cells by electroporation and subsequently the transfectant ES cell clones were induced to differentiate to ES-DC.

ES-DC carrying an epitope-presenting vector and expressing recombinant human invariant chain (Ii/CD74), which included GAD65p115-127 in the CLIP region, was generated. It was expected that the epitope could be efficiently targeted on MHC class II pathway [13]. The vector was introduced into KhES-1 ES cells, and a transfectant clone, KhES-1-Ii23, highly expressing transgene-derived recombinant CD74 was selected by a flowcytometric analysis at the pre-ES-DC stage. The expression of CD74 was detected even in the non-transfectant pre-ES-DC, reflecting intrinsic expression of CD74. The transfectant exhibited an increased expression of CD74 in comparison with the non-transfectants, thus indicating additional expression of the molecule derived from the transgene. The ability of the transfectant ES-DC, ES-DC-Ii23 to stimulate the GAD-epitope-specific T cell clone, SA32.5, in the absence of antigenic peptide or protein was next examined. As a result, ES-DC-Ii23 stimulated SA32.5 T cells and induced their proliferation, thus demonstrating functional expression of the epitope-presentation vector in the transfectant ES-DC. The in vivo transfer of ES-DC transfected with this antigen-presenting vector is therefore expected to be useful for controlling the immune response in an antigen-specific manner.

Su and colleagues [16] introduced mRNA for green fluorescence protein (GFP) or prostate-specific antigen (PSA) into DC generated from human ES cells. They showed induction of CTL specific to the antigens by in vitro stimulation of semi-allogeneic, HLA-A*0201^{+}, donor-derived T cells with the genetically modified DC.

5 Generation of DC from mouse iPS cells

As described so far, we can generate ES-DC from both mouse and human ES cells. Mouse systems have demonstrated the induction of anti-cancer immunity and the prevention of autoimmune disease by in vivo administration of genetically engineered ES-DC.

Considering the future clinical application of ES-DC technology, however, the unavailability of human ES cells genetically identical to the patients to be treated is a problem. Allogenicity caused by differences in the genetic background between human ES cell lines and the recipients is a critical problem in medical application of ES-DC. In addition, ethical concerns related to the use of human ES cells are anticipated to be serious obstacles, which will hinder the realization of the medical use of human ES-DC.

It was recently revealed that ES cell-like pluripotent stem cells, designated as iPS cells, can be generated by the simultaneous introduction of several genes for re-programming factors, such as Oct3/4, Sox2, Klf4, and c-Myc, into somatic cells, for both mouse and human [23–25]. The issue of histoincompatibility between patients to be treated and ES cells may be overcome by the generation of iPS cells from somatic cells of the patients such as fibroblasts or blood cells. The major ethical issues related to human ES cells would be avoided by aid of iPS cell-technology, because the use of human embryos is not necessary for the generation of iPS cells.

Differentiation of iPS cells into various cells belonging to the 3 germ layers has been demonstrated by the analysis of teratomas generated from mouse and human iPS cells. In addition, the pluripotency of iPS cells is obvious by the contribution of iPS cell-derived cells to various organs of the chimeric mice developed from iPS cell-introduced blastocysts. As for the in vitro generation of cells of mesodermal lineage from iPS cells, differentiation into cardiac myocytes and endothelial cells from mouse iPS cells has been reported.

We recently reported the generation and characterization of DC derived from mouse iPS cells [26]. The iPS cell-derived DC (iPS-DC) possessed the characteristics of DC including the capacity of T cell-stimulation, antigen-processing and presentation, and cytokine production. DNA microarray analyses revealed the up-regulation of genes related to antigen-presenting functions during differentiation into iPS-DC and similarity in gene-expression profile in iPS-DC and bone marrow cell-derived DC. There was some delay in the kinetics of differentiation process of iPS cells, as in comparison with most of mouse ES cell lines. On the other hand, the yield of differentiated cells was higher than that in the cases of most of mouse ES cell lines. Genetically modified iPS-DC expressing antigenic protein primed T cells specific to the antigen in vivo and elicited efficient antigen-specific anti-tumor immunity.

