Table II. Number of CTL-negative and -positive cases in chronic hepatitis, liver cirrhosis and HCC patients.

| | Healthy (n=5) | | Chronic hepatitis (n=33) | | Liver cirrhosis (n=23) | | HCC (n=54) | |
|---|---|---|---|---|---|---|---|---|
| Group | Negative (n=5) mean (±SD) | Positive (n=0) mean (±SD) | Negative (n=28) mean (±SD) | Positive (n=5) mean (±SD) | Negative (n=19) mean (±SD) | Positive (n=5) mean (±SD) | Negative (n=33) mean (±SD) | Positive (n=21) mean (±SD) |
| Age | 31.2±7.1 | - | 61.6±11.2 | 60.6±12.9 | 67.3±10.1 | 71.0±2.7 | 65.8±7.9 | 64.0±10.5 |
| Male | 4 | 0 | 16 | 3 | 12 | 3 | 28 | 15 |
| Female | 1 | 0 | 12 | 2 | 6 | 2 | 5 | 6 |
| HCV/HBV | | | | | | | | |
| +/- | ND | ND | 5 | 26 | 18 | 5 | 18 | 14 |
| -/+ | ND | ND | 2 | 0 | 0 | 0 | 4 | 2 |
| +/+ | ND | ND | 0 | 0 | 0 | 0 | 2 | 2 |
| -/- | ND | ND | 0 | 0 | 0 | 0 | 9 | 3 |
| AFP (ng/ml) | ND | ND | 9.5±18.9 | 9.6±7.3 | 21.2±25.4 | 8.8±7.7 | 26335.1±143782.5 | 1431.5±3574.9 |
| HLA- | | | | | | | | |
| $A02^+$ | 3 | 0 | 3 | 3 | 2 | 2 | 13 | 8 |
| $A24^+$ | 2 | 0 | 12 | 1 | 7 | 2 | 18 | 11 |
| $A02^+/24^+$ | 0 | 0 | 6 | 1 | 5 | 1 | 2 | 2 |
| $A02^-/24^-$ | 0 | 0 | 7 | 0 | 4 | 0 | 0 | 0 |

## Discussion

The oncofetal antigen GPC3 is known to be overexpressed in HCCs (3-10) and melanomas (6,8,9). We recently identified GPC3-specific peptides restricted to HLA-A24 ($A^*2402$) and H-$2K^d$, or HLA-A2 ($A^*0201$), both of which can induce GPC3-reactive cytotoxic T cells (CTLs) (19). We are currently conducting a phase I clinical trial of peptide vaccine prepared using these peptides against advanced HCC. In addition, in the near future, we propose to carry out a phase II clinical trial of the vaccine in HCC patients as well as CH and LC patients to evaluate its efficacy in preventing the onset of HCC. We report the finding of GPC3-specific CTLs in CH and LC patients for the first time in this study. Furthermore, the plasma titers of anti-GPC3 IgG in the CH and LC patients were also found to be significantly increased as compared with those in healthy donors.

It has been suggested that GPC3-specific CTLs may be derived from clinically invisible pre-neoplastic or neoplastic nodular lesions. In previous studies, expression of GPC3 was reported in 2/23 (8%) cirrhotic low-grade dysplastic nodules, and 2/9 (22%) (24), 2/22 (9%) (25) or 6/31 (19%) high-grade dysplastic nodules (26). In one study, among 5 adenomas with malignant characteristics, 3 (60%) showed immunoreactivity for GPC3 in the malignant regions (24). Other studies reported positive staining for GPC3 in 12/20 (60%) (24) and 22/32 (69%) cases (25) of early HCC. Meanwhile, the serum titers of the elevated GPC3 antigen in HCC cases were reported to be correlated with the clinical stage of HCC (19). In our study, we noted an increase of the plasma titers of anti-GPC3 IgG antibody in CH, LC and HCC patients. In addition, the frequency of patients with GPC3-specific CTLs appeared to increase with the stage of progression of the liver disease. These results suggest that GPC3 expression and the appearance of GPC3-specific CTLs may be prediagnostic markers of HCC.

On the other hand, the increase in the frequency of GPC3-specific CTLs and titers of anti-GPC3 IgG in the peripheral blood might be related to the expression of GPC3 in CH with high grade inflammation and LC. In this study, we did not perform immunohistochemical examination for GPC3, because needle biopsy of the liver in our patients was not conducted in our collaborative clinic. Previous studies have demonstrated GPC3 expression by immunohistochemistry in 25/30 (83%) cases of CH with high grade inflammation (27) and 11/95 (12%) cases of LC (26), indicating that GPC3 might be expressed in CH with high-grade inflammation and some LC patients, resulting in the appearance of GPC3-specific CTLs in the PBMCs of these patients.

During the 1-year follow-up of this study, onset of HCC was not observed in any of the 10 CH and LC patients who were positive for GPC3-specific CTLs in the peripheral blood; on the other hand, 2 (1CH and 1LC) patients who were negative for GPC3-specific CTLs showed development of HCC. It would, therefore, seem that the GPC3-specific CTLs might prevent the development of HCC or be predictive of a favorable prognosis of non-neoplastic liver lesions. However, our examination was limited to only HLA-A24- and A2-positive patients, and moreover, we followed up the patients for only one year. Therefore, careful long-term observation of a larger number of CH and LC cases is necessary to determine the role of GPC3-specific CTLs in patients with CH and LC.

In this study, we demonstrated an increase of GPC3-specific CTLs and high titers of anti-GPC3 IgG in CH and LC patients. Thus, GPC3-specific CTLs and anti-GPC3 IgG

may possibly be markers of early imaging-invisible HCC. In addition, active immunotherapy using GPC3 peptides may prevent the development of both non-neoplastic and neoplastic lesions of the liver.

## Acknowledgements

We thank Ms. Chinatsu Kojima for her help with the preparation of this manuscript. This study was supported in part by Health and Labor Sciences Research Grants for Research on Hepatitis from the Ministry of Health, Labour and Welfare, Japan, and a Grant-in-Aid for the Third-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour and Welfare, Japan, the Foundation for Promotion of Cancer Research in Japan, the Japan Research Foundation for Clinical Pharmacology, and a Research Resident Fellowship from the Foundation for Promotion of Cancer Research, Japan (E.H.).

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# Glypican-3 is a useful diagnostic marker for a component of hepatocellular carcinoma in human liver cancer

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Received September 10, 2008; Accepted October 27, 2008

DOI: 10.3892/ijo_00000190

**Abstract.** Primary liver cancers are classified into three types based on their morphology and cytogenetic characteristics hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (ICC) and combined hepatocellular and cholangiocarcinoma (CHC). It is often difficult to distinguish these liver tumors. Glypican-3 (GPC3) is serological and histochemical marker of hepatocellular carcinoma. In order to separate these three types of liver cancers, we analyzed the GPC3 expression in 85 liver resection specimens, including 46 HCCs, 28 ICCs and 11 CHCs. GPC3 immunohistochemical staining was used to distinguish HCC from ICC by comparing with the conventional biomarker, α-fetoprotein (AFP). The immunostaining of GPC3 was identified in 78.3% (36/46) of HCCs, 60% (9/15) of well differentiated, 88.9% (16/18) of moderately differentiated and 84.6% (11/13) of poorly differentiated HCCs. It was negative in the ICCs. We confirmed that GPC3 expression is specific to HCC component (8/11, 72.7%) but few samples also showed weakly in ICC component (2/11, 18.2%) of CHC sections among 11 cases compared with HCC biomarkers including AFP and hepatocyto paraffin 1 (HepPar1), and ICC biomarkers cytokeratin (CK) 7 and CK19. Three cases in which the macroscopic features resembled ICC did not express GPC3 even in the pathological HCC component. Most (10/11, 91%) of the pathological cholangiocarcinoma components in CHC showed positive staining for CK7 and CK19. The results of this study suggest that GPC3 is a biomarker that is sensitive and specific to HCC component of CHC, and CK7 and CK19 are markers for pathological cholangiocarcinoma component of CHC.

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*Abbreviations*: HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma; CHC, combined hepatocellular and chorangiocarcinoma; GPC3, glypican-3; AFP, α-fetoprotein; HepPar1, hepatocyto paraffin 1; CK, cytokeratin; CC, cholangiocarcinoma; cp, component

*Key words:* hepatocellular carcinoma, intrahepatic cholangiocarcinoma, combined hepatocellular and chorangiocarcinoma, glypican-3, CK7, CK19, immunohistochemical analysis

## Introduction

Liver cancer is one of the common malignancies that are rapidly increasing throughout the world. Primary liver cancers are classified into three types based on their morphology and cytogenetic characteristics, hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (ICC) and combined hepatocellular and cholangiocarcinoma (CHC). HCC is hepatocyto-origin, and ICC is from the epithelium of the intrahepatic bile duct. CHC is a rare type of liver cancer with features of both hepatocellular and biliary differentiation (1-3). The pathological structure of CHC is composed of hepatocellular element showing bile production, an intercellular bile canaliculi or trabecular growth pattern and cholangiocellular component showing mucin production or gland formation.

Because of their rapid growth rate and the lack of accurate ways of diagnosis in the early stages, the prognosis and the survival rate for liver cancer patients remain poor. Currently, ultrasound sonography (US), computed tomography (CT), magnetic resonance imaging (MRI), and histopathological examination for tumor biopsy are used for diagnosis. However, distinguishing the three different primary liver tumors is often a challenging task in diagnosis, for which immunohistochemical analysis for specific antigens is a helpful tool: α-fetoprotein (AFP) and hepatocyto paraffin 1 (HepPar1) for HCC (4-8) and cytokeratin (CK) 7 and CK19 for ICC (9-11).

