Identification of Aldo-Keto Reductase 1C3-derived Peptides Recognized by Cytotoxic T Cells in Hepatocellular Carcinoma Patients

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Abstract

Background & Aims: Identification of novel immunotherapy targets for hepatocellular carcinoma (HCC) is an urgent and important subject to improve the prognosis of patients. In the present study, using the data of complementary DNA (cDNA) microarray and immunological analyses, we identified aldo-keto reductase 1C3 (AKR1C3)-derived cytotoxic T cell (CTL) epitopes.

Methods: The study included 54 HCC patients. The expression level of AKR1C3 in HCC and non-HCC liver tissue was examined by cDNA microarray, real-time polymerase chain reaction (PCR) and immunohistochemistry. Immune responses were measured by interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) and CTL assays.

Results: The expression level of AKR1C3 was higher in HCC tissue than in non-HCC liver tissue (p=0.013). In immunological assays, AKR1C3-derived peptides containing human leukocyte antigen (HLA)-A*2402 binding motifs and showing binding affinity to HLA-A*2402 induced CTLs to produce IFN-γ and kill an AKR1C3-producing hepatoma cell line. The frequency of AKR1C3-specific CTLs in peripheral blood mononuclear cells (PBMCs) was 10 to 23 per 3×10⁵ PBMCs. The frequency of IFN-γ-producing AKR1C3-specific T cells in tumor-infiltrating lymphocytes (TILs) was higher than that in PBMCs, suggesting that AKR1C3-specific T cells infiltrate into the tumor and are functional. The analyses of the frequency of AKR1C3-specific CTLs before and after HCC treatments showed that AKR1C3-specific immune responses were enhanced by the treatments.

Conclusions: We identified HLA-A*2402-restricted T cell epitopes derived from AKR1C3. The newly identified AKR1C3 epitopes could be a valuable component of HCC immunotherapy and for analyzing host immune responses to HCC.

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Key words

epitope; CD8; HLA-A24; hepatitis

Introduction

Hepatocellular carcinoma (HCC) is the 6th most frequent type of cancer and the 3rd most common cause of death from cancer worldwide.¹ Several recent advances in therapeutic modalities such as surgical hepatic resection, percutaneous tumor ablation, transcatheter arterial embolization (TAE), chemotherapy, sorafenib, and liver transplantation have improved the prognosis of HCC patients.²,³ However, the survival of those who have advanced HCC is still not satisfactory.² Therefore, the development of new anti-tumor therapies for advanced HCC patients remains an urgent and important field of research.

Tumor antigen-specific immunotherapy is one of the attractive strategies. In several studies of cancer immunotherapy, effective induction of tumor-specific cytotoxic T lymphocytes (CTLs) by tumor-associated antigen (TAA) has shown anti-tumor effects.⁴,⁵ Although many TAAs and their epitopes have been identified in various cancers, the number of HCC-specific antigens is still limited and clinical data of immunotherapy using TAA-derived peptides have been reported in studies using alpha-fetoprotein (AFP)- or human telomerase reverse transcriptase (hTERT)-derived peptides.⁶,⁷ In human trials targeting AFP and hTERT, it is possible to raise a TAA-specific T cell response using peptides, but this has shown little anti-tumor effect; therefore, the identification of new TAA-derived epitopes is very important to develop more effective immunotherapy for HCC.

The recent advances of the technologies in the field of expression cloning, serological identification of antigens by recombinant expression cloning (SEREX) or complementary DNA (cDNA) microarray make the identification of new TAAs easy. Specially, the development of cDNA microarray technologies has provided comprehensive profiles of the gene expression of malignant and normal cells and become a powerful tool that allows the identification of molecular targets suitable for cancer immunotherapy.⁴ Using the techniques of cDNA microarray, several TAAs have been identified in some malignancies including leukemia, HCC, colon and pancreatic cancers over the last decades.⁹-¹²