6 Future direction

We recently succeeded in generating iPS-DC from human iPS cells. Human iPS-DC exhibited characteristics of DC, as human ES-DC do, in morphology, surface molecules, and T cell-stimulating capacity. Theoretically, we can now generate infinite number of DC genetically matched to the patients by using iPS cells generated from relatively small number of somatic cells of the patients, such as dermal fibroblasts or blood cells.

Considering clinical application of iPS-DC, the technical issue to be resolved is that we are still using mouse-derived OP9 feeder cells and culture medium containing fetal calf serum for the differentiation culture. A xeno-free culture method to maintain human iPS cells has been already developed, and we should make considerable effort

to establish xeno-free differentiation culture. Other issues to be considered may be the time and cost for production of iPS cells specific to the individual patients. This is not the issue specific to iPS-DC therapy, and in this regard, establishment of a bank of iPS cells derived from somatic cells of HLA haplotype-homozygous donors would be profoundly valuable [27].

References

1. Nakano T, Kodama H, Honjo T. Generation of lymphohematopoietic cells from embryonic stem cells in culture. Science. 1994;265:1098–101.
2. Fairchild PJ, Brook FA, Gardner RL, et al. Directed differentiation of dendritic cells from mouse embryonic stem cells. Curr Biol. 2000;10:1515–8.
3. Senju S, Hirata S, Matsuyoshi H, et al. Generation and genetic modification of dendritic cells derived from mouse embryonic stem cells. Blood. 2003;101:3501–8.
4. Matsuyoshi H, Senju S, Hirata S, et al. Enhanced priming of antigen-specific CTLs in vivo by embryonic stem cell-derived dendritic cells expressing chemokine along with antigenic protein: application to antitumor vaccination. J Immunol. 2004;172:776–86.
5. Matsuyoshi H, Hirata S, Yoshitake Y, et al. Therapeutic effect of alpha-galactosylceramide-loaded dendritic cells genetically engineered to express SLC/CCL21 along with tumor antigen against peritoneally disseminated tumor cells. Cancer Sci. 2005;96:889–96.
6. Fukuma D, Matsuyoshi H, Hirata S, et al. Cancer prevention with semi-allogeneic ES cell-derived dendritic cells. Biochem Biophys Res Commun. 2005;335:5–13.
7. Motomura Y, Senju S, Nakatsura T, et al. Embryonic stem cell-derived dendritic cells expressing glypican-3, a recently identified oncofetal antigen, induce protective immunity against highly metastatic mouse melanoma, B16-F10. Cancer Res. 2006;66:2414–22.
8. Fukushima S, Hirata S, Motomura Y, et al. Multiple antigen-targeted immunotherapy with alpha-galactosylceramide-loaded and genetically engineered dendritic cells derived from embryonic stem cells. J Immunother. 2009;32:219–31.
9. Matsunaga Y, Fukuma D, Hirata S, et al. Activation of antigen-specific cytotoxic T lymphocytes by beta 2-microglobulin or TAP1 gene disruption and the introduction of recipient-matched MHC class I gene in allogeneic embryonic stem cell-derived dendritic cells. J Immunol. 2008;181:6635–43.
10. Hirata S, Senju S, Matsuyoshi H, et al. Prevention of experimental autoimmune encephalomyelitis by transfer of embryonic stem cell-derived dendritic cells expressing myelin oligodendrocyte glycoprotein peptide along with TRAIL or programmed death-1 ligand. J Immunol. 2005;174:1888–97.
11. Hirata S, Matsuyoshi H, Fukuma D, et al. Involvement of regulatory T cells in the experimental autoimmune encephalomyelitis-preventive effect of dendritic cells expressing myelin oligodendrocyte glycoprotein plus TRAIL. J Immunol. 2007;178:918–25.
12. Senju S, Suemori H, Zembutsu H, et al. Genetically manipulated human embryonic stem cell-derived dendritic cells with immune regulatory function. Stem Cells. 2007;25:2720–9.
13. Fujii S, Senju S, Chen YZ, et al. The CLIP-substituted invariant chain efficiently targets an antigenic peptide to HLA class II pathway in L cells. Hum Immunol. 1998;59:607–14.
14. Okazaki T, Honjo T. The PD-1-PD-L pathway in immunological tolerance. Trends Immunol. 2006;27:195–201.
15. Zhan X, Dravid G, Ye Z, et al. Functional antigen-presenting leucocytes derived from human embryonic stem cells in vitro. Lancet. 2004;364:163–71.
16. Su Z, Frye C, Bae KM, et al. Differentiation of human embryonic stem cells into immunostimulatory dendritic cells under feeder-free culture conditions. Clin Cancer Res. 2008;14:6207–17.
17. Tseng SY, Nishimoto KP, Silk KM, et al. Generation of immunogenic dendritic cells from human embryonic stem cells without serum and feeder cells. Regen Med. 2009;4:513–26.
18. Suemori H, Tada T, Torii R, et al. Establishment of embryonic stem cell lines from cynomolgus monkey blastocysts produced by IVF or ICSI. Dev Dyn. 2001;222:273–9.
19. Suemori H, Yasuchika K, Hasegawa K, et al. Efficient establishment of human embryonic stem cell lines and long-term maintenance with stable karyotype by enzymatic bulk passage. Biochem Biophys Res Commun. 2006;345:926–32.
20. Slukvin II, Vodyanik MA, Thomson JA, et al. Directed differentiation of human embryonic stem cells into functional dendritic cells through the myeloid pathway. J Immunol. 2006;176:2924–32.
21. Choi KD, Vodyanik MA, Slukvin II. Generation of mature human myelomonocytic cells through expansion and differentiation of pluripotent stem cell-derived lin-CD34+CD43+CD45+progenitors. J Clin Invest. 2009;119:2818–29.
22. Tabata H, Kanai T, Yoshizumi H, et al. Characterization of self-glutamic acid decarboxylase 65-reactive CD4+ T-cell clones established from Japanese patients with insulin-dependent diabetes mellitus. Hum Immunol. 1998;59:549–60.
23. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006;126:663–76.
24. Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. Nature. 2007;448:313–7.
25. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007;131:861–72.
26. Senju S, Haruta M, Matsunaga Y, et al. Characterization of dendritic cells and macrophages generated by directed differentiation from mouse induced pluripotent stem cells. Stem Cells. 2009;27:1021–31.
27. Nakatsuji N, Nakajima F, Tokunaga K. HLA-haplotype banking and iPS cells. Nat Biotechnol. 2008;26:739–40.