Glypican-3 (GPC3) was discovered as a potential serological and histochemical marker whose expression is specific for HCC (12-16). GPC3 belongs to glypican family that is a group of heparan sulfate proteoglycans linked to the outer surface of cell membrane through a glycosylphosphatidylinositol anchor (17). In mammals, six members of GPCs have been reported, GPC1 to GPC6. GPCs are released from the cell surface by a lipase called Notum to regulate the signaling of Wnts, Hedgehogs, fibroblast growth factors (FGFs) and bone morphogenetic proteins (BMPs) (18-25). Depending on the cellular context, their function can be stimulatory or inhibitory activity, or signaling. The expression of GPC3 is detected in placenta and fetal liver, but not in other normal organs. During hepatic carcinogenesis, GPC3 have been reported to reappear in HCC and to be released into serum (12,13,15,26). Its expression is also detected in melanoma (27-29). The functions of GPC3 in cancer cells are still unclear.

In this study, we examined whether immunohistochemical analysis for GPC3 can be used to distinguish HCC from ICC, if so, how effectively GPC3 can be detected, compared to other biomarkers that are conventionally used. We demonstrate that distinguishing HCC from ICC by detecting the expression of GPC3 enables more accurate diagnosis.

## Materials and methods

*Case selection.* We selected 85 cases of liver tumors from the surgical pathology files from 1992 to 2006 of National Cancer Center Hospital East, Kashiwa, Chiba, Japan. The cases included 46 primary HCCs, 28 ICCs, and 11 CHCs that underwent hepatectomy. All identifiers were eliminated to protect patients' identities. Size of the tumor and any clinicopathologic factors (age, sex and grade of tumor) were matched between HCC and ICC. The 46 cases of HCCs occurred in 33 men and 13 women with a mean of age at 65.3 years (range, 44-80 years). HCC was subclassified into well (n=15), moderately (n=18), and poorly (n=13) differentiated types according to the World Health Organization classification criteria. The 28 cases of ICC consisted of 18 men and 10 women. Their mean age was 65.7 years (range, 51-82 years). All 28 resected cases of ICC were confirmed by hematoxylin-eosin (H.E.) staining.

The 11 cases of CHC included 7 men and 4 women with a mean age of 62.5 years (range, 47-76 years). All CHCs were pathologically confirmed after surgery.

*Tissue samples.* Liver tissue sections were retrieved from the files of the Department of Pathology in our institution. All liver specimens were prepared from surgically resected tumors and adjacent parenchyma. They were fixed in 10% formalin and paraffinized for routine histological examination.

*Immunohistochemical staining procedure.* Six-micrometer-thick sections were made from the paraffin-embedded blocks. Subsequently the sections were deparaffinized in xylene and rehydrated through ethanol to water. Endogenous peroxidase activity was blocked using 3% $H_2O_2$ in methanol for 20 min. For antigen retrieval, Sections were heated in 10 mM citrate buffer (pH 6.0) with microwave for 15 min in a water bath at 95°C. Only for CK7 immunostaining, sections were digested by Proteinase K (DakoCytomation, Carpenteria, CA) for 5 min at room temprature. Slides were then allowed to cool down. The prediluted primary antibodies, monoclonal anti-GPC3 (dilution 1:300, 1G12; Biomosaics, Inc., Burlington, VT), anti-AFP (dilution 1:400, DakoCytomation), anti-HepPar1 (dilution 1:100, DakoCytomation), anti-CK7 (dilution 1:100, DakoCytomation), and CK19 (dilution 1:200, DakoCytomation) were added to cover each slide, and the slides were incubated for 2 h at room temperature. Slides were washed 3 times in phosphate-buffered saline (PBS)/Tween for 5 min each. Mouse Envision Polymer (DakoCytomation) was used as a secondary antibody for 30 min at room temperature followed by washes in PBS/Tween 3 times for 5 min each. Diaminobenzidine chromagen (DakoCytomation) was added to each slide and incubated for 2 min. Slides were washed in distilled water, counterstained with hematoxylin and dehydrated in xylene. To analyze GPC3 expression, the immunohistochemical results were classified according to the number of positive cells as follows: -, negative (<10%); ±, weakly positive (10-30%); + positive (>30%). To validate the data in GPC3 as a marker for HCC, parallel staining for AFP of 46 cases were further analyzed. For 11 CHC cases, AFP, HepPar1, CK7 and CK19 were stained and compared with GPC3 staining pattern.

The slides were examined independently by 3 observers (Shirakawa H, Kuronuma T and Nakatsura T) and then collectively by 2 more pathologists (Hasebe T and Nakano M).

*Statistical analysis.* Differences in proportion were tested by the $\chi^2$ test. Differences in the means of each subgroup were tested using the Student's t-test. P-value of <0.05 was considered statistically significant.

## Results

*GPC3 was present in 80% of HCC and negative in ICC.* In order to examine the levels and pattern of GPC3 expression, 46 cases of HCC and 28 cases of ICC were immunohistochemically analyzed. GPC3 was detected in 36 cases (78%) of HCC (Fig. 1a), and no expression of GPC3 was found in any of the ICC patients (Fig. 1b). The GPC3 staining was diffused throughout (Fig. 1c) or localized in a granular pattern in the cytoplasm (Fig. 1d). In other cases, GPC3 was observed at the plasma membrane (Fig. 1e). Previously GPC3 is shown to bind to the cell membrane (16), however, those cases with membranous GPC3 had staining in the cytoplasm as well, but there was no case of GPC3 located only at the plasma membrane. When sensitivity of GPC3 was evaluated, 36 cases (78%) were positive for GPC3 when only 16 cases (35%; P<0.0001) were stained for AFP in HCC suggesting that GPC3 is more sensitive than AFP. Thus, GPC3 was confirmed to be specific and sensitive to HCC compared to AFP.

*GPC3 expression increased in moderately and poorly differentiated HCC.* In terms of GPC3 expression and tumor

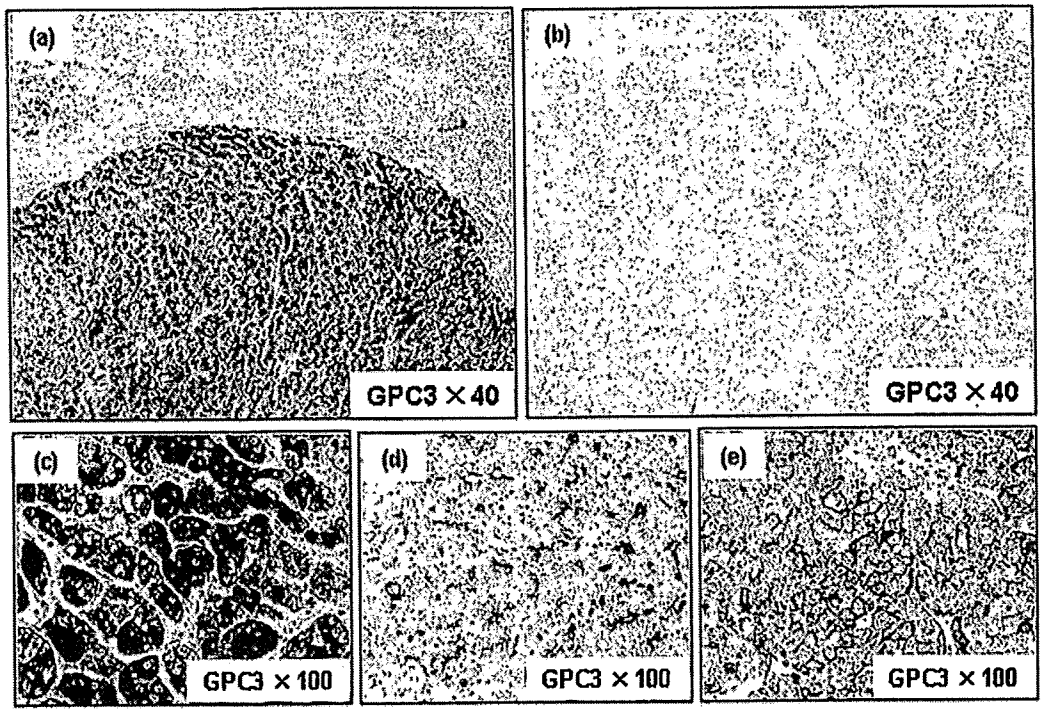


Figure 1. GPC3 expression was specific to HCC and absent in ICC. Immunohistochemical detection of GPC3 expression in HCC (a) and ICC (b) (magnification, x40). Immunostaining patterns of HCC: (c) diffuse in cytoplasm, gulanular in cytoplasm (d), and membranous (e).

Table I. Correlation of positive for GPC3 staining and tumor grade.

| Grade of tumor | HCC | | | | | ICC | | |
|---|---|---|---|---|---|---|---|---|
| | | GPC3 | | | | | | |
| | No. of case | - | ± | + | positivity | No. of case | GPC3 positivity | P-value |
| Well-differentiated | 15 | 6 | 5 | 4 | 9 (60%) | 8 | 0 (0%) | |
| Moderately differentiated | 18 | 2 | 4 | 12 | 16 (89%) | 10 | 0 (0%) | |
| Poorly differentiated | 13 | 2 | 5 | 6 | 11 (85%) | 10 | 0 (0%) | |
| Total | 46 | | | | 36 (78%) | 28 | 0 (0%) | <0.0001 |

-, negative (<10%); ±, weakly positive (10-30%); +, positive (>30%).

differentiation level, GPC3 was expressed in 9 (60%) of 15 well differentiated, 16 (89%) of 18 moderately differentiated and in 11 (85%) of 13 poorly differentiated HCC (Table I). AFP was expressed in 3 (20%) of 15 well differentiated, 6 (33%) of 18 moderately differentiated and in 7 (54%) of 13 poorly differentiated HCC (data not shown). The expression level of GPC3 was lower in well differentiated HCC than in the other HCC grades, though the difference was not statistically significant (well- vs. moderately differentiated: P=0.054, well- vs. poorly differentiated: P=0.150). Thus, GPC3 expression is also a good indicator for malignancy levels.