In the present study, using the data of cDNA microarray
and immunological analyses, we identified aldo-keto reductase 1C3 (AKR1C3)-derived CTL epitopes. AKR1C3 belongs to the aldo-keto reductase superfamily and shows preferences for 17-ketosteroid reduction and converts delta-4-androstene-3,17-dione to testosterone and to a lesser extent estrone to 17b-estradiol. It has been shown to be up-regulated in breast or prostate cancer and the high expression is significantly associated with a poor prognosis, making the enzyme a potential target in immunotherapy for cancers.1415 In this study, we clarified that the AKR1C3 expression level was higher in HCC tissue than in non-cancerous tissue, CTL lines reactive to AKR1C3-derived epitopes could be established from peripheral blood mononuclear cells (PBMCs) of HCC patients. Moreover, HCC treatment dramatically changed the strength of AKR1C3-specific immune responses, mostly by increasing the frequency of AKR1C3-specific CTL responses. These results provide a rationale for T-cell-based immunotherapy for HCC and suggest that the identified AKR1C3 epitopes could be a valuable component of HCC therapy and for analyzing host immune responses to HCC.

Materials and Methods

Patient population
In this study, 54 human leukocyte antigen (HLA)-A24-positive patients with HCC were examined (Table 1). The diagnosis of HCC was histologically confirmed in 38 patients. For the remaining 16 patients, diagnosis was made by dynamic computed tomographic (CT). The pathological grading of tumor cell differentiation was assessed according to the general rules for the clinical and pathologic study of primary liver cancer.17 The severity of liver disease was evaluated according to the criteria of Desmet et al. using biopsy specimens of liver tissue. All subjects provided written informed consent to participate in this study in accordance with the Helsinki declaration. Seventeen healthy blood donors and 26 patients with chronic hepatitis caused by hepatitis C virus (HCV) without HCC served as controls. This study was approved by the regional ethics committee (Medical Ethics Committee of Kanazawa University).

Gene expression analysis using cDNA microarray
HCC and non-cancerous tissue were obtained from 17 patients. Total RNA was isolated from liver tissue samples using an RNA extraction kit (Micro RNA Extraction Kit, Stratagene, La Jolla, CA, USA). The cDNA microarray analysis was performed as previously described using Liver chip 10 k consisting of 9614 non-redundant clones. As a reference for each microarray analysis, aRNA samples prepared from the normal liver tissue from one of the patients were used. Statistical analysis and hierarchical clustering were performed using BRB-Array Tools (http://linus.nci.nih.gov/BRB-ArrayTools.htm). A class comparison between HCC and non-HCC tissues based on univariate or paired t-tests was used to find differentially expressed genes (p<0.005). To confirm statistical significance, 2000 random permutations were performed, and all of the t-tests were re-computed for each gene.

Down-regulation of AKR1C3 by small interfering RNA (siRNA)
Huh6 cells were transfected with Ctrl (siGENOME Non-Targeting siRNA Pool #1, Thermo Fisher Scientific, Walum, MA) or AKR1C3 (AKR1C3 siGENOME SMART pool, Thermo Fisher Scientific) siRNA using DharmaFECT 4 siRNA Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions. Cell growth was monitored by 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenol)-2-(4-sulfoophenyl)-2H-tetrazolium (MTS) assay (Promega, Madison, WI).

Quantitative real-time detection polymerase chain reaction (RTD-PCR)
We performed quantitative RTD-PCR using TaqMan Universal Master Mix (PE Applied Biosystems, Foster City, CA, USA). Primer pairs and probes for AKR1C3 and b-actin were obtained from TaqMan assay reagent library. Total RNA was isolated from liver tissue samples using an RNA extraction kit (Micro RNA Extraction Kit, Stratagene, La Jolla, CA, USA). We reverse-transcribed 1 μg of isolated RNA to cDNA using SuperScript® II RT (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions, and the resultant cDNA was amplified with appropriate TaqMan assay reagents as previously described.19

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis
AKR1C3 expression of hepatoma cell lines was confirmed by western blot analysis. The cells were harvested and lysed in RIPA buffer. Samples containing 7 μg of cell lysate were separated using a 4% stacking gel and a 5%-20% separating gradient gel. The separated protein levels were detected using anti-human AKR1C3 (SIGMA, St. Louis, MO; diluted 1:200) and standard detection techniques (ECL system; Amersham). Signal intensity was quantified using Fujifilm imaging system (Multi Gauge, ver 3.1).