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Incidence of Hepatocellular Carcinoma in Patients With Chronic Hepatitis B Virus Infection Who Have Normal Alanine Aminotransferase Values

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The importance of alanine aminotransferase (ALT) levels in the progression of hepatitis B virus (HBV) infection remains a subject of debate. This study sought to identify independent risk factors involved in development of hepatocellular carcinoma (HCC), particularly in patients with chronic HBV infection who have normal ALT values. Data from 381 consecutive hepatitis B patients were analyzed with average ALT integration values $\leq$40 IU/L and follow-up periods of >3 years. Integration values were calculated from biochemical tests, and serological markers associated with the cumulative incidence of HCC were analyzed. HCC developed in 17 of the 381 patients (4.5%) during the follow-up period. Male sex (hazard ratio, 6.011 [95% confidence interval: 1.353–26.710], $P=0.018$), high HBV-DNA levels ($\geq$5.0 log copies/ml; 5.125 [1.880–13.973], $P=0.001$), low platelet counts ($<15.0\times10^4/mm^3$; 4.803 [1.690–13.647], $P=0.003$), and low total cholesterol levels (<130 mg/dl; 5.983 [1.558–22.979], $P=0.009$) were significantly associated with greater incidence of HCC development. High HBV-DNA levels and low platelet counts are associated with the development of HCC in patients infected with hepatitis B who have normal ALT values. Therefore, maintenance of low HBV-DNA levels is important for the prevention of HCC in patients with low platelet counts, particularly in patients whose ALT values fall within the current normal range. ***J. Med. Virol. 82:539–545, 2010.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: hepatitis B virus (HBV); HBV-DNA; normal alanine aminotransferase; platelet counts; hepatocellular carcinoma