*GPC3 expression was observed specifically in pathological HCC component in CHC.* There are discrepancies between preoperative diagnosis and pathological findings for CHC patients. Diagnostic results and the expression of tumor markers of 11 CHC patients are summarized in Table II. Initial diagnosis was carried out by H.E. staining. Among these 11 patients, 7 patients (63.6%) were diagnosed as HCC and 3 (27.3%) were ICC. Only 1 patient (9%) of the 11 CHC was correctly diagnosed as CHC. To seek the possibility to use GPC3 immunostaining to detect HCC component (cp) in CHC, combination of antibodies against GPC3, AFP, HepPar1, CK7 and CK17 were used. In addition to AFP, HepPar1 is frequently used as marker for HCC (4-8) and CK 7 and CK19 for ICC (9-11).

Among 11 CHC cases, 4 cases preoperatively diagnosed as HCC were chosen to represent the collision and transitional type of CHCs based on the macroscopic features

Table II. Correlation of immunostaining varieties and pathological components of CHC.

| Pt. no. | Preoperative diagnosis | Macroscopic diagnosis | Pathological hepatocellular carcinoma component | | | | | Pathological cholangiocarcinoma component | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | GPC3 | AFP | HepPar1 | CK7 | CK19 | GPC3 | AFP | HepPar1 | CK7 | CK19 |
| 1 | HCC | CHC | + | + | - | + | + | - | - | - | - | - |
| 2 | HCC | HCC | + | - | - | - | - | - | - | + | + | + |
| 3 | HCC | HCC | + | - | + | - | - | ± | - | - | + | + |
| 4 | CHC | HCC | + | + | + | - | - | ± | - | - | + | + |
| 5 | HCC | CHC | + | - | + | - | - | - | - | - | + | + |
| 6 | HCC | CHC | + | - | - | - | - | - | - | + | + | + |
| 7 | ICC | CHC | ± | - | - | ± | + | - | - | - | + | + |
| 8 | HCC | HCC | + | + | - | - | - | - | + | - | + | + |
| Total ± | | | 8/8 | 3/8 | 3/8 | 3/8 | 2/8 | 2/8 | 1/8 | 2/8 | 7/8 | 7/8 |
| positive rate (%) | | | 100 | 38 | 38 | 38 | 25 | 25 | 13 | 25 | 88 | 88 |
| 9 | ICC | ICC | - | - | - | - | - | - | - | - | + | + |
| 10 | HCC | ICC | - | - | - | + | ± | - | - | - | + | + |
| 11 | ICC | ICC | - | - | - | + | + | - | - | - | + | + |
| Total ± | | | 0/3 | 0/3 | 0/3 | 2/3 | 2/3 | 0/3 | 0/3 | 0/3 | 3/3 | 3/3 |
| positive rate (%) | | | 0 | 0 | 0 | 67 | 67 | 0 | 0 | 0 | 100 | 100 |

-, negative (<10%); ±, weakly positive (10-30%); +, positive (>30%); HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma; CHC, combined hepatocellular and cholangiocarcinoma; GPC3, glypican-3; AFP, α-fetoprotein; HepPar1, hepatocyto-paraffin 1; CK, cytokeratin; CC, cholangiocarcinoma.

in cut surface. In Fig. 2, macroscopic observation and the immunostained histological sections are shown. These sections include 2 elements with pathological HCC cp forming bile production and trabecular growth pattern by eosinophilic staining and cholangiocarcinoma (CC) cp forming mucin production or gland formation by basophilic staining. Cases 1-8 were GPC3 positive, and cases 9-11 were negative for GPC3 in the HCC cp. Macroscopic, histological and immunohistochemical features of cases 2, 6, 8 and 10 are shown in Fig. 2a, b, c and d. Case 2 had greenish white and yellow nodules within the same tumor mass in the cut surface. HCC subtypes such as simple nodular and confluent multinodular type exist. Case 2 exhibited the features of HCC with multinodular type (Fig. 2a-i). Pathological diagnosis by H.E. staining revealed CHC pathologically (Fig. 2a-ii and -iii), which was so-called 'collision'-type tumor as reported by Goodman *et al* (30). A 'collision'-type tumor is coincidental occurrence of HCC and CC within the same tumor mass (31). GPC3 was positive (Fig. 2a-iv), but AFP and HepPar1 were not detected in HCC cp (Fig. 2a-v and -vi). Although HepPar1 is generally used as HCC marker, it was unexpectedly stained in CC region as well as CK7 and CK19 (Fig. 2a-vii and -viii).

Case 6 showed pale and lobulated phenotype in the cut surface macroscopically (Fig. 2b-i), and pathological diagnosis was also confirmed by H.E. staining (Fig. 2b-ii and -iii). This was so-called 'transitional' type tumor (30). A 'transitional' type tumor has an area of HCC that appears to transform into CC (31). GPC3 was stained in pathological HCC cp (Fig. 2b-iv) where AFP was negative (Fig. 2b-v). The HCC region was surrounded by pathological CC cp with the staining for CK7 (Fig. 2b-vii). HepPar1 and CK19 were detected in the same region with CC cp (Fig. 2b-vi and -viii). HepPar1 stained the CC cp as in case 2. The immunoreactivity of CK19 was not consistent with that of CK7.

Case 8 was diagnosed as HCC similarly to cases 2 and 6, but mixed tumor masses with white and gray in the cut surface were observed (Fig. 2c-i and c-ii). Both GPC3 and AFP were positive in HCC cp (Fig. 2c-iv and -v). HepPar1 was stained in CC cp (Fig. 2c-vi). CK7 and CK19 were positive in CC cp (Fig. 2c-vii and -viii), especially CK19 was more specific for CC cp than CK7. These three cases (cases 2, 6 and 8) indicated that detecting GPC3 can compensate for AFP and enhance the ability to identify the presence of HCC cp in CHC.

Cases 9, 10 and 11 were negative for GPC3 expression in several tumors. Macroscopically, they had the features of ICC with irregular shaped, white solid tumor masses. As an example, case 10 is shown in Fig. 2d. Althogh case 10 was diagnosed as HCC preoperativerly, it showed macroscopic freatures of ICC with the presence of abundant fibrous stroma and indistinct tumor margin (Fig. 2d-i). This case was later diagnosed as CHC based on the pathological examination (Fig. 2d-ii and d-iii). GPC3, AFP and HepPar1 were not detected in either HCC cp or CC cp (Fig. 2d-iv, -v, and -vi). CK7 was stained diffusely in the tumor (Fig. 2d-vii), and CK19 expression was more specific in CC cp than CK7 (Fig. 2d-viii). These 3 cases showed positive staining

