients [10,11]. Recently, there are reports of clinical trials for various cancers, including colorectal cancer [12], esophageal cancer [13], pancreatic cancer [14], among others. However, most clinical trials did not demonstrate sufficient anti-tumor clinical responses. Thus, it is necessary to establish peptide-based immunotherapy that can induce sufficient clinical responses.

Survivin was initially isolated as one of the inhibitors of the apoptosis protein family with only one baculovirus inhibitor of apoptosis protein (IAP) repeat domain [15]. Survivin is aberrantly expressed in various cancer cells but is undetectable in normal differentiated adult tissues, with the exception of the testis, thymus and placenta. We have previously reported that survivin-2B, a splicing variant of survivin, is expressed in various tumor cell lines [16], and the survivin-2B80-88 (AYACNTSTL) peptide derived from the exon 2B-encoded region is recognized by CD8+ CTLs in the context of HLA-A24 molecules [16]. In addition, we recently reported further evidence that survivin-2B80-88 peptide might serve as a potent immunogenic cancer vaccine for various cancer patients [17]. In that report, we demonstrated that overexpression of survivin was detected in surgically resected primary tumor specimens of most breast cancers in an immunohistochemical study. In addition, HLA-A24/survivin-2B80-88 tetramer analysis revealed that there were an increased number of CTL precursors in peripheral blood mononuclear cells (PBMCs), and in vitro stimulation of PBMCs from 6 breast cancer patients with survivin-2B80-88 peptide led to increases of the CTL precursor frequency. Furthermore, CTLs specific for this peptide were successfully induced in PBMCs from all 7 HLA-A24+ patients (100%) with breast cancers and exhibited cytotoxicity against HLA-A24+/survivin+ adenocarcinoma cells [17]. On the basis of these studies, we started a phase I clinical study of vaccination with survivin-2B peptide for patients with advanced or recurrent breast cancer.

Methods

Patient selection

The study protocol was approved by the Clinic Institutional Ethical Review Board of the Medical Institute of Bioregulation, Sapporo Medical University, Japan. All patients gave informed consent before being enrolled. Patients enrolled in this study were required to conform to the following criteria: (1) to have histologically confirmed breast cancer, (2) to be HLA-A*2402 positive, (3) to be survivin-positive in the carcinomatous lesions by immunohistochemistry, (4) to be between 20 and 85 years old, (5) to be unresectable advanced cancer or recurrent cancer and (6) to have Eastern Cooperative Oncology Group (ECOG) performance status between 0 and 3. Exclusion criteria included (1) prior cancer therapy such as chemotherapy, radiation therapy, steroid therapy, or other immunotherapy within the past 4 weeks, (2) the presence of other cancers that might influence the prognosis, (3) immunodeficiency or a history of splenectomy, (4) severe cardiac insufficiency, acute infection, or hemat-

opoietic failure, (5) ongoing breast-feeding and (6) unsuitability for the trial based on clinical judgment. This study was carried out at the Department of Surgery, Sapporo Medical University Primary Hospital from July 2003 through November 2005.

Peptide preparation

The peptide, survivin-2B80-88 with the sequence **AYACNTSTL**, was prepared under good manufacturing practice conditions by Multiple Peptide Systems (San Diego, CA). The identity of the peptide was confirmed by mass spectrometry analysis, and the purity was shown to be more than 98% as assessed by high pressure liquid chromatography analysis.

The peptide was supplied as a freeze-dried, sterile white powder. It was dissolved in 1.0 ml of physiological saline (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) and stored at -80°C until just before use.

Incomplete Freund's Adjuvant (IFA) preparation

Montanide ISA 51 (SEPPIC Inc., NJ, USA) was used as incomplete Freund's adjuvant (IFA).

Patient treatment

In this phase I clinical study, two protocols were used. One was a basic protocol, namely, with survivin-2B peptide alone, and the other used survivin-2B peptide mixed with IFA. In this trial, the primary endpoint was safety. The second endpoint was investigations about anti-tumor effects and clinical and immunological monitoring.

In the first protocol, the vaccination schedule was as follows. Vaccinations with survivin-2B peptide were given subcutaneously (s.c.) four times at 14-day intervals. To set up a dose-escalation trial, the patients were separated into the following two groups: in group 1 patients were vaccinated with 0.1 mg of the peptide and in group 2 patients were vaccinated with 1.0 mg of the peptide. Each group included five patients. Escalation to the next dose was allowed if side effects did not exceed grade 3. If patients whose disease was not far advanced hoped for continuation of this peptide vaccination therapy, we vaccinated them in the same manner after the fourth vaccination.

In the second protocol, survivin-2B peptide at a dose of 1 mg/1 ml and IFA at a dose of 1 ml were mixed immediately before vaccination. Then the patients were vaccinated subcutaneously (s.c.) four times at 14-day intervals.

Delayed-type hypersensitivity (DTH) skin test

A DTH skin test was performed at each vaccination. The peptide (10 μg) solution in physiological saline (0.1 ml) or physiological saline alone (0.1 ml) was separately injected intradermally (i.d.) into the forearm opposite the vaccination site. A positive reaction was defined as a more than 4 mm diameter area of erythema and induration 48 hr after the injection.

Toxicity evaluation

Patients were examined closely for signs of toxicity during and after vaccination. Adverse events were recorded using the National Cancer Institute Common Toxicity Criteria (NCI-CTC).

Clinical response evaluation

Physical examinations and hematological examinations were conducted before and after each vaccination. Tumor markers (CEA, CA15-3, NCC-ST-439, and ICTP) were examined monthly.

Tumor size was evaluated by computed tomography (CT) scans or MRI in comparison with the size before the first vaccination and that after the fourth vaccination. A complete response (CR) was defined as complete disappearance of all measurable and evaluable disease. A partial response (PR) was defined as a ≧30% decrease from the baseline in the size of all measurable lesions (sum of maximal diameters). Progressive disease (PD) was defined as an increase in the sum of maximal diameters by at least 20% or the appearance of new lesions. Stable disease (SD) was defined as the absence of criteria matching those for CR, PR, or PD. Patients who received less than four vaccinations were excluded from all evaluations in this study.