INTRODUCTION

Worldwide, an estimated 350 million individuals are infected chronically with hepatitis B virus (HBV), and 1 million die each year from HBV-related liver disease [EASL Jury, 2003]. Chronic HBV infection is a major risk factor for the development of hepatocellular carcinoma (HCC) [Beasley, 1988; EASL Jury, 2003]. Patients who test positive for the hepatitis B surface antigen (HBsAg) have a 70-fold greater risk of developing HCC compared with HBsAg-negative patients [Szmuness, 1978; Beasley et al., 1981]. HBV infection is endemic in Southeast Asia, China, Taiwan, Korea, and sub-Saharan Africa, where up to 85–95% of patients with HCC are HBsAg-positive [Rustgi, 1987]. HCC is the third and fifth leading cause of death from malignant neoplasms in Japanese men and women, respectively, and the death rate from HCC has increased markedly in Japan since 1975 [Kiyosawa et al., 2004]. Hepatitis C virus (HCV)-related HCC accounts for 75% of all cases of HCC in Japan, while HBV-related HCC accounts for 15% of such cases [Kiyosawa et al., 2004].

Although an increasing body of epidemiological and molecular evidence suggests that HBV is associated with the development of HCC, the exact role of HBV in carcinogenesis is unclear [Ikeda et al., 2005; Wong et al., 2006]. HBV elicits a chronic necroinflammatory hepatic disease [Yu and Chen, 1994], and liver injury associated with HBV infection is mediated by viral factors in addition to the host immune response. Patients who are positive for the hepatitis B e antigen (HBeAg) commonly have increased hepatic inflammatory activity and an increased risk of developing HCC [Yang et al., 2002]. HBeAg-negative HBsAg carriers who retain high levels of HBV-DNA and show persistent necroinflammation of the liver have an increased risk of acquiring HCC [Yu et al., 2005; Chen et al., 2006].

The authors report no conflicts of interest.

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Alanine aminotransferase (ALT) activity is the most widely used laboratory test for the evaluation of necroinflammatory activity in liver disease [Prati et al., 2002]; however, it is well known that HCC occurs in some HBsAg carriers with normal ALT values. Recently, Chen et al. [2006] conducted a large cohort study in Taiwan and found that elevated serum HBV-DNA levels are strong predictive factors for the development of HCC, independent of the ALT values. It is an important problem for early detection of HCC that general practitioners are sometimes unaware of those patients with normal ALT as high-risk subjects for HCC. There is little information about how many patients with normal ALT develop HCC. It is important that ALT values should be expressed with integration values to ensure a valid analysis, since ALT values fluctuate frequently [Kumada et al., 2007]. Therefore, this study sought to identify the independent risk factors, involving mainly serological markers, associated with the development of HCC in patients infected chronically with HBV with average ALT integration values ≤40 IU/L.

MATERIALS AND METHODS

Patient Selection

A total of 1,861 consecutive patients who were positive for HBsAg visited the Department of Gastroenterology at Ogaki Municipal Hospital, Japan, between September 1994 and August 2003. After assessing each patient's long-term prognosis, 381 consecutive patients were selected for further study who (1) were positive for HBsAg for at least 6 months; (2) displayed no evidence of HCV infection; (3) had no other possible causes of chronic liver disease (i.e., alcohol consumption lower than 80 g/day, no history of hepatotoxic drug use, and negative tests for autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, and Wilson's disease); (4) had a follow-up period of >3 years; (5) had no evidence of HCC for at least 3 years from the start of the follow-up period; (6) had no history of therapy involving interferons, nucleosides, or nucleotide analogues; (7) had ALT measurements taken more than twice in a year; and (8) had average ALT integration values ≤40 IU/L (Fig. 1).