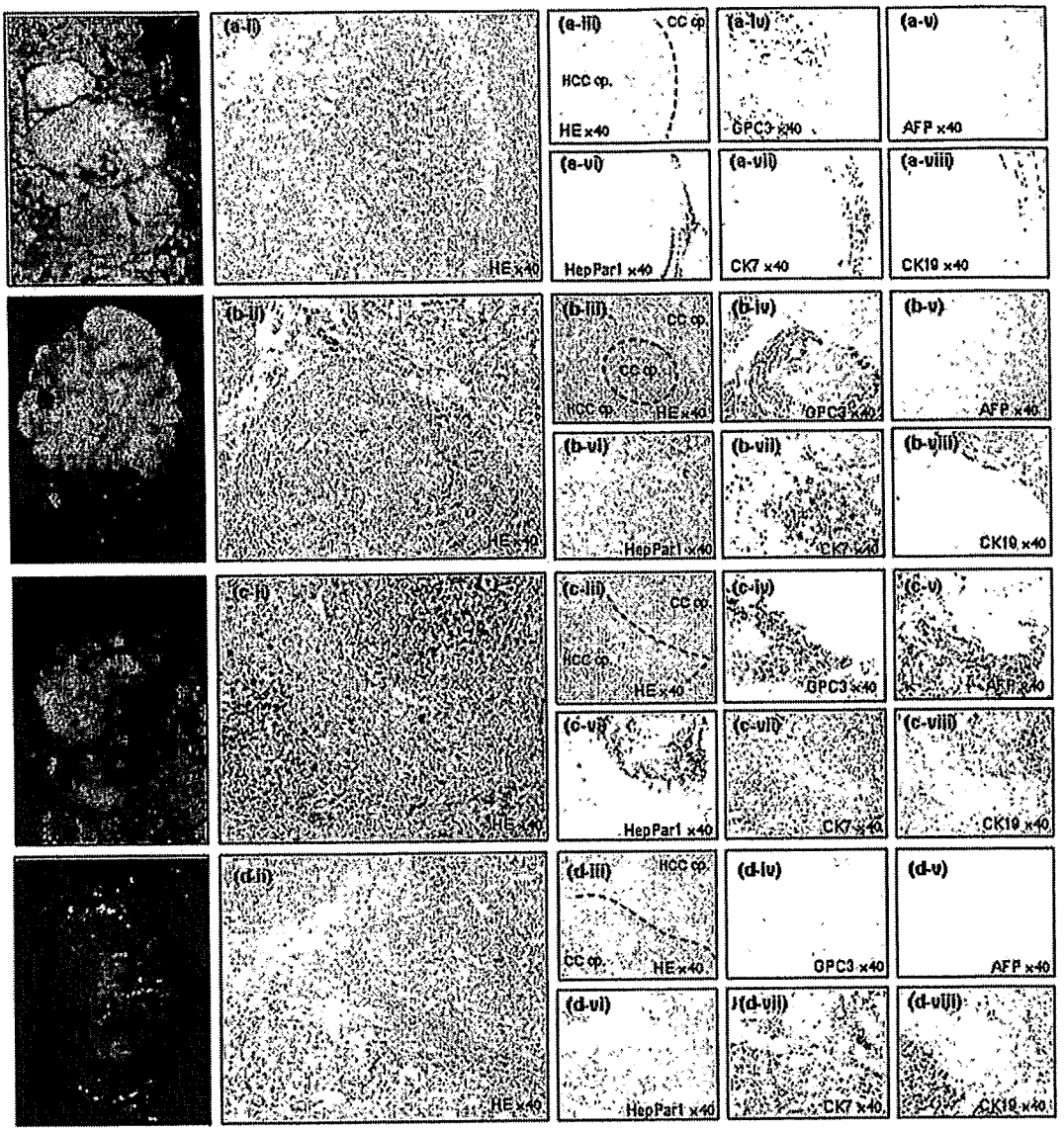


Figure 2. Macroscopic, histological and immunohistochemical features of four cases of CHC, a, case 2; b, case 6; c, case 8; d, case 10 in Table II. (a-i) Macroscopic feature in cut surface of case 2 tumor. (a-ii) The histological structure can be also devided into 2 types. HCC component showed expansive growth oppressing the cholangiocarcinoma component. (a-iii) Collision border between hepatocellular carcinoma and cholangiocarcinoma component are indicated as dots. The tumor cells within mainly hepatocellular carcinoma component showed only expression of GPC3 (a-iv) without expression of AFP (a-v). In the opposite side, the glandular area with cholangiocarcinoma component shows HepPar1 (a-vi), CK7 (a-vii) and CK19 expression (a-viii). (b-i) Case 6 shows macroscopic CHC feature in tumor cut surface that was suspected out HCC preoperatively. (b-ii) The histological cholangiocarcinoma component forming trabeculae with columnar appearance was surrounded by HCC component forming hepatoid structure. (b-iii) A dotted line is a boundary of HCC in the H.E. staining. The tumor cells within transitional region were positive for GPC3 (b-iv), CK 7 (b-vii) and CK 19 (b-viii). The difference was recognized between hepatocellular carcinoma component and cholangiocarcinoma component because GPC3 positive area encircled the CK7 area. The expressions of AFP (b-v) and HepPar1 (b-vi) were not observed. (c-i) Though case 8 was also suspected to be HCC preoperatively, the macroscopic features showed atypical HCC with mixed white and gray and indistinct tumor border. (c-ii) The cholangiocarcinoma component was obviously composed of structural gland formation. (c-iii) Collision area was distinguished histopathologically by a dotted line. The tumor cells of HCC component showed not only GPC3 (c-iv) but also AFP expression (c-v). In the glandular area of cholangiocarcinoma component, HepPar1 was expressed (c-vi), but CK7 not at all (c-vii) and CK19 shows weak positive expression (c-viii). (d-i) Case 10 shows macroscopic ICC features in tumor cut surface that was suspected as HCC preoperatively. (d-ii) The histological structure can be devided into 2 types with cholangiocarcinoma component forming trabeculae with columnar appearance and HCC component forming hepatocellular structures. (d-iii) A dotted line is a boundary of HCC in the H.E. GPC3 (d-iv), AFP (d-v) and HepPar1 (d-vi) were not stained, but CK7 (d-vii) and CK19 (d-viii) stained the cholagiocarcinoma component.

for CK7 and CK19 in CC cp, but not AFP or HepPar1 in HCC cp. Therefore, accuracy of CHC diagnosis can be achieved by combination of multiple tumor markers in addition to morphological characteristics: GPC3 that is specific for pathological HCC cp of CHC, and CK7 and CK19 that are specific for pathological CC cp of CHC.

## Discussion

The diagnosis for HCC, ICC and CHC has been routinely performed by histopathological examination. Additionally, diagnosis of HCC is done by supplementary immunohistochemical analysis for AFP and HepPar1. Until now, though

the sensitivity is limited, AFP has been regarded as the most useful marker for HCC (4,32-34). HepPar1 is also widely used for HCC to distinguish between primary HCC and ICC. However, both markers are limited for the ability to discriminate different levels of malignancy in HCC because its sensitivity drops substantially in poorly differentiated HCC, and it does not discriminate between benign and malignant liver cancers (35). As these biomarkers frequently results in misdiagnosis, in this study, we showed that GPC3 is more sensitive to detect HCC compared to AFP. Due to the fact that GPC3 was downregulated in ICC (36), GPC3 may help to separate HCC from ICC.

CHC is the least common primary cancer of the liver but followed by an aggressive growth, it tends to metastasize to many organs leading to significantly poorer prognosis than HCC and ICC (31,37,38). Correct diagnosis leads to both appropriate treatment and better outcome for the patients. Nishie, *et al* reported that one third (nine of 27 cases) of patients with CHC were correctly diagnosed by enhanced computed tomography (39). In our study, only one of the 11 (9.1%) patients with CHC was correctly diagnosed before operation without fine needle aspiration biopsy. The difficulty to pathologically distinguish CHC from HCC and ICC comes from glandular or pseudoglandular structures in HCC and solid or trabecular patterns in CC (37,38). We believe that combination with histopathological examination with GPC3 immunostaining and radiological examination can bring an accurate diagnosis and improved clinical therapies for the patients leading to a better prognosis.

We showed that the immunostaining for GPC3 is specific for HCC patients and not detected in ICC patients. This confirmed that detecting GPC3 may improve the method to diagnose CHC. Of the 11 cases of CHC, 8 displayed GPC3 expression in restricted area of HCC cp. We demonstarated that immunohistochemical staining of GPC3 in liver tumor helps to recognize the pathological HCC cp more precisely. GPC3 expression was observed with high frequency in the HCC cp compared with AFP and HepPar1. HepPar1 was unexpectedly stained in CC cp, but this has been observed previously as well (7,40). This could be due to a transition from HCC to ICC where HepPar1 is one of the molecules that is downregulated at later stages in the process. CK7 and CK19 have been already reported as good markers of biliary epithelial differentiation (41). These were highly expressed in pathological CC cp (10/11, 91%) in CHC. The positive immunoreactivity of CK19 was more distinct than that of CK7 whose staining was weaker. Our immunohistochemical data disclosed that GPC3 can be a better marker specific for HCC leading to a better confirmation for HCC component of CHC as well as for HCC. Moreover, it provided evidence of the biologic behavior of such combined tumors, which are phenotypically and genetically leaning toward either ICC with predominant biliary differentiation or HCC with hepatocellular differentiation (42,43).

Employing multiple tumor markers may also allow the accurate diagnosis of CHC containing both hepatocellular and biliary differentiation. Concerning sensitivity and specificity, the combination of GPC3 for HCC cp and CK19 for ICC cp seems to be useful in the diagnosis of liver cancer. For CHC, GPC3 positive/CK19 negative profile suggests HCC, GPC3 positive/CK19 positive indicates CHC, and GPC3 negative/CK19 positive essentially rules out HCC and suggests the possibility of CC or CHC.

We developed a new anti-cancer immunotherapy with GPC3 as a target (44-47), and the phase I clinical trial of GPC3-derived peptide vaccination for advanced HCC is now on going. Because this new immunotherapy is not indicated for ICC, immunohistochemical staining of GPC3 is a useful method to select eligible patients. Furthermore, if CHC would be justified as a target of our immunotherapy in future, immunohistochemical analysis for GPC3 expression is indispensable for the process of patient selection.

GPC3 is expressed in the group of cells that are AFP-positive and/or CK7/19-positive in injured livers with activation of oval cell compartment; an indication for liver repair and regeneration (48). In addition, CK7, CK19 and AFP are frequently expressed in biliary epithelial cells (49,50) and in immature fetal hepatoblasts (51,52). Liver progenitor cells originate from the canal of Hering, lined by both hepatocytes and biliary ductular epithelial cells (53). It is not clear whether GPC3 is expressed in hepatic embryonic progenitor cells or cancer stem cells, but GPC3 may be a marker for hepatic progenitor/stem cells. In CHC cases of 2, 3 and 4, GPC3, CK7 and CK19 coincided in the regions of HCC and CC. Although HCC and ICC are two different kinds of primary liver malignancies arising from different cell types as hepatocytes and cholangiocytes, co-localization of GPC3 and CK7/19 suggest that the CHC is originated from progenitor or oval cell. In addition, case 6 showed an HCC lesion with GPC3 positive immunostaining surrounded by CC (Fig. 2b). This finding suggests that GPC3-positive HCC tumor cells are derived from GPC3-negative CC mass. Moreover, we predict from the fact that GPC3 is expressed in embryonic liver and downregulated after birth in normal liver but reappears in cancer is due to its regulatory role in proliferative and dedifferentiated cells, like cancer cells that acquired a progenitor- or cancer stem cell-like characteristics.

In summary, we confirmed that GPC3 is a marker sensitive and specific for HCC, but not ICC. Moreover, we revealed that GPC3 was expressed specifically in the HCC cp in the CHC. Therefore, GPC3 is a molecule that is significant not only in clinical but also biological field. It is clinically an important biomarker that can be used for accurate diagnosis leading to a better treatment and prognosis. Also, biologically, it may be an indicator for the identity and the origin of the cancer cells.

## Acknowledgments

This study was supported in part by Health and Labor Sciences Research Grants for Research on Hepatitis from the Ministry of Health, Labor, and Welfare, Japan, and a grant-in-aid for the Third-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour and Welfare, Japan. Foundation for Promotion of Cancer Research in Japan, Japan Research Foundation for Clinical Pharmacology and Research Resident Fellowship from the Foundation for Promotion of Cancer Research, Japan (H.S.). We thank Dr Chinatsu Kojima (Section for Cancer Immunotherapy, Investigative Treatment Division,

Research Center for Innovative Oncology, National Cancer Center Hospital East) for technical assistance.