In vitro stimulation of PBMC

PBMCs were isolated from blood samples by Ficoll-Conray density gradient centrifugation. Then they were frozen and stored at -80°C. As needed, frozen PBMCs were thawed and incubated in the presence of 30 μl/ml survivin-2B peptide in AIM-V medium containing 10% human serum at room temperature. Next, interleukin-2 was added at a final concentration of 50 U/ml 1 hr, 2 days, 4 days and 6 days after the addition of the peptide. On day 7 of culture, the PBMCs were analyzed by tetramer staining and ELISPOT assay.

Tetramer staining

FITC-labeled HLA-A*2402-HIV peptide (RYLRDQQLL) tetramer and PE-labeled HLA-A*2402-Survivin-2B80-88 peptide tetramer were purchased from MBL Inc.(Japan). For flow cytometric analysis, PBMCs, which were stimulated *in vitro* as above, were stained with the tetramers at 37°C for 20 min, followed by staining with FITC- or PerCP-conjugated anti-CD8 mAb (Beckton Dickinson Biosciences) at 4°C for 30 min. Cells were washed twice with PBS before fixation in 1% formaldehyde. Flow cytometric analysis was performed using FACSCalibur and CellQuest software (BD Biosciences). The frequency of

CTL precursors was calculated as the number of tetramer-positive cells divided by the number of CD8-positive cells.

ELISPOT assay

ELISPOT plates were coated sterilely overnight with an IFN-γ capture antibody (Beckton Dickinson Biosciences) at 4°C. The plates were then washed once and blocked with AIM-V medium containing 10% human serum for 2 hr at room temperature. CD8-positive T cells separated from patients' PBMCs (5×10^3 cells/well), which were stimulated *in vitro* as above, were then added to each well along with HLA-A24-transfected CIR cells (CIR-A24) (5×10^4 cells/well), which had been preincubated with or without survivin-2B80-88 peptide (10 μg/ml) and with an HIV peptide as a negative control. After incubation in a 5% CO_2 humidified chamber at 37°C for 24 hours, the wells were washed vigorously five times with PBS and incubated with a biotinylated anti-human IFN-γ antibody and horseradish peroxidase-conjugated avidin. Spots were visualized and analyzed using KS ELISPOT (Carl Zeiss, Germany).

Results

Patient profiles

In the first protocol with survivin-2B peptide alone, 12 patients were initially enrolled in the study (Additional file 1), but two (cases 7 and 10) discontinued halfway through the protocol. One patient (case 7) had local recurrence, brain and lung metastases from bilateral breast cancer and was removed from the study after 3 vaccinations since new brain metastasis appeared and she required radiation therapy. Another patient (case 10) had lymph node metastases from right breast cancer. She was removed from the study after 3 vaccinations because of enlargement of lymph node metastases. Neither of the treatment interruptions was due to adverse effects of the vaccination. Ten patients received the complete regimen including four vaccinations and were evaluated. They were all women, whose average age was 49 years (range, 34–71).

In the second protocol with survivin-2B peptide mixed with IFA, five patients were initially enrolled in the study (Additional File 2), but one (case 2) discontinued halfway through the protocol. This patient had lung and liver metastases from right breast cancer and was removed from the study after 3 vaccinations because of exacerbated liver function resulting from advanced liver metastases. In this protocol, there were no patients who dropped out because of adverse events due to the peptide vaccination. Four patients received the complete regimen including four vaccinations and were evaluated. They were all women, whose average age was 52 years (range, 36–71).

Safety

Peptide vaccination was well tolerated in all patients. In patients vaccinated with the peptide alone, no adverse events were observed during or after vaccination (Additional File 3). Of the patients vaccinated with the peptide mixed with IFA, two (cases 1 and 3) had induration at the injection site (Additional File 4). One (case 4) had general malaise (grade 1) and one (case 5) had general malaise (grade 1) and fever (grade 1). No other severe adverse events were observed during or after vaccination.

Clinical responses

Table 3 summarizes the clinical outcomes for the 10 patients in the first protocol with survivin-2B peptide alone. In all patients except two (cases 1 and 9), the tumor marker levels were increased. In one patient (case 1), the levels of CEA, CA15-3 and NCC-ST-439 were within the normal range during this clinical trial. The level of ICTP was decreased from 7.2 ng/ml just before the first vaccination to 5.5 ng/ml after the fourth vaccination. However, this change was not considered a significant decrease. In case 9, all tumor marker levels were within the normal range during this clinical trial. As for tumor size, two patients (cases 3 and 5) were considered to have SD. In one patient (case 9) who had bone metastases, the area of bone metastases did not increase in bone scintigraphy, and new metastatic foci did not appear during this trial. In this patient, although there was no aggravation of the clinical condition, we could not estimate the clinical response by our criteria because bone metastases were not able to be evaluated in CT images. The other patients were considered to have progressive disease (PD). In this protocol, if patients whose disease was not far advanced hoped for the continuation of this peptide vaccination therapy, we vaccinated them in the same manner after the fourth vaccination. There were 2 patients (cases 3 and 9) who were vaccinated for more than one year. In case 3, with bone and lymph node metastases, vaccination continued 42 times for 20 months (Fig. 1). For this period, new metastatic foci did not appear and there was almost no increase in the size of the metastatic lesions in this patient. Tumor marker levels did not increase rapidly (Fig. 1). In addition, she maintained good quality of life because there was no adverse effect.

Table 4 summarizes the clinical outcomes for the 4 patients in the second protocol with survivin-2B peptide mixed with IFA. In all patients, the tumor marker levels were increased. As for tumor size, all patients were considered to have PD.