Patients were evaluated at the hospital at least every 6 months. During each follow-up examination, platelets, ALT, aspartate aminotransferase (AST), gamma glutamyl transpeptidase (gamma-GTP), total bilirubin, cholinesterase, alkaline phosphatase (ALP), albumin, total cholesterol, HBeAg, anti-HBe, HBV-DNA, and alpha-fetoprotein (AFP) were measured at least every 6 months. Commercial radioimmunoassay kits were used to test blood samples for HBsAg, HBeAg, and anti-HBe (Abbott Japan Co., Ltd, Tokyo, Japan). Before July 2001, serum HBV-DNA concentrations were monitored using the amplification-hybridization protection assay (DNA probe, Chugai-HBV; Chugai Pharmaceutical Co., Ltd, Tokyo, Japan) with a lower detection limit of ~5,000 viral genome copies/ml (3.7 log copies/ml). After August 2001, serum HBV-DNA levels were monitored using the polymerase chain reaction (PCR) (COBAS Amplicor HBV monitor test, Roche Diagnostics K.K., Tokyo, Japan) with a lower detection limit of ~400 viral genome copies/ml (2.6 log copies/ml). HBV genotyping was carried out as described previously [Kato et al., 2001]. ALT, AST, gamma-GTP, ALP, and AFP were expressed as integration values [Kumada et al., 2007]. When ALT was used as an example, the integration value of ALT was calculated as follows: $(y_0+y_1)\times x_1/2+(y_1+y_2)\times x_2/2+(y_2+y_3)\times x_3/2+(y_3+y_4)\times x_4/2+(y_4+y_5)\times x_5/2+(y_5+y_6)\times x_6/2+(y_6+y_7)\times x_7/2+(y_7+y_8)\times x_8/2$ (Fig. 2). The area of a trapezoid with ALT value was calculated and the measurement interval and added the values. The

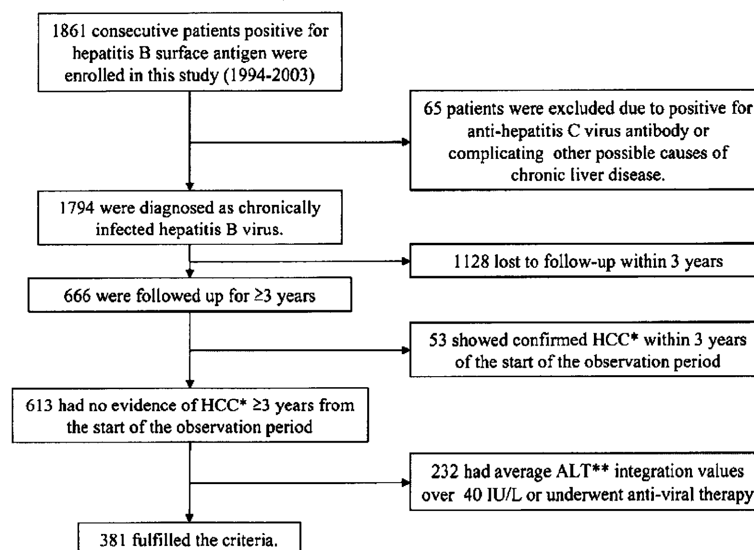

Fig. 1. Schematic flowchart of enrolled patients. *, hepatocellular carcinoma (HCC); **, alanine aminotransferase (ALT).

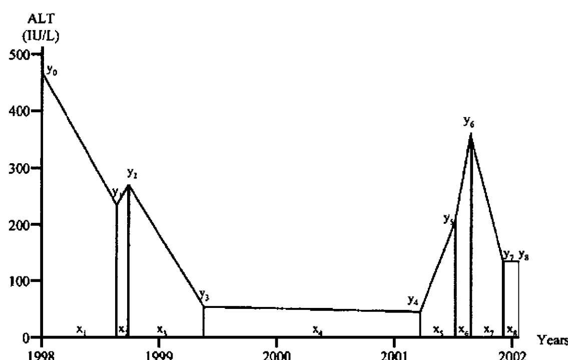

Fig. 2. Integration value of alanine aminotransferase (ALT). The integration value of ALT was calculated as follows: $(y_0+y_1) \times x_1/2 + (y_1+y_2) \times x_2/2 + (y_2+y_3) \times x_3/2 + (y_3+y_4) \times x_4/2 + (y_4+y_5) \times x_5/2 + (y_5+y_6) \times x_6/2 + (y_6+y_7) \times x_7/2 + (y_7+y_8) \times x_8/2$. The integration value of ALT was divided by the observation period and expressed as an average integration value.

integration value of ALT was divided by the observation period to obtain the average integration value (Fig. 3). In addition, patients were classified into two groups according to the change of pattern of ALT: persistently normal ALT group and intermittently normal ALT group. The persistently normal ALT group included patients with persistently normal ALT values ≤40 IU/L during follow-up period. The intermittently normal ALT group included patients with temporary ALT fluctuations but the average integration value was ≤40 IU/L. Platelet counts, total bilirubin, cholinesterase, albumin, total cholesterol, HBeAg, anti-HBe, and HBV-DNA were analyzed at the time of entry into the study.