Immunohistochemical analysis
The liver tissues were fixed in buffered zinc formalin (Anatech Ltd., Battle Creek, MI), embedded in paraffin, sectioned (at 3 μm), and stained with hematoxylin and eosin. The sections were deparaffinized, treated in a pressure cooker for 1-4 min, and incubated with anti-human AKR1C3 (SIGMA, St. Louis, MO; diluted 1:200) or AFP (DakoCytomation, Inc., Carpinteria, CA) antibody for immunohistochemical analysis. The tissue sections were counterstained with hematoxylin before mounting.

Synthetic peptides
To identify potential HLA-A24-binding peptides within AKR1C3, a computer-based program was employed by accessing the World Wide Web site Bioinformatics and Molecular Analysis Section (BIMAS) for HLA peptide binding predictions. The HLA-A24 restricted epitopes derived from human immunodeficiency virus (HIV) envelope protein20 and cytomegalovirus (CMV) pp6521 were used as control peptides to test for T cell responses, and the HLA-A2 restricted epitope derived from AFP was used as a control peptide for HLA-A24 stabilization assay. Peptides were synthesized at Sumitomo Pharmaceuticals (Osaka, Japan). They were identified using mass spectrometry, and their purities were determined to be >90% by analytical high-pressure liquid
Table 1. Characteristics of the patients studied

<table>
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<th>Clinical diagnosis</th>
<th>Normal donors</th>
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<th>HCC</th>
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<tr>
<td>No. of patients</td>
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<td>54</td>
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<td>Sex (male/female)</td>
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<td>ND</td>
<td>99±92</td>
<td>63±35</td>
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<tr>
<td>AFP (ng/mL)</td>
<td>ND</td>
<td>15±15</td>
<td>955±4078</td>
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<td>24/2/0</td>
<td>33/19/2</td>
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<tr>
<td>Etiology (HCV/HBV/others)</td>
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<td>26/0/0</td>
<td>41/9/4</td>
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<tr>
<td>Differentiated degree of HCC (wel/mod/por/ND)</td>
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<td>ND</td>
<td>16/20/2/16</td>
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<td>Tumor size (large/small)</td>
<td>ND</td>
<td>ND</td>
<td>31/23</td>
</tr>
<tr>
<td>Tumor multiplicity (multiple/solitary)</td>
<td>ND</td>
<td>ND</td>
<td>28/26</td>
</tr>
<tr>
<td>Vascular invasion (+/-)</td>
<td>ND</td>
<td>ND</td>
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<td>TNM stage (I/II/IIIa/IIIb/IV)</td>
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<td>ND</td>
<td>24/21/7/0/1/1</td>
</tr>
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</table>

Data are expressed as the mean±SD. HCC, hepatocellular carcinoma; ALT, alanine transaminase; ND, not determined; AFP, alpha-fetoprotein; Child-Pugh score: Class A: 5-6; Class B: 7-9; Class C: 10-15; HCV, hepatitis C virus; HBV, hepatitis B virus; wel, well differentiated; mod, moderately differentiated; por, poorly differentiated; ‘Histological degree of HCC; ‘Tumor size was divided into either ‘small’ (≤2 cm) or ‘large’ (>2 cm); TNM, tumor, node, metastasis.

Chromatography (HPLC).

Cell lines
Four human hepatoma cell lines, HepG2, SKHep1, Alex, and HuH6, were cultured in DMEM (Gibco, Grand Island, NY) with 10% fetal calf serum (FCS) (Gibco, Grand Island, NY).