DTH skin test

A DTH skin test was performed at each vaccination and assessed 48 hr later. A positive reaction was defined as erythema and induration more than 4 mm in diameter. In

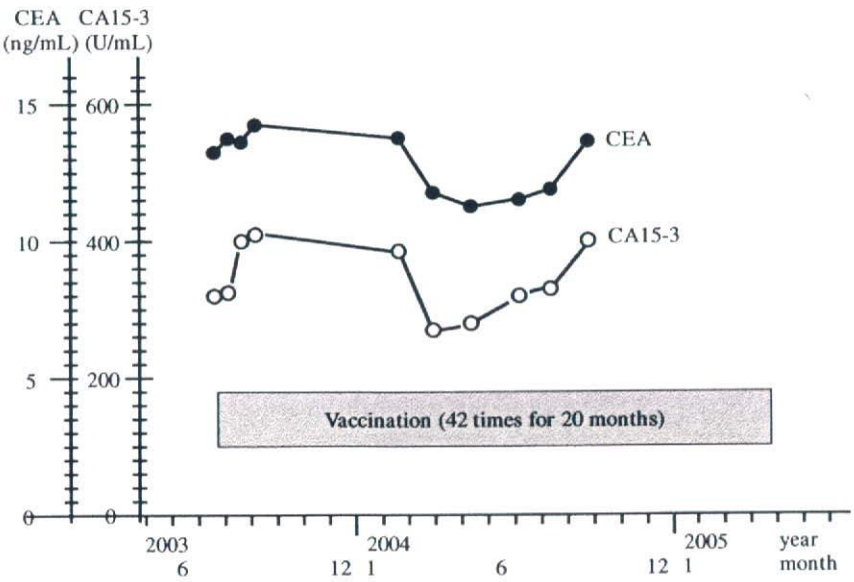

Figure 1
The changes in tumor marker levels in case 3 in the first protocol. For case 3, with bone and lymph node metastases, vaccination continued 42 times for 20 months. In this period new metastatic foci did not appear and there was almost no increase in size of the metastatic lesions. Tumor marker levels did not increase rapidly.

the first protocol with survivin-2B peptide alone, 2 of the 10 patients (20%) exhibited a positive DTH reaction at least once during the study. In the second protocol with peptide mixed with IFA, a positive DTH reaction was observed in 1 of the 4 patients (25%).

Tetramer staining assay and ELISPOT assay

To determine if the survivin-2B peptide vaccination could bring about specific immune responses in the patients, we analyzed the peptide-specific CTL frequency by using the HLA-A24/peptide tetramer. The change of tetramer-positive CTL frequency was evaluated by comparison with that before the first vaccination and that after the fourth vaccination as follows: detected and undetected. Detected was defined as an increase of twofold or more. Undetected was defined as a less than twofold increase. In the first protocol with the peptide alone, a change was considered to be detected in 3 patients (30%) (Table 3). On the other hand, in the second protocol with peptide mixed with IFA, it was considered to be detected in all 4 patients (100%) (Table 4). In Figure 2, the peptide-specific CTL frequency in the second protocol is indicated as the percentage of tetramer-positive CTL cells among CD8-positive T cells before the first vaccination and after the fourth vaccination.

ELISPOT assay of CD8-positive T cells separated from the patients' PBMCs showed peptide-specific IFN-γ responses in 2 patients, one of whom (case 8) was in the first protocol while the other (case 5) was in the second protocol. To be more precise, in case 5 in the second protocol, in the wells that were preincubated without the survivin-2B peptide and with the HIV peptide, spots were almost not visualized. On the other hand, in the wells that had been preincubated with survivin-2B peptide, many spots were visualized (Fig. 3). In these two patients, the peptide-specific CTL frequency was also increased by the vaccination with survivin-2B peptide.

Discussion

Recently, a large number of tumor antigens and epitopes recognized by CTLs have been identified, and reports of clinical trials utilizing peptide vaccination are increasing [10,18-20]. We demonstrated that survivin was expressed in a large proportion of various cancer specimens, and the survivin-2B-derived peptide could induce a CTL response in the context of HLA-A24 [16,17]. In addition, we showed an elevation in CTL precursor frequencies in PBMCs of HLA-A24+ cancer patients by using an HLA-A24/survivin-2B peptide tetramer. On the basis of the above studies, we started a phase I clinical study of survivin-2B peptide vaccine therapy for patients with advanced or recurrent colorectal cancer in 2003 [12]. In