Ultrasonograpy was performed in all patients at the start of the follow-up period for the evaluation of liver fibrosis. The diagnosis of cirrhosis was made according to typical ultrasound findings, for example, superficial nodularity, a coarse parenchymal echo pattern, and

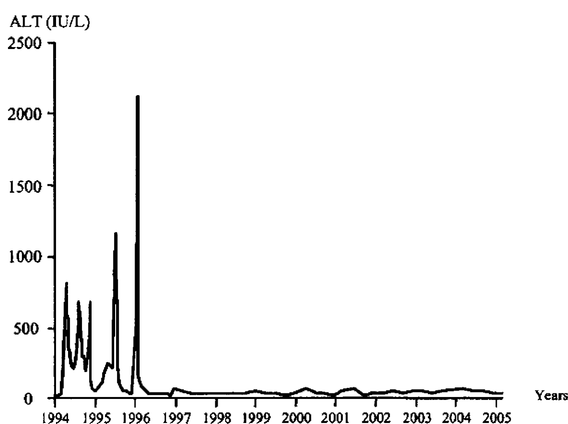

Fig. 3. Average integration value and arithmetic mean value of alanine aminotransferase (ALT) in a 26-year-old patient with hepatitis B virus (HBV). The patient was followed-up for 11.2 years. The number of ALT examinations was 96. The integration value of ALT was 955.2 IU/L × years. The average integration value was 85.3 IU/L, whereas the arithmetic mean value was 255.6 IU/L. This difference is due to the number of ALT measurements between a period of high ALT level and low ALT level.

signs of portal hypertension (splenomegaly >120 mm, dilated portal vein diameter >12 mm, patent collateral veins, or ascites) [Caturelli et al., 2003; Iacobellis et al., 2005; Shen et al., 2006].

To detect early-stage HCC, ultrasonography, computed tomography, magnetic resonance imaging, and/or measurement of tumor markers (i.e., AFP, *Lens culinaris* agglutinin-reactive AFP, and des-γ-carboxyprothrombin) were performed for all patients, at least every 6 months. Blood biochemistry data used in this study were obtained over 1 year prior to HCC development. The study ended in December 31, 2007 or on the date of HCC identification, whichever was earlier. The diagnosis of HCC was based on histological examination (n = 9). In the remaining eight patients, the diagnosis was based on clinical criteria [Kudo, 1999; Torzilli et al., 1999].

Statistical Analysis

Statistical analyses were performed using the Statistical Program for Social Science (SPSS version 17.0 for Windows; SPSS Japan, Inc., Tokyo, Japan). Continuous variables are expressed as median (range). The Kruskal–Wallis test was used to assess continuous variables with a skewed distribution, and the chi-square test was used to assess categorical variables. An actuarial analysis of the cumulative incidence of HCC was performed using the Kaplan–Meier method, and differences were tested by a log-rank test. The Cox proportional hazard model and forward selection method were used to estimate the relative risk of HCC development associated with age (i.e., ≤40 years or >40 years), sex (i.e., male or female), HBeAg (i.e., positive or negative), HBV-DNA level (i.e., <5.0 or ≥5.0 log copies/ml), average ALT integration value (i.e., ≤20 or >20 IU/L), the change pattern of ALT (persistently normal ALT group or intermittently normal ALT group), average AST integration value (i.e., ≤40 or >40 IU/L), platelet count (i.e., <15.0 or ≥15.0 × 10^4/mm^3), average gamma-GTP integration value (i.e., ≤56 or >56 IU/L), total bilirubin (i.e., ≤1.2 or >1.2 mg/dl), average ALP integration value (i.e., ≤338 or >338 IU/L), cholinesterase (i.e., <431 or ≥431 IU/L), albumin (i.e., <3.5 or ≥3.5 g/dl), total cholesterol (i.e., <130 or ≥130 mg/dl), and average AFP integration value (i.e., ≤10 or >10 ng/ml). The lower and upper limits of the reference values at our institution were used as cut-off values for AST, platelet count, gamma-GTP, total bilirubin, ALP, cholinesterase, albumin, and total cholesterol. Statistical significance was defined as $P < 0.05$.