T2-A24 cells, which were transfected with HLA-A*2402 molecule into T2 cells, were cultured in RPMI 1640 medium containing 10% FCS and 800 μg/mL G418 (GibcoBRL, Grand Island, NY). The HLA-A*2402 gene-transfected C1R cell line (C1R-A24) was cultured in RPMI 1640 medium containing 10% FCS and 500 μg/mL hygromycin B (Sigma, St. Louis, MO), and K562 was cultured in RPMI 1640 medium containing 10% FCS. All medium contained 100 U/mL penicillin and 100 μg/mL streptomycin (GibcoBRL, Grand Island, NY).

Major histocompatibility complex (MHC) binding assay
Peptide binding assays were performed as previously described. The data are expressed as % mean fluorescence intensity (MFI) increase, which was calculated as follows: % MFI increase = (MFI with the given peptide - MFI without peptide) / (MFI without peptide) × 100.

Enzyme-linked immunospot (ELISPOT) assay
ELISPOT assays were performed as reported previously. Responses to peptides were considered positive if more than 10 specific spots were detected and if the number of spots in the presence of an antigen was at least more than twofold that in its absence. In 7 patients treated by radiofrequency ablation (RFA) or TAE, ELISPOT assays were performed using the PBMCs obtained before and 2 weeks after HCC treatments.

Cytotoxicity assay
AKR1C3-derived peptide-specific T cells were expanded from PBMC and cytotoxic assay was performed as previously described. The percent cytotoxicity was determined from the following formula: % cytotoxicity = [100 × (experimental release - spontaneous release) / (maximum release - spontaneous release)], and maximum release was determined by lysis of 51Cr-labeled targets with 5% Triton X-100 (Sigma Chemical Co., St. Louis, MO). Spontaneous release was <15% of maximum release for all experiments.

Statistical analysis
Data are expressed as the mean±SD. Differences of AKR1C3 expression levels between HCC and non-HCC tissues were analyzed for statistical significance by the Mann-Whitney's U test. Fisher's exact test (2-sided p-value) was used to analyze differences of AKR1C3-specific T cell responses among the 3 groups consisting of normal donors, patients with HCV, and patients with HCC.

Results

Patient profiles
The clinical profiles of the patients are shown in Table 1. The tumors of 38 patients were histologically diagnosed as HCC, and their differentiation was well, moderate, and poor for 16, 20, and 2 cases, respectively. The tumor size was categorized as ‘small’ (≤2 cm) for 23 cases or ‘large’ (>2 cm) for 31 cases, and tumor multiplicity was categorized as ‘multiple’ for 28 cases or ‘solitary’ for 26 cases. Vascular invasion of HCC was observed in 6 cases. The tumor, node, metastasis (TNM) stage was classified according to the Union Internationale Contre Le Cancer (UICC) classification system (6th version), where 24, 21, 7, 0, 1, and 1 patients had stage I, II, IIIa, IIIb, IIIc, and IV tumors, respectively. The etiology of liver disease in HCC patients consisted of HCV, hepatitis B virus (HBV) and others including alcohol and nonalcoholic steatohepatitis as shown in Table 1.

Gene expression profile of HCC and non-tumor liver tissue
By comparing gene expression between HCC and non-cancerous tissues, 149 genes were found to be up-regulated and 227 genes were down-regulated in HCC compared with those in each paired non-cancerous tissue (p<0.005) (data not shown). Representative genes among 25 mostly up-regulated genes are shown in Table 2. Genes related to cell proliferation, metabolism, and secretory
proteins were up-regulated. Among metabolism-related genes, 2 aldo-keto reductase superfamily genes (AKR1B10 and AKR1C3) were up-regulated. Recent studies have shown that AKR1C3 is overexpressed in breast or prostate cancer and the high expression is significantly associated with a poor prognosis. Therefore, we focused on AKR1C3 in this study.

Expression of AKR1C3 in hepatoma cell lines and human HCC tissues
The expression of AKR1C3 was observed in 3 hepatoma cell lines (HepG2, Alex, and Huh6) (Fig. 1A). SKHep1, PBMC, C1RA24, and K562 did not show AKR1C3 expression. The expression was also examined in human HCC tissue. The AKR1C3 expression level examined by quantitative RTD-PCR was higher in HCC tissue than that in non-cancerous tissue (Fig. 1B). In immunohistochemical analysis, AKR1C3 protein was expressed in all HCC tissues examined including HCC without AFP expression (Fig. 1C). The AKR1C3 expression level examined by immunohistochemical analysis was also higher in HCC tissue than that in non-cancerous tissue.