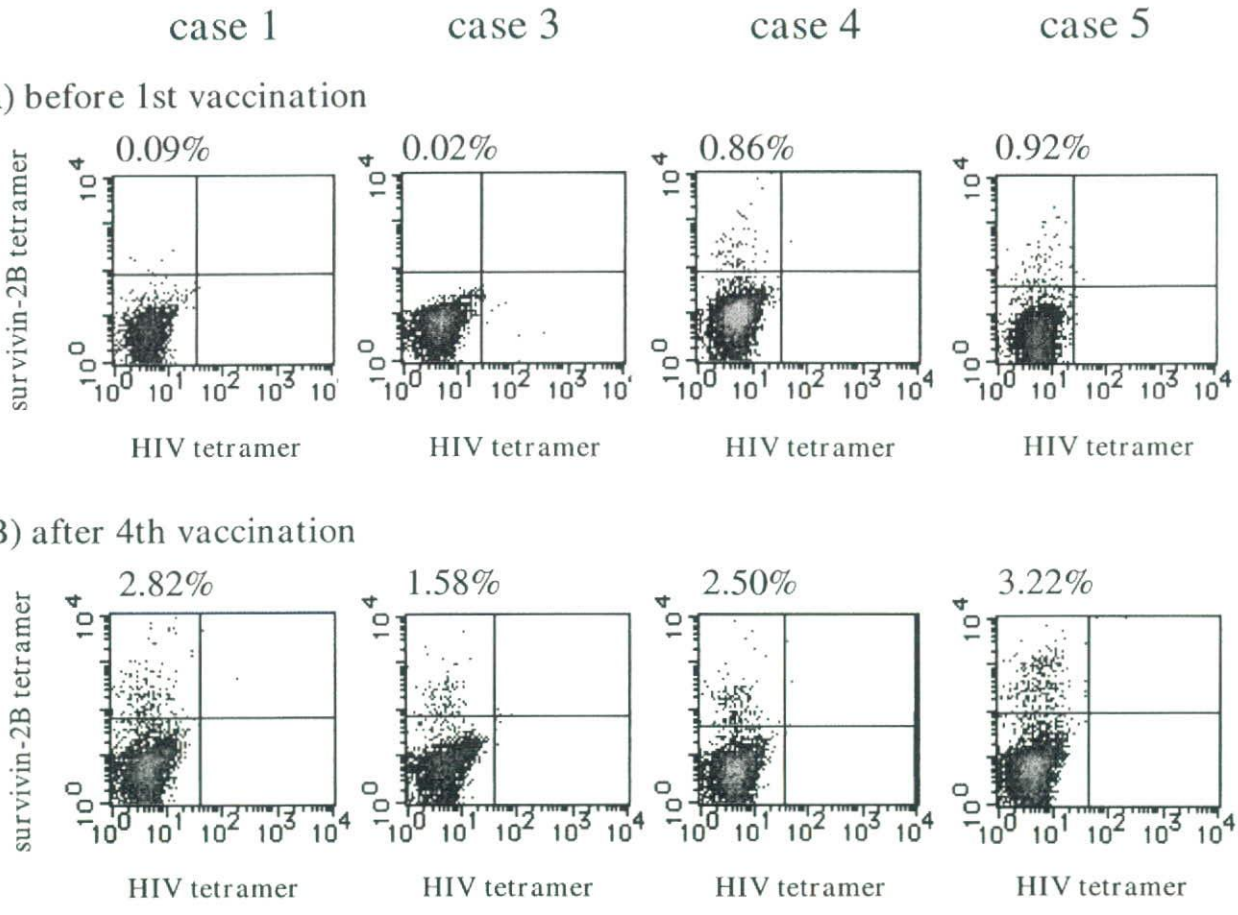

Figure 2

Tetramer staining before the first vaccination and after the fourth vaccination in the second protocol. FITC-labeled HLA-A*2402-HIV peptide (RYLRDQQLL) tetramer and PE-labeled HLA-A*2402-Survivin-2B80-88 peptide tetramer were uesd. For flow cytometric analysis, PBMCs, which were stimulated *in vitro*, were stained with the tetramers at 37°C for 20 min, followed by staining with FITC- or PerCP-conjugated anti-CD8 mAb (Beckton Dickinson Biosciences) at 4°C for 30 min. Cells were washed twice with PBS before fixation in 1% formaldehyde. Flow cytometric analysis was performed using FACSCalibur and CellQuest software (BD Biosciences). The frequency of CTL precursors was calculated as the number of tetramer-positive cells divided by the number of CD8-positive cells. The peptide-specific CTL frequency is indicated as the percentage of tetramer-positive CTL cells among CD8-positive T cells before the first vaccination and after the fourth vaccination. The peptide-specific CTL frequency after the fourth vaccination (B) was compared with that before the first vaccination (A). In the second protocol with the peptide mixed with IFA, the peptide-specific CTL frequency was increased in all 4 patients (100%).

this study, we vaccinated patients with survivin-2B peptide alone, and reported the safety of this peptide vaccination and the potency of anti-tumor effects induced by the peptide vaccination. At that time, we started this phase I clinical study of peptide vaccine therapy for patients with advanced or recurrent breast cancer as well. In this study, we vaccinated patients with not only survivin-2B peptide alone but also survivin-2B mixed with IFA in order to induce greater anti-tumor effects. We recently established immunological monitoring methods using a tetramer staining assay and ELISPOT assay. Thus, in this study, we also conducted immunological monitoring using these techniques.

Survivin is an ideal tumor-associated antigen expressed in a broad variety of malignancies and recognized by specific cytotoxic T cells [21]. The first survivin-derived peptides were characterized in 2000 [22,23]. Subsequently, there

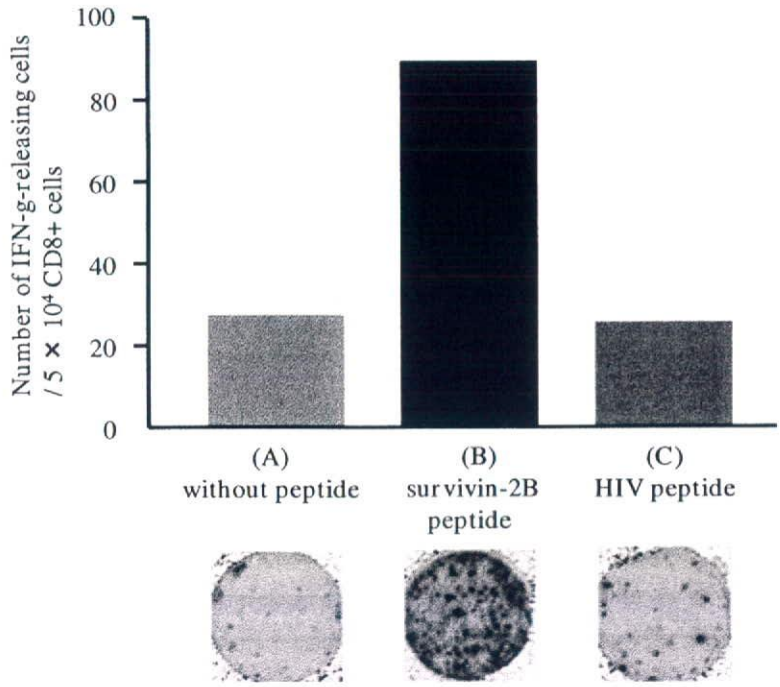

Figure 3
ELISPOT assay after the fourth vaccination of case 5 in the second protocol. In the wells that were preincubated without survivin-2B peptide (A) or with an HIV peptide (C), spots were almost not visualized. On the other hand, in the wells that were preincubated with survivin-2B peptide (B), many spots were visualized. These findings demonstrate that CD8-positive T cells separated from the patients' PBMCs had a peptide-specific IFN-γ response.