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# Identification of the H2-K$^d$-restricted cytotoxic T lymphocyte epitopes of a tumor-associated antigen, SPARC, which can stimulate antitumor immunity without causing autoimmune disease in mice

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(Received August 12, 2008/Revised September 17, 2008/Accepted September 22, 2008/Online publication December 5, 2008)

**We previously reported that the secreted protein acidic and rich in cystein (SPARC) was overexpressed in melanoma in humans, and the serum SPARC level was useful as a novel tumor marker for melanoma. SPARC was also reported to be overexpressed in various human cancers. In this study, we asked whether SPARC-specific cytotoxic T lymphocytes (CTL) could induce antitumor immunity to SPARC-expressing tumor in mice or not as a preclinical study of SPARC-directed anticancer immunotherapy. Because of similarities in the structural motifs of major histocompatibility complex-binding peptides between H2-K$^d$ and HLA-A24 (*A*2402*), the most common human leukocyte antigen class I allele in the Japanese population, we attempted to identify the H2-K$^d$-restricted SPARC epitope for CTL in BALB/c mice and we found that the mouse SPARC$_{143-151}$ (DYIGPCKYI) and SPARC$_{225-234}$ (MYIFPVHWQF) peptides could induce peptide-reactive CTL in BALB/c mice without causing autoimmune diseases. The immunization of mice with SPARC$_{225-234}$ peptide-pulsed bone marrow-derived dendritic cells (BMDC) inhibited the growth of s.c. inoculated mouse mammary cancer cell line, N2C, expressing SPARC and these mice lived longer than the mice immunized with peptide-unpulsed BMDC. In conclusion, our study indicated that SPARC peptide-based cancer immunotherapy was effective and safe at least in a mouse tumor prevention model. (*Cancer Sci* 2009; 100: 132–137)**

Secreted protein acidic and rich in cystein (SPARC), also called osteonectin or BM-40, was identified in 1981 as a major non-collagenous constituent of bovine bone.[1] SPARC is a matricellular glycoprotein secreted by many cells types,[2] that modulates cellular interaction with extracellular matrix during tissue remodeling.[3] SPARC plays an important role in wound repair, cell proliferation, cell migration, morphogenesis, cellular differentiation and angiogenesis.[2–5] Targeted disruption of the *SPARC* gene in mice results in early cataractogenesis,[6,7] osteopenia[6] and curly tails.[2]

SPARC was reported to be overexpressed in various human cancers,[9–12] including primary and metastatic melanomas. The overexpression of SPARC by melanoma cells was associated with an invasive phenotype *in vivo*.[13,14] We previously reported that serum SPARC levels observed in melanoma patients were higher than those observed in healthy donors.[15] Increased level of serum SPARC was observed in 33% of all melanoma patients, irrespective of the clinical stages and even in the sera of patients with stage 0 *in situ* melanoma. Moreover, the combined use of SPARC and glypican-3, which was reported by us as a novel tumor marker for melanoma,[16] enabled a 66.2% detection rate of melanoma patients at an early stage (0–II). Thus, SPARC is considered to be a useful tumor marker for melanoma. However, the usefulness of SPARC as a target for cancer immunotherapy has not been previously investigated.

One of the actual methods of the immunotherapy for cancer was vaccination of epitope peptides derived from tumor-associated antigen. Recently, several investigators have reported the effect of peptide vaccination on cancer.[17,18] However, the effect was partial, and more useful antigens were required. We previously identified several tumor-associated antigens, including glypican-3, heart shock protein 105, proliferation potential-related protein, KM-HN-1, cell division cycle associated 1 and cadherin-3/P-cadherin.[16,19–27] In addition, we identified several HLA-A2- or HLA-A24-restricted cytotoxic T lymphocytes (CTL) epitopes derived from these antigens. The immunization with these epitopes was effective in a mouse tumor model and some of these were applied to phase I clinical trials of cancer immunotherapy.

In this study, we identified the H2-K$^d$-restricted and SPARC-derived CTL epitopes useful for SPARC-directed immunotherapy, and the vaccination with these peptides elicited effective antitumor immunity with no evidence of autoimmune diseases in mice.

## Materials and Methods

**Cell lines.** Mouse cancer cell lines, B16, B16F1, B16F10, EL4, MCA, NIH3T3, 3LL, BALB/3T3, Colon26, A20, RL male1 and MethA were provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer Tohoku University (Sendai, Japan). A mouse mammary cancer cell line, N2C, was provided by Dr Sangaletti Sabina of the National Institute of Tumors (Milan, Italy). T2K$^d$, a TAP-deficient T2 cell transfected with *K$^d$*-gene expression vector, was provided by Dr Paul M. Allen of Washington University School of Medicine (St Louis, MO, USA). These cells were maintained *in vitro* in RPMI-1640 or Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS). Expression of H2-K$^d$ was examined with flow cytometry analysis by using a fluorescein isothiocyanate (FITC)-conjugated antimouse H2-K$^d$-specific antibody (clone SF1-1.1, mIgG2ak; BD Biosciences Pharmingen, San Diego, CA, USA).

**Mice.** Seven-week-old female BALB/c mice (H-2$^d$), purchased from Charles River Laboratories Japan (Yokohama, Japan),

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doi: 10.1111/j.1349-7006.2008.01016.x

were kept in the Center for Animal Resources and Development of Kumamoto University and handled in accordance with the animal care policy of Kumamoto University.

**Identification of the CTL epitopes of SPARC in BALB/c mice.** Mouse SPARC-derived peptides (purity, >90%), sharing the amino acid sequences with human SPARC and carrying binding motifs for both H2-K$^d$ and HLA-A24 (*A*2402*), were searched for using BIMAS software (BioInformatics and Molecular Analysis Section, Center for Information Technology, NIH, Bethesda, MD, USA), and we purchased four kinds of peptides (Table 1) from AnyGen (Gwangju, Korea). Identification of the CTL epitopes of SPARC was done using BALB/c mice as described.[19] In brief, the BALB/c mice were immunized i.p. with bone marrow-derived dendritic cells (BMDC) pulsed with the mixture of SPARC candidate peptides once a week for 2 weeks. Seven days after the last immunization, CD4$^-$ spleen cells collected from immunized BALB/c were stimulated with syngeneic BMDC pulsed with each peptide *in vitro*. Then, 6 days later, CD4$^-$ T cells were collected from the culture and the CTL-producing γ-interferon (IFN-γ) was detected by an enzyme-linked immunospot (ELISPOT) assay. Moreover, after 5 days culture *in vitro* under the same conditions, cytotoxic activities of these cells directed against target cells were tested by standard 6-h $^{51}$Cr release assays as described previously.[19]

**Table 1. SPARC-derived peptides conserved between human and mouse SPARC and predicted to bind to H2-K$^d$ and HLA-A24**

| Designation | Position | Subsequence residue listing | Binding score | |
|---|---|---|---|---|
| | | | H2-K$^d$ | HLA-A24 |
| SPARC-1 | 143–151 | DYIGPCKYI | 4000 | 75 |
| SPARC-2 | 123–131 | HFFATKCTL | 1382 | 20 |
| SPARC-3 | 161–170 | EFPLRMRDWL | 960 | 30 |
| SPARC-4 | 225–234 | MYIFPVHWQF | 120 | 210 |

These peptides were searched for using BIMAS (BioInformatics and Molecular Sections, Center for Information Technology, NIH, Bethesda, MD, USA) software (http://www-bimas.cit.nih.gov/molbio/hla_bind/).

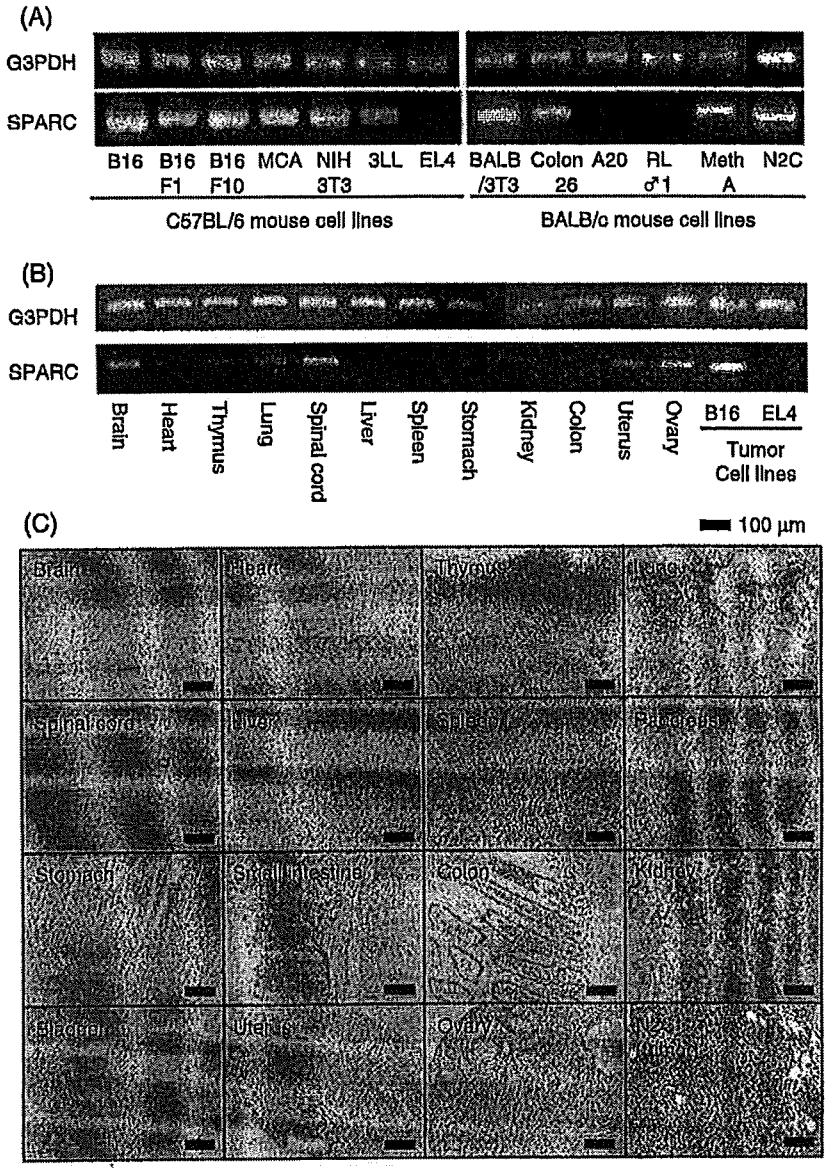


**Fig. 1.** The reverse transcription polymerase chain reaction (RT-PCR) analysis of *SPARC* mRNA expression in mouse cancer cell lines and normal tissues. (A) Various C57BL/6 and BALB/c mouse-derived cancer cell lines showed strong expression of *SPARC* except EL4, A20 and RL male 1. (B) Mouse *SPARC* gene was expressed in the normal tissues including ovary, spinal cord, brain and uterus by the RT-PCR analysis. (C) SPARC was not detected in the normal tissues by immunohistochemical staining.