Down-regulation of AKR1C3 by siRNA
To examine the functional relevance of AKR1C3 to tumor cell growth, we knocked down the expression of AKR1C3 in Huh6 cells using specific siRNA and evaluated the cell growth by MTS assay. The expression of AKR1C3 was successfully knocked down to less than 10% of that of control cells that were transfected with non-specific scramble siRNA (Fig. 1D). In this condition, cell growth was significantly repressed at 75% of that of the control (p=0.013, Fig. 1E). The results indicated that AKR1C3 participated in tumor cell growth.

Selection of potential HLA-A24-binding peptides within AKR1C3
Nine peptides were selected according to the order of high half-time dissociation scores (Table 3). Next, major histocompatibility complex (MHC) stabilization assays were performed to test these peptides for HLA-A*2402 binding capacity using T2-A24 cells. Peptides AKR1C3_80, AKR1C3_113, AKR1C3_183, and AKR1C3_195...
showed a strong binding affinity (Fig. 2). In contrast, peptides AKR1C3_{45}, AKR1C3_{91}, AKR1C3_{204}, and AKR1C3_{294} showed a weak binding affinity. Peptide CMVpp65_{328}, which is identified as a strong binder of the HLA-A^{*}2402 molecule, showed a strong binding affinity, but peptide AFP_{137}, which is HLA-A2-restricted, did not show it, suggesting that the assay was specific for HLA-A24.

**Immunogenicity of AKR1C3-derived peptides assessed by interferon (IFN)-γ ELISPOT analysis**

An overview of all IFN-γ ELISPOT responses is shown in Figure 3. Five of 9 AKR1C3-derived peptides were recognized by PBMC of at least one patient and 11 of 54 patients (20%) responded to at least one of the analyzed AKR1C3-derived peptides. Single AKR1C3 epitope-specific IFN-γ-producing cells were detected in 1 of 54 (1.9%), 3 of 54 (5.6%), 6 of 54 (11.1%), 2 of 54 (3.7%), and 2 of 54 (3.7%) patients for peptides AKR1C3_{45}, AKR1C3_{133}, AKR1C3_{294}, AKR1C3_{204}, and AKR1C3_{294}, respectively. Peptides AKR1C3_{91}, AKR1C3_{180}, AKR1C3_{183}, and AKR1C3_{294} were not recognized by any patient.

Regarding the strength of the AKR1C3-specific T cell responses assessed by the frequency of IFN-γ-producing cells in the PBMC population, most patients displayed between 10 and 23 specific cells per 3 \times 10^{5} PBMCs. The frequency of positive T cell
Figure 3. Direct ex vivo analysis (IFN-γ ELISPOT assay) of peripheral blood T cell responses to AKR1C3-derived peptides or control peptides in HCC patients. Responses were considered positive if more than 10 specific spots were detected and if the number of spots in the presence of antigen was at least twofold greater than the number of spots in the absence of antigen. The peptide sequences are described in Table 3. *denotes more than 40 specific spots. AKR1C3, aldo-keto reductase 1C3; HCC, hepatocellular carcinoma; PBMC, peripheral blood mononuclear cell; HIV, human immunodeficiency virus; CMV, cytomegalovirus; INF, interferon; ELISPOT, enzyme-linked immunospot.