have been many reports about survivin peptide responses. Grube et al. [24] reported that HLA-A2.1-restricted survivin peptide induced CD8+ T cell reactivity in patients with multiple myeloma. Andersen et al. [25] reported the detection of HLA-A24 restricted and survivin peptide-specific CD8-positive cells by IFN-γ ELISPOT assay and perforin ELISPOT assay in patients with breast cancer, melanoma and renal cancer. Abrogating the function of survivin not only limits the proliferative potential and viability of tumor cells directly [26], but also inhibits tumor angiogenesis [27]. Xiang et al. [28] reported that a DNA vaccine targeting survivin lead to eradication of pulmonary metastases by a combinational effect inducing tumor cell apoptosis and suppressing tumor angiogenesis in a murine model. Thus, survivin is a suitable target for immune therapy for cancer [26,29]. Recently, a number of survivin epitopes restricted to several additional HLA-molecules have been identified [22,25,29-34], and several clinical trials of immunotherapy based on survivin-derived peptides have been initiated. Wobser et al. [35] reported complete remission of liver metastasis of pancreatic cancer under vaccination with an HLA-A2 restricted survivin peptide. In addition, a phase I/II trial with HLA-A1, -A2 and -B35 restricted survivin peptides for patients with advanced cancer is ongoing. Fuessel et al. [27] reported a phase I clinical trial for patients with prostate cancer in which they evaluated a vaccination with DCs loaded with five different prostate cancer-associated antigens (survivin, prostate-specific antigen [PSA], prostate-specific membrane antigen [PSMA], and prostein, transient receptor potential p8 [trp-p8]) and concluded that the concept was safe and feasible. Besides the above-mentioned investigations, various clinical trials are ongoing now.

At present, 4 splicing variants of survivin (survivin-ΔEx3, survivin-2α, survivin-2B, and survivin-3B) have been identified. Espinosa et al. [36] reported that the expression of survivin-ΔEx3 and survivin-2B was higher in cervical cancer samples. There is also a report that survivin-2B was dominantly expressed in gastric cancer [37]. Futakuchi et al. [38] reported that the ratios of survivin-2B/survivin and survivin-ΔEx3/survivin in malignant cervical tissue samples were significantly higher than those in normal cervical tissue templates. Moreover, the ratio of survivin-2B/survivin was increased in patients with higher stages and with pelvic lymph node metastasis. These reports might support the idea that survivin-2B is the ideal

target of immunotherapy for cancer patients [17,39], especially for those with advanced or recurrent cancer. On the other hand, Mahotka et al. [40] reported that the ratio of survivin-2B/survivin was decreased in the late stages of renal cell carcinoma. Yamada et al. [41] reported that there was no significant difference in the ratio of survivin-2B/survivin in malignant brain tumors and gliomas compared with nonglioma. There is a hypothesis that the relevant ratios of the survivin and splicing variants may regulate the ultimate apoptotic activities of cancer cells and determine their biological behaviours and responses to apoptosis-inducing treatment [37,40]. Nevertheless, the exact roles and expression of survivin splicing variants and their interplay in various cancers are as yet unclear because of the high complexity of its regulation [36,42]. We previously demonstrated that the expression of survivin-2B was detected in a variety of tumor cell lines but not in normal tissues except in the thymus, although low levels of survivin expression were detected by reverse transcription-PCR analysis [16]. In addition, we reported that survivin-2B-specific CTLs could be induced efficiently from PBMCs of HLA-A24-positive survivin-positive cancer patients [17]. As described above, we are sure that survivin-targeting immunotherapy with survivin-2B peptide should be a reasonable strategy.

A dose-escalation trial was chosen to estimate a safe and optimal dose in the first protocol with survivin-2B peptide alone. We used 0.1 mg and 1.0 mg dosage groups, each consisting of five patients. No adverse events were observed in either group. In addition, for the patients (cases 3 and case 9) who were vaccinated 42 times and 38 times respectively, adverse events were not observed during or after the vaccination. Thus, we concluded that the survivin-2B peptide vaccine was safe and could be repeatedly injected into patients without severe adverse events. In comparison between patients who were vaccinated with 0.1 mg and 1.0 mg of the peptide, there was almost no difference in clinical responses. However, peptide-specific immune responses in tetramer staining and ELISPOT assay were frequently induced in patients vaccinated with 1.0 mg of the peptide in comparison with patients vaccinated with 0.1 mg of the peptide. Therefore, we decided that the optimal dose of the peptide was 1.0 mg. IFA has sustained-release effect, which can enhance the anti-tumor effect of the peptide injected subcutaneously. So, in the second protocol which its purpose was to induce the more effective anti-tumor effect by the survivin-2B peptide, IFA was used mixed with 1.0 mg of the peptide. In this protocol, two patients (cases 1 and 3) had induration. This was due to IFA trapped in the subcutaneous lesion. Patient 4 had general malaise (grade 1), and patient 5 had general malaise (grade 1) and fever (grade 1). No other severe adverse events were observed during or after vaccination. Therefore, we concluded that the vaccine using survivin-2B peptide mixed with IFA was safe, as was the peptide alone.

Positive delayed-type hypersensitivity (DTH) reactions were observed in 2 of the 10 patients (20.0%) in the first protocol and in 1 of the 4 patients (25.0%) in the second protocol at least once during the vaccination. Some reports have suggested a positive correlation between DTH and clinical [43] or immunological responses [44]. In this study, in case 5 in the first protocol a positive DTH reaction was observed and the change of tumor size was considered to indicate SD, while the tumor marker level was considered to have increased, although immunological responses were not induced. However, neither clinical nor immunological responses were totally associated with a positive DTH reaction in this study.