**Histological and immunohistochemical analysis.** Immunohistochemical detections of SPARC was done as described previously.[21] We purchased Human, Normal Organs, and Cancers, Tissue Array, BC4 (SuperBioChips Laboratories, Seoul, Korea) for immunohistochemical analysis. Immunohistochemical staining of CD8 or CD4 was done as described previously.[28]

***In vivo* tumor prevention model.** BMDC ($5 \times 10^5$ cells/body) loaded with or without SPARC-$4_{225-234}$ peptide or phosphate-buffered saline (PBS) were transferred i.p. into BALB/c mice ($n = 8$, each group) twice on days −14 and −7, and N2C cells ($3 \times 10^4$/body) were challenged s.c. into the shaved back region on day 0. The tumor sizes were determined biweekly using a caliper square, and the tumor volume ($mm^3$) was calculated as long diameter × squared short diameter.

**Statistical analysis.** We analyzed all data with the StatView statistical program for Macintosh (SAS Institute, Cary, NC, USA) and evaluated the statistical significance with an unpaired Student's *t*-test. $P < 0.05$ was considered significant. The percentage of overall survival rate was calculated by the Kaplan–Meier method, and statistical significance was evaluated with the Wilcoxon rank sum test.

## Results

**Expression of *SPARC* mRNA in mouse cancer cell lines and normal tissues.** We examined the expression level of *SPARC* mRNA using reverse transcription polymerase chain reaction (RT-PCR). The mouse fibroblast cell line NIH/3T3 and various mouse cancer cell lines, including melanoma (B16, B16F1 and B16F10), fibrosarcoma (MCA) and lung cancer (3LL) originated from C57BL/6 mice, and sarcoma (BALB/3T3 and MethA), colon cancer (colon26) and breast cancer (N2C) originated from BALB/c mice, showed strong expression of *SPARC* (Fig. 1A). C57BL/6 mouse leukemia/lymphoma cell line, EL4, and BALB/c mouse lymphoma cell line, A20 and RL male 1, did not express *SPARC*. Although *SPARC* mRNA was expressed in the ovary, spinal cord, brain and uterus, the expression levels observed in these tissues were lower than those observed in cancer cell lines (Fig. 1B). Moreover, normal tissues including ovary, spinal cord, brain and uterus did not express SPARC at protein level investigated by the immunohistochemical analysis (Fig. 1C).

**Identification of the SPARC-derived and H2-K$^d$-restricted CTL epitopes in BALB/c mice.** Structural motifs of peptides bound to human HLA-A24 (*A*2402*) and mouse H2-K$^d$ are similar. The amino acid sequences of human and mouse SPARC have a 92% homology.[4] Thereby, we searched for SPARC-derived and H2-K$^d$- or HLA-A24 (*A*2402*)-restricted peptides of which amino acid sequences were completely shared between human and mouse SPARC, and prepared four different synthetic peptides (Table 1). CD4$^-$ spleen cells isolated from BALB/c mice immunized twice with BMDC pulsed with mixture of these four peptides were stimulated *in vitro* with BMDC pulsed with each peptide for 5–6 days. Subsequently, we collected these CD4$^-$ T cells from the culture and we found that CD4$^-$ T cells stimulated with the SPARC-$1_{143-151}$ (DYIGPCKYI) or SPARC-$4_{225-234}$ (MYIFPVHWQF) peptides produced a large amount of IFN-γ in a peptide-specific manner in ELISPOT assays (Fig. 2A). Moreover, we tested cytotoxic activities of these cells directed against target cells by standard 6-h $^{51}$Cr release assays. CTL induced by SPARC-$1_{143-151}$ or SPARC-$4_{225-234}$ peptides showed specific cytotoxicity against T2K$^d$ (H2-K$^d$+, TAP negative) cells pulsed with each SPARC peptide but not against T2K$^d$ cells unpulsed with SPARC peptide (Fig. 2B). In addition, those CTL had cytotoxic activities directed against MethA (SPARC$^+$, H-2$^d$) but not against RL male 1 (SPARC$^-$, H-2$^d$). These findings suggest that these SPARC-$1_{143-151}$ and SPARC-$4_{225-234}$ peptides had the capacity to induce the H2-K$^d$-restricted peptide-reactive CTL and that the CTL killed the tumor naturally expressing both SPARC and H2-K$^d$.

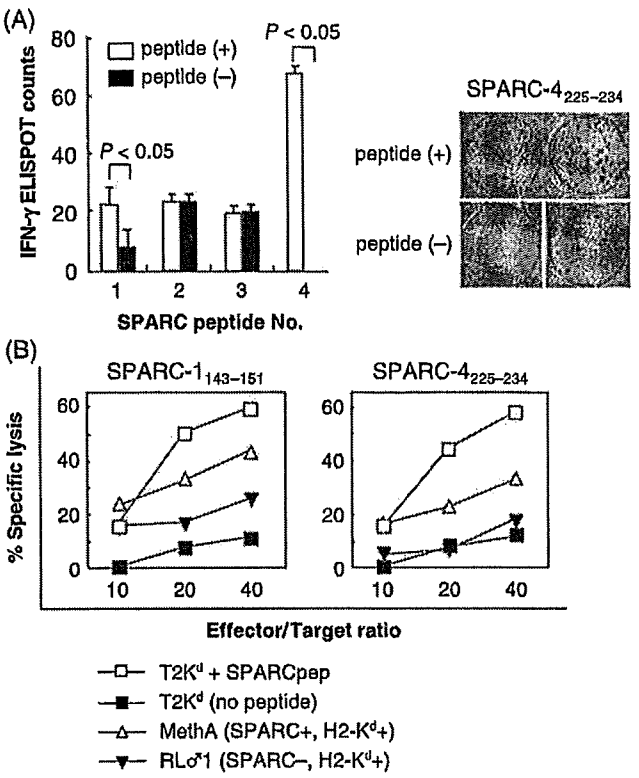


**Fig. 2.** Identification of the SPARC-derived and H2-K$^d$-restricted cytotoxic T lymphocyte (CTL) epitopes in BALB/c mice. We immunized the BALB/c mice with syngeneic bone marrow-derived dendritic cells (BMDC) ($5 \times 10^5$/mouse) pulsed with the mixture of candidate peptides *in vivo* once a week for 2 weeks. Seven days after the last immunization, CD4$^-$ spleen cells isolated from immunized mice ($2 \times 10^6$/well) were stimulated with BMDC ($2 \times 10^5$/well) pulsed with each peptide *in vitro*. (A) Six days after the stimulation *in vitro*, the CTL-producing γ-interferon (IFN-γ) in response to the peptide-pulsed BMDC were detected by an enzyme-linked immunospot (ELISPOT) assay. (B) Five days after the stimulation *in vitro* under the same conditions, cytotoxic activities of these cells directed against indicated target cells was tested with standard $^{51}$Cr release assays. We found that CD4$^-$ spleen cells stimulated with the SPARC-$1_{143-151}$ or SPARC-$4_{225-234}$ peptide produced a large amount of IFN-γ, and had cytotoxic activities directed against both H2-K$^d$ and SPARC-expressing MethA tumor cell line or T2K$^d$ pulsed with each peptide, but not to SPARC-negative RL male 1 and peptide-unpulsed T2K$^d$. These assays were done twice with similar results.

**Immunization of SPARC-$4_{225-234}$ peptide did not induce the autoimmune diseases in BALB/c mice.** To investigate whether the immunization of mice with the SPARC-derived H2-K$^d$-restricted peptide causes autoimmune diseases, the immunohistochemical staining of several important organs with anti-CD4 and anti-CD8 monoclonal antibody was performed in BALB/c mice immunized with BMDC pulsed with a mixture of four SPARC peptides once a week for 2 weeks. Tissue specimens of these mice were removed and analyzed 7 days after the second dendritic cell (DC) vaccination. As shown in Fig. 3, we could not find any pathological changes, such as lymphocyte infiltration or tissue destruction, in brain, heart, lung, liver, kidney, uterus, ovary and spinal cord of BALB/c mice. Although SPARC was expressed in spinal cord and brain by RT-PCR, the BALB/c mice immunized with BMDC pulsed with SPARC-$4_{225-234}$ peptide did not show any neurological disorders such as paralysis or abnormal behavior. No sign of autoimmune diseases



doi: 10.1111/j.1349-7006.2008.01016.x

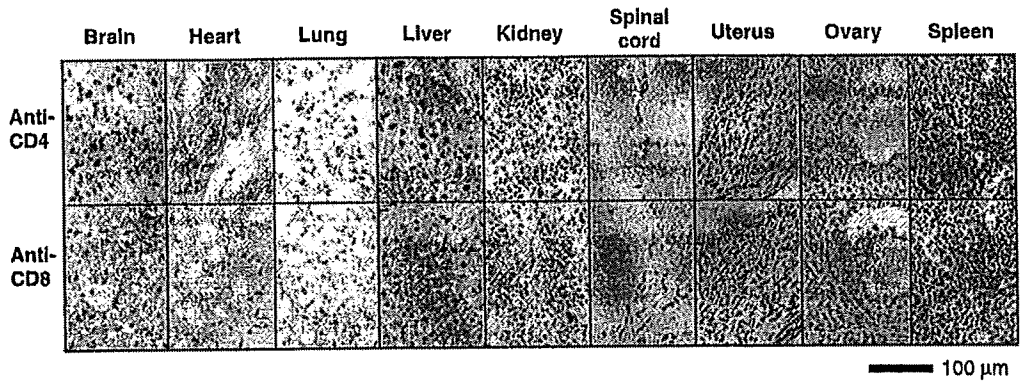


Fig. 3. Immunohistochemical staining with anti-CD4 or anti-CD8 monoclonal antibody in tissue specimens of BALB/c mice twice immunized with BMDC pulsed with the mixture of H2-K$^d$-restricted SPARC-derived peptides twice. These tissue specimens were removed and analyzed 7 days after the second dendritic cells (DC) vaccination (original magnification, ×200).

such as weight loss, diarrhea and skin abnormalities was observed (data not shown). These results indicate that the immunization of mice with SPARC-$4_{225-234}$ peptide does not induce autoimmune diseases.