Figure 2. MHC binding affinity. TAP-deficient T2-A24 cells were cultured for 16 h at 26°C to enhance the expression of peptide-receptive cell surface molecules. They were incubated with individual peptides at 10 µg/mL at 37°C for 2 h, washed, and stained with anti-HLA-A24 monoclonal antibody, anti-mouse immunoglobulin-conjugated FITC, and 1 µg of propidium iodide per milliliter. The data are expressed as the % mean fluorescence intensity (MFI) increase for live, propidium iodide-negative cells. AKR1C3, aldo-keto reductase 1C3; MHC, major histocompatibility complex; TAP, transporter for antigen presentation; HLA, human leukocyte antigen; FITC, fluorescein isothiocyanate.
responses was lower than that of peptide CMVpp65_328, which was derived from CMVpp65 protein and is strongly immunogenic. No patients exhibited positive T cell responses against peptide HIVenv_584 derived from the HIV envelope protein, suggesting that these T cell responses were antigen-specific.

Identification of AKR1C3-derived peptides that elicit a primary CTL response

The 5 selected AKR1C3-derived peptides that showed positive T cell responses in ELISPOT assay were tested for their potential to induce HLA-A24-restricted CTL using PBMC from HCC patients (Fig. 4A). Each peptide was tested on at least 5 patients. After 3 rounds of stimulation, responder cells that had been stimulated with peptides AKR1C3_45, AKR1C3_113, AKR1C3_195, AKR1C3_204, and AKR1C3_294 lysed the peptide-pulsed C1R-A*2402 cells. As shown in Fig. 3A, the CTL induced with AKR1C3_195 showed high-level cytotoxicity against C1RA24 cells pulsed with the corresponding peptides.

To examine whether the identified epitope were produced from AKR1C3 protein by intracellular natural processing in cancer cells, we tested the cytotoxicity of AKR1C3_195 peptide-specific CTLs against several hepatoma cell lines. The CTLs exhibited cytotoxicity against hepatoma cell lines with the HLA-A24 molecule and high expression of AKR1C3, HepG2, but not against AKR1C3-hypoexpressing SKHep1 and AKR1C3-overexpressing Alex without HLA-A24 molecule (Fig. 4B).

Characteristics of AKR1C3-specific T cell responses

To evaluate the characteristics of AKR1C3-specific T cell responses, we examined the responses in normal donors and patients with chronic hepatitis C using 2 peptides, AKR1C3_113 and AKR1C3_195, which showed strong cytotoxicity in CTL assay and frequent responses in ELISPOT assay with PBMCs of HCC patients. AKR1C3_113-specific CTLs were observed in both normal donors and patients with chronic hepatitis C (Fig. 5A). The frequencies of patients with AKR1C3_113-specific CTLs were not different among the 3 groups. AKR1C3_195-specific CTLs were also observed in patients with chronic hepatitis C and the frequencies of patients with AKR1C3_195-specific CTLs were not different between the patient groups with chronic hepatitis C and HCC.
Next, to analyze the specificity and function of AKR1C3-specific CTLs against tumor in HCC patients, we examined the number of AKR1C3-specific T cells among tumor infiltrating lymphocytes (TILs) and compared the results with those for PBMCs. We performed IFN-γ ELISPOT assay in another 7 patients from whom samples of both PBMCs and TILs could be obtained (Table 4). In the assay using PBMCs, 1/7 (14.3%) and 1/7 (14.3%) of the patients showed significant responses to AKR1C3 and AKR1C3, respectively. In contrast, in the assay using TILs, 2/7 (28.6%) and 5/7 (71.4%) of the patients showed significant responses to AKR1C3 and AKR1C3, respectively. A positive T cell response in TILs was observed even in the patients without a positive T cell response in PBMCs.

Furthermore, to analyze the specificity of AKR1C3-specific CTLs against tumor in HCC patients, we evaluated the T cell responses before and after HCC treatment for peptides AKR1C3 and AKR1C3 in 7 patients from whom PBMCs could be obtained. The frequency of AKR1C3-specific T cells increased significantly in all of the patients after treatments (Fig. 5C). HIV-specific T cell responses did not increase in all patients. Regarding CMV-specific T cell responses, only 2 patients (Patients 40 and 49) showed a significant increase. These results suggest that the effect of anti-cancer treatment on the T cell response is specific for AKR1C3.