In the first protocol with survivin-2B peptide alone, none of patients in the 0.1 mg peptide group had tetramer response and that 3 of the 5 patients (30%) in the 1.0 mg peptide group had increased the tetramer-specific CTL frequency. On the other hand, in the second protocol with survivin-2B peptide mixed with IFA, all patients had a significant increase of the tetramer-positive CTL frequency. These results might demonstrate that the addition of IFA could enhance the immunological responses to the survivin-2B peptide. In addition, these findings might also indicate that the addition of another effectual adjuvant such as a cytokine, heat shock protein [45], etc. to the vaccination with survivin-2B peptide mixed with IFA could more effectively enhance the immunological and clinical responses to the peptide. At present a phaseII clinical study of survivin-2B peptide vaccine therapy, in which the peptide is combined with IFA and IFN-α, is ongoing in our group.

In the second protocol with the peptide mixed with IFA, although all patients had an increase of the tetramer-positive CTL frequency, only one patient had a peptide specific IFN-γ response in the ELISPOT assay. In tetramer staining, the frequency of the peptide-specific CTL was investigated. In the ELISPOT assay, the function of the peptide-specific CTL was investigated. It is possible that the peptide-specific CTL induced by the vaccination might not function well due to immune escape mechanisms in the effector phase. Thus, this might be one of the reasons why the CTL response to the vaccination was not sufficient to induce clinical responses. This also could imply a dysfunction of the host immune system or an immunosuppressive effect of the tumor microenvironment, including the down-regulation of HLA-class I molecules on tumor cells. Therefore, we have recently begun to investigate a novel strategy to overcome the immune escape in peptide vaccine therapy.

Conclusion

In conclusion, this phase I clinical study revealed that the administration of not only survivin-2B peptide alone but also the peptide in combination with IFA for patients with advanced or recurrent breast cancer was well tolerated. Vaccination with survivin-2B peptide mixed with IFA increased the frequency of the peptide-specific CTL more effectively than vaccination with the peptide alone, although neither vaccination could induce an efficient clinical response. Thus, the addition of IFA might enhance the immunological response to the peptide vaccination. Considering the above, the addition of another effectual adjuvant such as a cytokine, heat shock protein [45], etc. to the vaccine using survivin-2B peptide mixed with IFA might induce improved immunological and clinical responses.

List of abbreviations

PBMCs: peripheral blood mononuclear cells, CTL: cytotoxic T lymphocyte, IAP: inhibitor of the apoptosis proteins, ECOG: Eastern Co-operative Oncology Group, IFA: incomplete Freund's adjuvant, DTH: delayed-type hypersensitivity, CR: complete response, PR: partial response, SD: stable disease, PD: progressive disease.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TT[1] performed peptide vaccine preparation, and contributed largely to designing the phase I clinical study, coordination of this study and analysis of all results. TT[1] and YI contributed to the medical examination and vaccination of patients, and interpretation of clinical data. TO, TK, FH, TF, KY, YK and NT contributed to the medical care. TT2 and YH contributed to designing the peptide vaccine. TT[2] contributed to interpretation of immunological data. TT[2], AY, HA and KS contributed to analysis of immunological responses, such as tetramer staining, ELISPOT assay, etc. MO, YM, NN contributed to registration of patients. NS and KH contributed largely to control over the clinical system and immunological study, respectively, and they also contributed to reviewing the manuscript. All authors have read and approved the final manuscript. (TT[1]: Tetsuhiro Tsuruma, TT[2]: Toshihiko Torigoe)

Additional material

Additional file 1

Table 1: Profiles of patients enrolled in the first protocol with survivin-2B peptide alone. The data showed profiles of patients enrolled in the first protocol with survivin-2B peptide alone.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1479-5876-6-24-S1.pdf]

Additional file 2

Table 2: Profiles of patients enrolled in the second protocol with survivin-2B peptide mixed IFA. The data showed profiles of patients enrolled in the second protocol with survivin-2B peptide mixed IFA.
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[http://www.biomedcentral.com/content/supplementary/1479-5876-6-24-S2.pdf]

Additional file 3

Table 3: Outcome in the first protocol with survivin-2B peptide alone. This data showed the clinical and immunological evaluation in the first protocol.
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[http://www.biomedcentral.com/content/supplementary/1479-5876-6-24-S3.pdf]

Additional file 4

Table 4: Outcome in the second protocol with survivin-2B peptide mixed IFA. This data showed the clinical and immunological evaluation in the second protocol.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1479-5876-6-24-S4.pdf]

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Written consent for publication was obtained from the patient or their relative.

References

1. Talback M, Stenbeck M, Rosen M, Barlow L, Glimelius B: **Cancer survival in Sweden 1960–1998 – developments across four decades.** *Acta Oncol* 2003, **42**:637-659.
2. Edwards BK, Brown ML, Wingo PA, Howe HL, Ward E, Ries LA, Schrag D, Jamison PM, Jemal A, Wu XC, Friedman C, Harlan L, Warren J, Anderson RN, Pickle LW: **Annual report to the nation on the status of cancer, 1975–2002, featuring population-based trends in cancer treatment.** *J Natl Cancer Inst* 2005, **97**:1407-1427.
3. Sant M, Francisci S, Capocaccia R, Verdecchia A, Allemani C, Berrino F: **Time trends of breast cancer survival in Europe in relation to incidence and mortality.** *Int J Cancer* 2006, **119**:2417-2422.
4. Gralow JR: **Breast cancer 2004: Progress and promise on the clinical front.** *Phys Med* 2006, **21(Suppl 1)**:2.
5. Boon T, Coulie PG, Eynde B Van den: **Tumor antigens recognized by T cells.** *Immunol Today* 1997, **18**:267-268.
6. Rosenberg SA: **A new era for cancer immunotherapy based on the genes that encode cancer antigens.** *Immunity* 1999, **10**:281-287.
7. Marchand M, van Baren N, Weynants P, Brichard V, Dreno B, Tessier MH, Rankin E, Parmiani G, Arienti F, Humblet Y, Bourlond A, Vanwijck R, Lienard D, Beauduin M, Dietrich PY, Russo V, Kerger J, Masucci G, Jager E, De Greve J, Atzpodien J, Brasseur F, Coulie PG, Bruggen P van der, Boon T: **Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1.** *Int J Cancer* 1999, **80**:219-230.
8. Rosenberg SA, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM, Topalian SL, Restifo NP, Dudley ME, Schwarz SL, Spiess PJ, Wunderlich JR, Parkhurst MR, Kawakami Y, Seipp CA, Einhorn JH, White DE: **Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma.** *Nat Med* 1998, **4**:321-327.
9. Kawaguchi S, Wada T, Ida Y, Sato Y, Nagoya S, Tsukahara T, Kimura S, Sahara H, Ikeda H, Shimozawa K, Asanuma H, Torigoe T, Hiraga H, Ishii T, Tatezaki SI, Sato N, Yamashita T: **Phase I vaccination trial**