**Inhibition of tumor growth in BALB/c mice by vaccination of SPARC-$4_{225-234}$ peptide-pulsed BMDC.** We investigated whether the immunization of mice with the SPARC epitope peptide inhibit the growth of tumor expressing SPARC *in vivo* or not. The BALB/c mice were injected i.p. twice at 7-day intervals with SPARC-$4_{225-234}$ peptide-pulsed BMDC, peptide-unpulsed BMDC or PBS ($n = 8$, each group). During the vaccination period, none of the treated mice showed any abnormalities. Subcutaneous inoculation of N2C cells ($3 \times 10^4$) into the right flank was given 7 days after the last vaccination. Growth curves of N2C tumor mass are shown in Fig. 4(A,B). The N2C tumor appeared 25 days after the inoculation in the PBS-injected group. Measurement of tumor size was continued until 62 days after inoculation of the tumor cells when one mouse in the PBS-injected group died. Mean tumor size ($1024.8 \pm 1820.7$ mm$^3$) on day 62 observed in the mouse group inoculated with SPARC-$4_{225-234}$ peptide-pulsed BMDC was significantly smaller ($5343.6 \pm 3117.2$ mm$^3$, $P < 0.01$) than that observed in the mice inoculated with peptide-unpulsed BMDC and in those injected with PBS ($6623.1 \pm 3883.9$ mm$^3$, $P < 0.01$). Complete tumor rejection was observed in four out of the eight mice in the SPARC-$4_{225-234}$ peptide-pulsed BMDC group. Although one out of the eight mice in the peptide-unpulsed BMDC group also completely rejected the tumor growth, there was no statistical significance in difference of tumor growth between the mice inoculated with peptide-unpulsed BMDC and those injected with PBS ($P = 0.48$). All mice injected with PBS died within 125 days after inoculation of the tumor cells. Mice inoculated with the SPARC-$4_{225-234}$ peptide-pulsed BMDC lived significantly longer than the mice of the other two groups (Fig. 4C). No significant abnormalities including neurological disorders were observed in four tumor-free mice in the SPARC-$4_{225-234}$ peptide-pulsed BMDC group for over 150 days after vaccination. This experiment was repeated twice with similar results. These findings indicate that vaccination of mice with BMDC pulsed with the SPARC epitopes resulted in significant inhibition of tumor growth and prolongation of overall survival *in vivo*.

## Discussion

In this study, we demonstrated that: (i) *SPARC* was overexpressed in mouse cancer cell lines; (ii) the CTL induced by the SPARC-derived peptides were reactive to tumor overexpressing SPARC; and (iii) the immunization of BALB/c mice with SPARC peptide-pulsed BMDC protected mice from tumor growth and induced prolonged survival without causing autoimmune diseases.

Although immunohistochemical staining of these tissues with anti-SPARC antibody was negative (Fig. 1C), *SPARC* mRNA was expressed in several important normal tissues including brain and spinal cord using RT-PCR. Therefore, we needed to investigate whether induction of SPARC-specific CTL could induce antitumor immunity without causing autoimmune diseases in the mouse model system. One of the reasons for lack of autoimmune diseases might be that the H2-K$^d$-positive SPARC epitope peptide complexes are more densely expressed on tumor cells in comparison to those expressed on normal tissues including brain and spinal cord, if any. These possibilities must be evaluated in a future study.

The HLA-A24 is positive in 60% of the Japanese population (95% of whom are genotypically *A*2402*), 20% of Caucasians and 12% of Africans.[29] It is important especially for the Japanese to identify HLA-A24-restricted CTL epitope peptides. Structural motifs of peptides bound to human HLA-A24 and BALB/c mouse H2-K$^d$ are similar,[30-32] and the amino acid sequences of human and mouse SPARC protein exhibit a 92% homology.[33] SPARC-derived and H2-K$^d$-restricted CTL epitopes identified in BALB/c mice may well be applicable to induce human HLA-A24-restricted CTL. Therefore, in this study, we used BALB/c mice, and searched for SPARC-derived peptides having amino acid sequences shared between mouse and human SPARC. We could identify the SPARC-derived and H2-K$^d$-restricted CTL epitopes. According to these findings, we tried to induce the HLA-A24-restricted human CTL reactive to these peptides by stimulating peripheral blood mononuclear cells (PBMC) of healthy donors or various cancer patients *in vitro* with the peptides. In a preliminary study, these SPARC peptide-specific and HLA-A24-restricted human CTL were also generated *in vitro* (unpublished observation, 2008).

We observed the inhibitory effect of immunization of mice with BMDC pulsed with SPARC-derived epitope peptide on growth of the inoculated N2C tumor cell line in BALB/c mice. N2C expressing SPARC is a mouse breast cancer cell line originating from Her-2/neu transgenic BALB/c mice.[34] It was reported that SPARC was overexpressed in human breast cancers, and associated with poor prognosis or invasive phenotype type.[35-38] N2C tumors grew as solid nests forming lobules embedded in dense, well-vascularized, connective tissue and surrounded by the stromal septa.[34] The stromal cells in N2C tumors also expressed SPARC. N2C tumor grown in SPARC

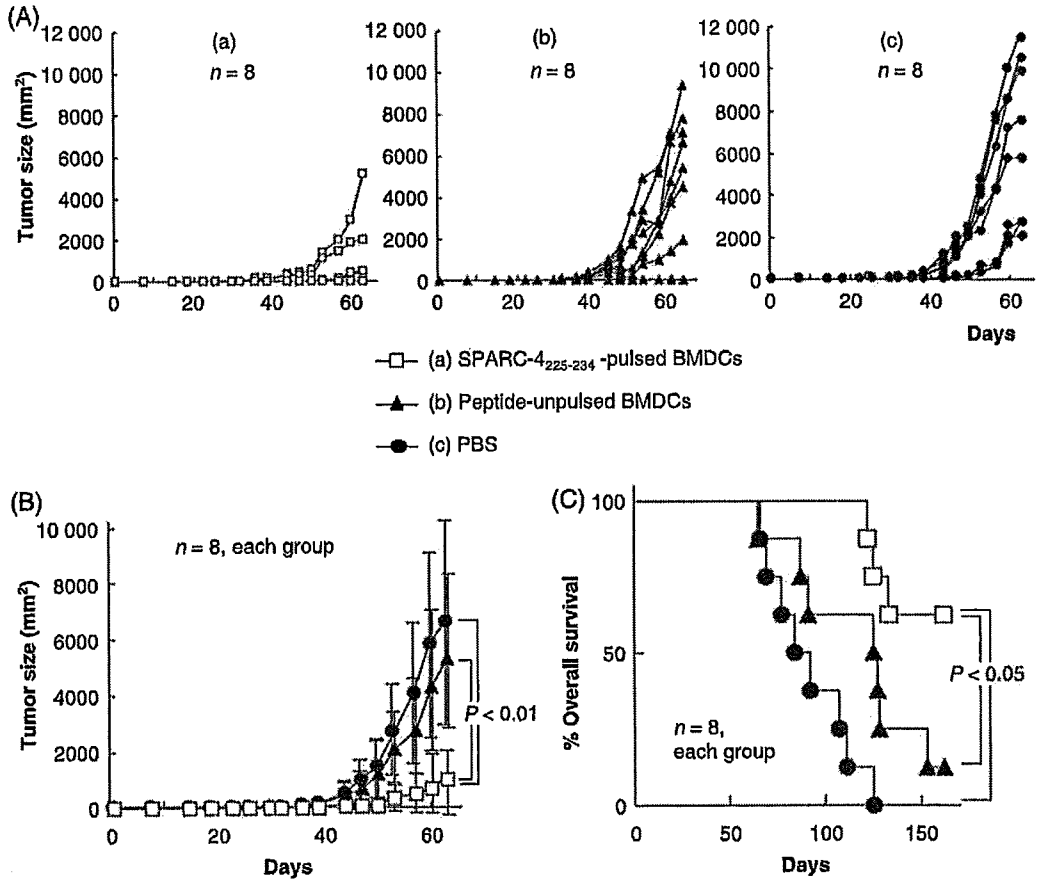


**Fig. 4.** Inhibition of tumor growth in BALB/c mice by vaccination of SPARC-$4_{225-234}$ peptide-pulsed bone marrow-derived dendritic cells (BMDC) *in vivo*. The BALB/c mice were injected i.p. twice at 7-day intervals with SPARC-$4_{225-234}$ peptide-pulsed BMDC, peptide-unpulsed BMDC or phosphate-buffered saline (PBS) only. Subcutaneous inoculation of N2C cells ($3 \times 10^4$/mouse) into the right flank was given 7 days after the last vaccination. (A) Growth curves of N2C tumor mass in individual mice in each group: (a) SPARC-$4_{225-234}$ peptide-pulsed BMDC inoculated group; (b) peptide-unpulsed BMDC inoculated group; (c) PBS injected group ($n = 8$, each group). (B) The mean tumor volumes ± standard deviation in three groups were compared in this panel. Statistical significance of the differences between each group were evaluated using the unpaired Student's *t*-test. (C) Survival rate of mice in each group. Mice in the SPARC-$4_{225-234}$ peptide-pulsed BMDC group lived significantly longer than the mice in the other two groups. Statistical significance of the differences between each group were evaluated using the Wilcoxon rank sum test.

knockout mice were smaller and histologically characterized by undefined lobules, frequently presenting necrotic central areas. The lobules were not completely delineated by the stromal septa, which appeared generally thin and sometimes heavily infiltrated by leukocytes. Therefore, the destruction of tumor stromal cells by immunity directed against SPARC may well be a possible mechanism for inhibition of N2C tumor cell proliferation observed in this study. This possibility awaits evaluation in a future study.