**Discussion**

The development of cDNA microarray technologies has provided comprehensive profiles of the gene expression of malignant and normal cells and become an effective approach to identify immunological target molecules for cancer immunotherapy. In the present study, we focused on AKR1C3 on the basis of the results of cDNA microarray analysis of the gene expression profile of HCC.

AKR1C3 belongs to the aldo-keto reductase superfamily and shows preferences for 17-ketosteroid reduction and converts delta-androstene-3,17-dione to testosterone and to a lesser extent estrone to 17b-estradiol. Recent studies have shown that AKR1C3 is overexpressed in breast or prostate cancer and the high expression is significantly associated with a poor prognosis. On the basis of results of gene expression analysis and the above background, we first examined the expression of AKR1C3 in human HCC tissue by real-time PCR and immunohistological staining and confirmed that its expression level is higher in HCC tissue than that in non-cancerous tissue. In addition, the result that the hepatoma cell with down-regulated AKR1C3 shows slow growth suggests that the molecule participates in tumor cell growth. The mechanism of cell growth suppression by down-regulation of AKR1C3 gene is still not fully understood. However, AKR1C3 possesses 3α-hydroxysteroid dehydrogenase (HSD), 3β-HSD, 17β-HSD, and 11-ketoprostaglandin reductase activities, and catalyzes estrogen, progesterone, androgen, and prostaglandins (PG) metabolism. As a result, AKR1C3 is capable of indirectly governing ligand access to various nuclear receptors, including estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR), and peroxisome proliferator-activated receptor (PPAR), and regulating trans-activation activities of these nuclear receptors through intracellular actions. In addition to our results, it has been reported that AKR1C3 promotes prostate cancer cell proliferation and survival. These results suggest that AKR1C3 is a favorable candidate as a target of immunotherapy for HCC.

Next, we attempted to identify AKR1C3 epitopes restricted by HLA-A24, which are present in 60% of Japanese, using a combined computer-based and immunological approach. Analysis of amino acid sequences of AKR1C3 using a computer revealed a number of potential HLA-A24 binding peptides, and 5 of 9 peptides (AKR1C3, AKR1C3, AKR1C3, AKR1C3, and AKR1C3) functionally stabilized HLA-A*2402 molecules expressed in the peptide transporter-deficient cell line T2-A24. In another immunological assay, 5 AKR1C3-derived peptides (AKR1C3, AKR1C3, AKR1C3, AKR1C3, and AKR1C3) induced IFN-γ production in PBMCs and T cell lines that showed cytotoxicity against the peptide-pulsed C1R-A24 cells. In addition, T cell lines induced by AKR1C3, which revealed high cytotoxicity against the peptide-pulsed C1R-A24
cells, showed cytotoxicity against hepatoma cell lines that expressed HLA-A*2402 and AKR1C3, but did not show it against other hepatoma cell lines without HLA-A*2402 or AKR1C3 expression, suggesting that the cytotoxicity was HLA-A24-restricted and AKR1C3-specific. Taken together with these results, we confirmed that AKR1C3 contained HLA-A24-restricted epitopes that were endogenously processed within the AKR1C3-producing cells and was most immunogenic because it showed the highest rate of patients with positive response in ELISPOT assay and the highest cytotoxicity in CTL assay. In the analysis of the characteristics of AKR1C3-specific immune responses, AKR1C3-specific CTLs were detected in both normal donors and patients with chronic hepatitis C. The frequencies of patients with AKR1C3-specific CTLs were not different between the patient groups with chronic hepatitis C and HCC. Furthermore, the frequency of AKR1C3-specific CTLs in PBMCs was independent of etiology of liver disease in HCC patients.

On the other hand, the frequency of IFN-γ-producing CTLs was higher in patients with chronic hepatitis C than in normal donors. This result suggests that hepatitis C virus infection may lead to the release of AKR1C3 into the environment, which could be recognized by CD8+ T cells. In addition, the frequency of AKR1C3-specific CTLs was higher in patients with chronic hepatitis C than in normal donors, suggesting that hepatitis C virus infection may lead to the release of AKR1C3 into the environment, which could be recognized by CD8+ T cells.

