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, RYAMTV-WYF) 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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and/or Pro564, are hydroxylated (11–15). The site-specific HLA-A24-restricted manner. These data highlight HIFPH3 as a novel target in the therapy. 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, Pro402, and/or Pro564, 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 SMKRT-1 (HLA-A*2402+), SMKRT-2 (HLA-A*2402+), SMKRT-3 (HLA-A*2402+), and SMKRT-R-4 (HLA-A*2402+), lung cancer line LHK2, gastric cancer line SSTW, and pancreatic cancer line PNJ 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 HC20, and erythroblatemia 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 and 500 ng/mL puromycin (Sigma). T2-A*2402, a stable transfectant of HLA-A*2402 cDNA of SMKRT-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 k 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'-CATCCCTTGTGCTGTTGG-3' and 5'-CCAACAGCCCTGATTAGA-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'-ACCACAGCTTCCATGCCATCAC-3' and reverse primer 5'-TCCACACCTGTTGCTGTA-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 (IMRLEKLI), HIFPH3-2 (NWDAKHGGI), HIFPH3-3 (IFPEGSFSI), HIFPH3-4 (SFIADVEPI), HIFPH3-5 (GFCYLDNFL), HIFPH3-6 (SFLLSLIDRL), HIFPH3-7 (YYVKERSKAM), HIFPH3-8 (RYAMTVWYF), EBV LMIP2-derived HLA-A24-binding peptide (TYGPVFMSI; 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 Pharma- ceutical, 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, the cells were cultured in AIM-V medium supplemented with 10 mmol/L HEPES, 50 μmol/L 2-mercaptoethanol, 100 μg/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 (Miltenyi Biotech) and anti-CD14 monoclonal antibody coupled with magnetic microbeads according to the manufacturer’s instruction. Human recombinant IL-2, IL-4, and granulocyte-macrophage colony-stimulating factor were added to the culture. On the next day, the cells were washed with AIM-V medium once and then irradiated (100 Gy). The cells were then cultured 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. 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 effector/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

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**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 88BME and LG2M2E, colon cancer line SW480, lung cancer lines LNY-1, AS649, LH2K2, 8-87, LK79, and Lu65, pancreatic cancer lines HS777B and PUN, gastric cancer line SSTW, and hepatic cell cancer line CHC20.
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 effector 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) / (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 (SMKTR-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. 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.

**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 on T2-A’2402 cells that were pulsed with each peptide. HLA-A24-bound EBV LMP2-derived peptide (TYGPVFMKL) and HIV env-derived peptide (RYLADQOQLLGI) were used as positive controls. SL-8 peptide (SIINFEKL) was used as a negative control. Histograms of MFI were displayed for each peptide.
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 HIFPH3 positive by immunostaining (Fig. 2A and B).

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<th>Stage*</th>
<th>Peptides</th>
<th>HLA-A*2402</th>
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*Age: 15-86 years; Sex: M, male; F, female; Stage: T1aN0M0, tumor; node; metastasis.

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

*<sup>1</sup>*Tumor-node-metastasis classification.

<sup>2</sup> <sup>1</sup><sup>×</sup>20% specific lytic activity against HIFPH3 peptide-pulsed T2-A*2402 target cells was indicated as +.

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 51Cr release assay at the indicated E:T ratio. Peptide-pulsed T2-A*2402 cells and nonpulsed cells were used as target cells.
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 react 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.

### Discussion

The process of tumor progression (proliferation, local invasion, and distant metastasis) is characterized by rapid cellular growth accomplished 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.

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