of SYT-SSX junction peptide in patients with disseminated synovial sarcoma. *J Transl Med* 2005, **3**:1-9.
10. Bruggen P van der, Traversari C, Chomez P, Lurquin C, De Plasen E, Eynde B Van den, Knuth A, Boon T: **A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma.** *Science* 1991, **254**:1643-1647.
11. Kawakami Y, Eliyahu S, Delgado CH, Robbins PF, Rivoltini L, Topalian SL, Miki T, Rosenberg SA: **Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor.** *Proc Natl Acad Sci USA* 1994, **91**:3515-3519.
12. Tsuruma T, Hata F, Torigoe T, Furuhata T, Idenoue S, Kurotaki T, Yamamoto M, Yagihashi A, Ohmura T, Yamaguchi K, Katsuramaki T, Yasoshima T, Sasaki K, Mizushima Y, Minamida H, Kimura H, Akiyama M, Hirohashi Y, Asanuma H, Tamura Y, Shimozawa K, Sato N, Hirata K: **Phase I clinical study of anti-apoptosis protein, survivin-derived peptide vaccine therapy for patients with advanced or recurrent colorectal cancer.** *J Transl Med* 2004, **2**:19-29.
13. Uenaka A, Wada H, Isobe M, Saika T, Tsuji K, Sato E, Sato A, Noguchi Y, Kawabata R, Yasuda T, Doki Y, Kumon H, Iwatsuki K, Shiku H, Monden M, Jungbluth AA, Ritter G, Murphy R, Hoffman E, Old LJ, Nakayama E: **T cell immunomonitoring and tumor responses in patients immunized with a complex of cholesterol-bearing hydrophobized pullulan (CHP) and NY-ESO-1 protein.** *Cancer Immun* 2007, **7**:9-19.
14. Yanagimoto H, Mine T, Yamamoto K, Satoi S, Terakawa N, Takahashi K, Nakahara K, Honma S, Tanaka M, Mizoguchi J, Yamada A, Oka M, Kamiyama Y, Itoh K, Takai S: **Immunological evaluation of personalized peptide vaccination with gemcitabine for pancreatic cancer.** *Cancer Sci* 2007, **98**:605-611.
15. Ambrosini G, Adida C, Altieri DC: **A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma.** *Nat Med* 1997, **3**:917-921.
16. Hirohashi Y, Torigoe T, Maeda A, Nabeta Y, Kamiguchi K, Sato T, Yoda J, Ikeda H, Hirata K, Yamanaka N, Sato N: **An HLA-A24-restricted cytotoxic T lymphocyte epitope of a tumor-associated protein, survivin.** *Clin Cancer Res* 2002, **8**:1731-1739.
17. Idenoue S, Hirohashi Y, Torigoe T, Sato Y, Tamura Y, Hariu H, Yamamoto M, Kurotaki T, Tsuruma T, Asanuma H, Kanaseki T, Ikeda H, Kashiwagi K, Okazaki M, Sasaki K, Sato T, Ohmura T, Hata F, Yamaguchi K, Hirata K, Sato N: **A potent immunogenic general cancer vaccine that targets survivin, an inhibitor of apoptosis proteins.** *Clin Cancer Res* 2005, **11**:1474-1482.
18. Yang D, Nakao M, Shichijo S, Sasatomi T, Takasu H, Matsumoto H, Mori K, Hayashi A, Yamana H, Shirouzu K: **Identification of a gene coding for a protein possessing shared tumor epitopes capable of inducing HLA-A24-restricted cytotoxic T lymphocytes in cancer patients.** *Cancer Res* 1999, **59**:4056-4063.
19. Nishizaka S, Gomi S, Harada K, Oizumi K, Itoh K, Shichijo S: **A new tumor-rejection antigen recognized by cytotoxic T lymphocytes infiltrating into a lung adenocarcinoma.** *Cancer Res* 2000, **60**:4830-4837.
20. Gjertsen MK, Buanes T, Rosseland AR, Bakka A, Gladhaug I, Soreide O, Eriksen JA, Moller M, Baksaas I, Lothe RA, Saeterdal I, Gaudernack G: **Intradermal ras peptide vaccination with granulocyte-macrophage colony-stimulating factor as adjuvant: Clinical and immunological responses in patients with pancreatic adenocarcinoma.** *Int J Cancer* 2001, **92**:441-450.
21. Schmidt SM, Schag K, Muller MR, Weck MM, Appel S, Kanz L, Grunebach F, Brossart P: **Survivin is a shared tumor-associated antigen expressed in a broad variety of malignancies and recognized by specific cytotoxic T cells.** *Blood* 2003, **15**:571-576.
22. Schmitz M, Diestelkoetter P, Weigle B, Schmachtenberg F, Stevanovic S, Ockert D, Rammensee HG, Rieber EP: **Generation of survivin-specific CD8+ T effector cells by dendritic cells pulsed with protein or selected peptides.** *Cancer Res* 2000, **60**:4845-4849.
23. Andersen MH, Becker JC, Straten P: **Identification of a cytotoxic T lymphocyte response to the apoptosis inhibitor protein survivin in cancer patients.** *Cancer Res* 2001, **61**:869-872.
24. Grube M, Moritz S, Obermann EC, Rezvani K, Mackensen A, Andressen R, Holler E: **CD8+ Tcell reactive to survivin antigen in patients with multiple myeloma.** *Clin Cacncer Res* 2007, **13**:1053-1060.
25. Andersen MH, Soerensen RB, Becker JC, Straten P: **HLA-A24 and survivin: possibilities in therapeutic vaccination against cancer.** *J Transl Med* 2006, **4**:38.