Figure 5. Characteristics of AKR1C3-specific immune responses. A: Comparative analysis of AKR1C3-, HIV-, and CMV-derived peptide-specific T cell responses among 3 groups of subjects: normal donors, patients with chronic hepatitis C not complicated by HCC, and HCC patients; B: Direct ex vivo analysis (IFN-γ ELISPOT assay) of responses of TILs to AKR1C3-derived peptides (peptides AKR1C3_113 and AKR1C3_195) in 7 HCC patients from whom samples of both PBMCs and TILs were obtained. Open and solid bars show the frequencies of AKR1C3-specific T cells in PBMCs and TILs, respectively; C: The induction of AKR1C3-specific T cell responses in HCC patients after HCC treatment. Direct ex vivo analyses (IFN-γ ELISPOT assay) of peripheral blood T cell responses to AKR1C3-, HIV-, and CMV-derived peptides were performed before (open bar) and after (solid bar) HCC treatment. *denotes more than 30 specific spots. AKR1C3, aldo-keto reductase 1C3; HIV, human immunodeficiency virus; CMV, cytomegalovirus; INF, interferon; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; ELISPOT, enzyme-linked immunospot; TILs, tumor infiltrating lymphocytes; PBMC, peripheral blood mononuclear cell.
AKR1C3-specific T cells in TILs was higher than that in PBMCs, suggesting that AKR1C3-specific T cells infiltrate into the tumor and are functional. Furthermore, to examine the tumor specificity of AKR1C3-specific T cell responses, the newly identified immunogenic AKR1C3 epitopes (AKR1C3\textsubscript{111} and AKR1C3\textsubscript{193}) were then used to analyze the immunological effects of HCC treatments including RFA and TAE. The frequency of AKR1C3-specific T cells was increased in all patients after HCC treatments and in 4 patients the immunological enhancement was observed for only AKR1C3 but not for viral antigens. These results suggest that the effect of treatments on the host immune response is specific for HCC-associated antigens and AKR1C3-specific T cell responses reflect it.

Consistent with our findings, increased numbers of TAA-specific T cells after HCC treatment have been reported in studies of immunogenic TAA-derived epitopes.\textsuperscript{39} The increased numbers of AKR1C3-specific T cells are similar to those in previous studies, suggesting that these AKR1C3-derived peptides are immunogenic.

Although further studies are necessary to determine whether the newly identified AKR1C3 epitopes could be useful for HCC immunotherapy, the present results may provide a rationale for T-cell-based immunotherapy against HCC, and suggest that the identified AKR1C3 epitopes could be a valuable component for HCC immunotherapy and for analyzing host immune responses.

**Contributors**

Eishiro Mizukoshi was responsible for conception, design, analysis of the data and drafting of the article; Masao Honda and Kazumi Fushimi were responsible for analysis and interpretation of the data; Kuniai Ari and Tatsuya Yamashita were responsible for critical revision of the article for important intellectual content; Shuichi Kano was responsible for final approval of the article.

**Conflicts of interest**

The authors declared no conflicts of interest.

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### Summary Box

**What is already known:**
- The prognosis of advanced hepatocellular carcinoma (HCC) patients is not satisfactory and the development of new anti-tumor therapies is necessary.
- Although many tumor-associated antigens (TAAs) and their epitopes have been identified in various cancers, the number of HCC-specific antigens suitable for immunotherapy is still limited.
- Aldo-keto reductase 1C3 (AKR1C3) is overexpressed in breast or prostate cancer and the high expression is significantly associated with a poor prognosis.

**What the new findings are:**
- AKR1C3 is expressed in HCC cells and participates in tumor cell growth.
- Human leukocyte antigen (HLA)-A*2402-restricted cytotoxic T cell (CTL) epitopes derived from AKR1C3 is identified in this study.
- AKR1C3-specific CTLs are detectable in HCC patients, infiltrate into HCC tissues and kill tumor cells.

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### References

20. Ikeda-Moore Y, Tomiyama H, Mitaka K, et al. Identification and characterization of...