Taken together, these findings indicate that the antitumor immunity stimulated with SPARC-derived peptide is effective and safe at least in a preclinical study using a mouse cancer-prevention model system.

## Acknowledgments

This work was supported in part by Grants-in-Aid (nos. 17015035 and 18014023) from the Ministry of Education, Culture, Sports, Science and Technology, Japan; a Research Grant for Health Sciences from the Ministry of Health, Labor and Welfare, Japan; funding from: Onco Therapy Science; the Sagawa Foundation for the Promotion of Cancer Research; and the Foundation for the Promotion of Cancer Research in Japan.

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*Pediatric Hematology and Oncology*, 26:74–83, 2009

ISSN: 0888-0018 print / 1521-0669 online
DOI: 10.1080/08880010802435500

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# WT1 (WILMS TUMOR 1) PEPTIDE IMMUNOTHERAPY FOR CHILDHOOD RHABDOMYOSARCOMA: A Case Report

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□ *Immunotherapy using a Wilms tumor (WT1) peptide has been undergoing clinical trials for adulthood leukemia and solid cancer with promising results. In this study, the authors used WT1 peptide vaccination to treat a 6-year-old girl with metastatic alveolar rhabdomyosarcoma. She received weekly intradermal injection with HLA-A* 2404-restricted, 9-mer WT1 peptide against residual bone disease. After 3 months her bone disease disappeared, concurrent with an increase in the frequency of WT1-specific cytotoxic T lymphocytes (CTLs). A high proportion of WT1-specific CTLs with effector or effector memory phenotype were detected in peripheral blood of this patient. She is currently still on continued WT1 peptide immunotherapy in a disease-free condition for 22 months. WT1 peptide-based immunotherapy should be a promising option forhigh-risk rhabdomyosarcoma in childhood.*

**Keywords** childhood, rhabdomyosarcoma, WT1 peptide immunotherapy



The Wilms tumor gene WT1 was first identified as a gene responsible for Wilms tumor, a childhood renal cancer. This gene encodes a zinc finger transcription factor and plays an important role in cell proliferation, differentiation, apoptosis, and organ development by the positive or negative regulation of the expression of various kinds of genes [1]. Although the WT1 gene was first categorized as a tumor suppressor gene, recent studies showed the overexpression of WT1 mRNA in various kinds of solid tumors [2], the growth inhibition of WT1-expressing cells by WT1 antisense oligomers [2, 3], and a correlation between a high level of WT1 and a poor prognosis [15] in patients with certain kinds of tumors [4, 5], suggesting that WT1 plays an oncogenic role in human cancers. Furthermore, a sequencing study revealed the absence of mutations in the WT1 gene in tumors [2], indicating wild-type WT1 could be oncogenic.

WT1 is often overexpressed in leukemias and various types of solid tumors. Nakatsuka et al. examined overexpression of WT1 in 494 cases of human cancers and found overexpression in 30–70% of tumors of the gastrointestinal and pancreatobiliary system, urinary tract, male and female genital organs, breast, lung, brain, skin, and bone [6]. They also showed WT1 expression in 3 of 7 patients with PNET/Ewing sarcoma and in all 6 patients with rhabdomyosarcoma.

WT1 is now regarded as a molecular target for immunotherapy in various malignant tumor types. Clinical trials of WT1 peptide-based cancer immunotherapy are ongoing: WT1 peptide vaccination has been shown to be safe and clearly effective against several kinds of malignancies [7–10]. The trial for pediatric cancer is currently limited. Here, we describe the case report of a 6-year-old girl with rhabdomyosarcoma who was successfully treated with WT1 peptide-based immunotherapy.

## PATIENT AND METHODS

### Clinical Study

The WT1 peptide-based phase I/II clinical study was approved by the Institutional Review Board of Osaka University Hospital. Patients aged

<20 years with pediatric cancer or leukemia were eligible if they were resistant to conventional multimodal therapy. Other inclusion criteria were WT1 protein expression in solid cancer tissues or WT1 mRNA expression in leukemic cells determined by immunohistochemistry and RT-PCR, respectively; HLA-A*2402-positive; and performance status 0 to II (Eastern Cooperative Oncology Group). Patients were excluded if they had severely impaired organ function or had received chemotherapy or radiotherapy between the confirmation of residual disease and WT1 peptide vaccination.

### Immunohistochemistry Determination of WT1 Expression in Solid Cancer Tissue

Formalin-fixed tissue sections (3-$\mu$m thickness) were cut from each paraffin block. After being dewaxed with xylene and rehydrated through a graded series of ethanol, the sections were microwaved for 15 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval. These sections were incubated in phosphate-buffered saline containing goat serum albumin, reacted with anti-WT1 6F-H2 mouse monoclonal antibody (mAb) (Dako Cytomation. Carpinteria, CA, USA) diluted 1:50 at 4°C overnight, and then reacted with EnVision kit (Dako Cytomation) according to the manufacturer's instructions. After treatment with 3% $H_2O_2$ solution to reduce endogenous peroxidase activity, immunoreactive WT1 protein was visualized with diaminobenzidine tetrahydrochloride. The sections were then counterstained with hematoxylin.

### WT1 Peptide Treatment Plan

The 9-mer WT1 peptide (a.a. 235–243, CYTWNQMNL) was used for immunization [11]. GMP grade WT1 peptide was purchased from Multiple Peptide Systems (San Diego, CA) as a lyophilized peptide, which was dissolved just prior to injection. After written informed consent was obtained from the patient and her parents, a skin test for an HLA-A*2402-restricted, 9-mer WT1 peptide was performed and confirmed to be negative. WT1 peptide (1 mg) was emulsified with Montanide ISA51 adjuvant (SEPPIC S.A., Paris, France) [7, 8, 12], and the emulsion was injected intradermally at several different regions, including upper arms and lower abdomen. The WT1 peptide vaccination was scheduled to be performed weekly.

### Analysis of WT1-specific Cytotoxic T-lymphocytes

The procedure for cell staining was performed as described elsewhere [13]. Peripheral blood mononuclear cells were stained with phycoerythrin (PE)-conjugated HLA-A*2402-WT1 235–243 tetramer (WT1-Tet) (MBL, Tokyo, Japan). The cells were then stained with fluorescein

isothiocyanate-labeled anti-CD4, CD14, CD16, CD19, and CD5 mAbs (eBioscience, San Diego, CA), APC-Cy7-labeled anti-CD8 mAb (BD Pharmingen, San Diego, CA), ECD-labeled anti-CD45RA mAb (2H4LDH11LDB9, Beckeman Coulter, Fullerton, CA), and PE-Cy7-labeled CCR7 mAb (3D12, BD Biosciences, San Jose, CA). After this procedure, cells were analyzed with FACS Aria (BD Biosciences). CD4, CD14, CD16, CD19, and CD56-negative WT1-Tet$^+$ CD8$^+$ T cells were considered to be the WT1 peptide-specific CD8$^+$ T cells. We measured the frequency (%) of WT1-Tet$^+$ CD8$^+$ T cells among the CD8$^+$ T cells, and defined it as the WT1-specific cytotoxic T-lymphocyte (CTL) frequency. In addition, we analyzed the phenotype of WT1-Tet$^+$ CD8$^+$T cells according to their expression of CD45RA and CCR7. The WT1-Tet$^+$ CD8$^+$ T cells were phenotypically classified into four differentiation stages: naïve (CD45RA$^+$CCR7$^+$), central memory (CD45R$^-$CCR7$^+$), effector memory (CD45RA$^-$CCR7$^-$), and effector(CD45RA$^+$CCR7$^-$) [13].

## CASE REPORT

A 6-year-old girl presented with a mass on her lower left leg (Figure 1A). A diagnosis of alveolar rhabdomyosarcoma was made by histopathology with presence of left inguinal PAX3-FKHR. Image studies showed a lymph node metastasis from the right axial to para-aortic legion (Figure 1B) and multiple bone metastases located on right parietal, right 4th rib, and thoracic vertebrae (Figure 1C). Bone marrow aspiration revealed aggregation of tumor cells (Figure 1D). The disease status was stage 4 and group IV.

Combination chemotherapy was started. The combination consisted of cyclophosphamide, etoposide, THP-adriamycin, cisplatin, and vincristine (course 1), followed by ifosfamide, etoposide, actinomycin-D, and vincristine (course 2). However, a new bone metastasis lesion was observed on lumbar vertebrae (Figure 2A) after two courses of chemotherapy. At this point, bone marrow aspiration showed no residual tumor cells. She then received two further courses of chemotherapy intensified with nogitecan (2 mg/m$^2$ × 3–5). She subsequently underwent operation on the primary site and additional two courses of chemotherapy with radiotherapy on the primary site, high-dose chemotherapy consisting of thio-TEPA and L-PAM with autologous bone marrow and peripheral blood stem cell rescue, and radiotherapy on the metastatic lymph node site. The metastatic lesions of the vertebrae and right 4th rib were not irradiated. After all these therapies were completed, no residual disease was observed at the primary site or the metastatic para-aortic site. However, the bone disease on lumbar vertebrae remained (Figure 2B). We did not perform a biopsy of the uptake region.