26. Andersen MH, Becker JC, Straten PT: **Regulators of apoptosis: suitable targets for immune therapy of cancer.** *Nat Rev Drug Discov* 2005, **4**:399-409.
27. Fuessel S, Meye A, Schmitz M, Zastrow S, Linne C, Richter K, Lobel B, Hakenberg OW, Hoelig K, Rieber EP, Wirth MP: **Vaccination of hormone-refractory prostate cancer patients with peptide cocktail-loaded dendritic cells: results of a phase I clinical trial.** *Prostate* 2006, **66**:811-821.
28. Xiang R, Mizutani N, Luo Y, Chiodoni C, Zhou H, Mizutani M, Ba Y, Becker JC, Reisfeld RA: **A DNA vaccine targeting survivin combines apoptosis with suppression of angiogenesis in lung tumor eradication.** *Cancer Res* 2005, **65**:553-561.
29. Andersen MH, Pedersen LO, Capeller B, Brocker EB, Becker JC, Straten PT: **Spontaneous cytotoxic T-cell responses against survivin-derived MHC class I-restricted T-cell epitopes in situ as well as ex vivo in cancer patients.** *Cancer Res* 2001, **61**:5964-5968.
30. Reker S, Becker JC, Svane IM, Ralfkiaer E, Straten PT, Andersen MH: **HLA-B35-restricted immune responses against survivin in cancer patients.** *Int J Cancer* 2004, **108**:937-941.
31. Shangary S, Johnson DE: **Recent advances in the development of anticancer agents targeting cell death inhibitors in the Bcl-2 protein family.** *Leukemia* 2003, **17**:1470-1481.
32. Reker S, Meier A, Holten-Andersen L, Svane IM, Becker JC, Straten P, Andersen MH: **Identification of novel survivin-derived CTL epitopes.** *Cancer Biol Ther* 2004, **3**:173-179.
33. Siegel S, Steinmann J, Schmitz N, Stuhlmann R, Dreger P, Zeis M: **Identification of a survivin-derived peptide that induces HLA-A0201-restricted antileukemia cytotoxic T lymphocytes.** *Leukemia* 2004, **18**:2046-2047.
34. Andersen MH, Becker JC, Straten P: **Regulations of apoptosis: suitable targets for immune therapy of cancer.** *Nat Rev Drug Discov* 2005, **4**:399-409.
35. Wobser M, Keikavoussi P, Kunzmann V, Weininger M, Andersen MH, Becker JC: **Complete remission of liver metastasis of pancreatic cancer under vaccination with a HLA-A2 restricted peptide derived from the universal tumor antigen survivin.** *Cancer Immunol Immunother* 2006, **55**:1294-1298.
36. Espinosa M, Cantu D, Herrera N, Lopez CM, De la Garza JG, Maldonado V, Melendez-Zajgla J: **Inhibitors of apoptosis proteins in human cervical cancer.** *BMC Cancer* 2006, **6**:45-54.
37. Cheng Z, Hu L, Fu W, Zhang Q, Liao X: **Expression of survivin and its splice variants in gastric cancer.** *J Huazhong Univ Sci Technolog Med Sci* 2007, **27**:393-398.
38. Futakuchi H, Ueda M, Kanda K, Fujino K, Yamaguchi H, Noda S: **Transcriptional expression of survivin and its splice variants in cervical carcinoma.** *Int J Gynecol Cancer* 2007, **17**:1092-1098.
39. Ichiki Y, Hanagiri T, Takenoyama M, Baba T, Fukuyama T, Nagata Y, Mizukami M, So T, Sugaya M, Yasuda M, So T, Sugio K, Yasumoto K: **Tumor specific expression survivin-2B in lung cancer as a novel target of immunotherapy.** *Lung Cancer* 2005, **48**:281-289.
40. Mahotka C, Krieg T, Krieg A, Wenzel M, Suschek CV, Heydthausen M, Gabbert HE, Gerharz CD: **Distinct in vivo expression patterns of survivin splice variants in renal cell carcinomas.** *Int J Cancer* 2002, **100**:30-36.
41. Yamada Y, Kuroiwa T, Nakagawa T, Kajimoto Y, Dohi T, Azuma H, Tsuji M, Kami K, Miyatake S: **Transcriptional expression of survivin and its splice variants in brain tumors in humans.** *J Neurosurg* 2003, **99**:738-745.
42. Vegran F, Boidot R, Oudin C, Defrain C, Rebucci M, Lizard-Nacol S: **Association of p53 gene alterations with the expression of antiapoptosis survivin splice variants in breast cancer.** *Oncogene* 2007, **11**:290-297.
43. Nestle FO, Alijagic S, Gilliet M, Sun Y, Grabbe S, Dummer R, Burg G, Schadendorf D: **Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells.** *Nat Med* 1998, **4**:328-332.
44. Hildenbrand B, Sauer B, Kalis O, Stoll C, Freudenberg MA, Niedermann G, Giesler JM, Juttner E, Peters JH, Haring B, Leo R, Unger C, Azemar M: **Immunotherapy of patients with hormone-refractory prostate carcinoma pre-treated with interferon-gamma and vaccinated with autologous PSA-peptide loaded dendritic cells – a pilot study.** *Prostate* 2007, **67**:500-508.

45. Wu Y, Wan T, Zhou X, Wang B, Yang F, Li N, Chen G, Dai S, Liu S, Zhang M, Cao X: **Hsp70-like protein 1 fusion protein enhances induction of carcinoembryonic antigen-specific CD8$^+$ CTL response by dendritic cell vaccine.** *Cancer Res* 2005, **65**:4947-4954.