The study protocol was approved by the Ethics Committee at Ogaki Municipal Hospital and performed in compliance with the Helsinki Declaration.

RESULTS

Patient Characteristics

The median follow-up period was 8.6 years (range, 3.0–14.0 years). HCC developed in 17 of 381 patients

(4.5%) during the follow-up period. The 5- and 10-year cumulative incidence of HCC was 0.8% and 6.5%, respectively. Profiles and data from the 381 patients with normal ALT values are summarized in Table I.

Factors Associated With the Incidence of HCC

Factors associated with the incidence of HCC, as determined by univariate analysis, are listed in Table II. Male sex, high HBV-DNA levels, intermittently normal ALT, high AST levels, low platelet counts, low cholinesterase levels, low albumin levels, low total cholesterol levels, high AFP levels, and presence of cirrhosis were significantly associated with HCC development. The cumulative incidence of HCC was significantly higher in patients with platelet counts $<15.0 \times 10^4/mm^3$ (n = 70) than in patients with platelet counts $\geq 15.0 \times 10^4/mm^3$ (n = 311, $P < 0.001$, Fig. 4). The cumulative incidence of HCC was significantly higher in patients with HBV-DNA levels ≥ 5.0 log copies/ml (n = 90) than in patients with HBV-DNA levels <5.0 log copies/ml (n = 291, $P < 0.001$, Fig. 5).

Factors associated with incidence of HCC, as determined by the Cox proportional hazard model and the forward selection method, are listed in Table III. Male sex, high HBV-DNA levels, low platelet counts, and low total cholesterol levels were significantly associated with the development of HCC.

Baseline of patients with normal ALT according to HBV-DNA level and platelet counts.

HBV carriers with normal ALT levels were divided into four groups (A: HBV-DNA levels <5.0 log copies/ml and platelet counts $\geq 15.0 \times 10^4/mm^3$ [n = 257]; B: HBV-DNA levels <5.0 log copies/ml and platelet counts $<15.0 \times 10^4/mm^3$ [n = 45]; C: HBV-DNA levels ≥ 5.0 log copies/ml and platelet counts $\geq 15.0 \times 10^4/mm^3$ [n = 54]; and D: HBV-DNA levels ≥ 5.0 log copies/ml and platelet counts $<15.0 \times 10^4/mm^3$ [n = 25]). Positive rates of HBeAg were highest in Group C, total cholesterol levels were lowest in Group D, and ALT level, frequency of intermittently normal ALT, AFP levels, and presence

TABLE I. Patient Characteristics

Age (years)	49 (12–84)
Sex (F/M)	201/180
BMI (kg/m²)	22.4 (17–36)
HBV genotype (A/B/C/D)	8/24/149/2
HBeAg (positive/negative)	59/322
HBV-DNA (log copies/ml)	3.7 (2.6–9.6)
ALT (IU/L)	22.6 (8.7–39.9)
Persistently normal ALT (+/−)[a]	182/199
AST (IU/L)	23.4 (13.3–74.3)
Platelet (×10⁴/mm³)	19.3 (3.3–39.5)
Gamma-GTP (IU/L)	19.5 (7.4–441.0)
Total bilirubin (mg/dl)	0.6 (0.3–4.7)
ALP (IU/L)	214.8 (82.4–621.3)
Cholinesterase (IU/L)	314.0 (99.6–483.9)
Albumin (g/dl)	4.2 (2.4–4.9)
Total cholesterol (mg/dl)	186.5 (102.0–332.1)
AFP (ng/ml)	2.4 (0.8–303.6)
Cirrhosis (−/+)[b]	341/40
Hepatocarcinogenesis (+/−)	17/364

F, female; M, male; BMI, body mass index; HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase: AST, aspartate aminotransferase; GTP, glutamyl transpeptidase; ALP, alkaline phosphatase; AFP, alpha-fetoprotein.
Values are expressed as median (range).
[a]Persistently normal ALT values includes patients with ≤40 IU/L.
[b]Cirrhosis diagnosed by ultrasound findings.

TABLE II. Factors Associated With Hepatocarcinogenesis (Univariate Analysis)

	Hazard ratio (95% CI)	P-value
Sex		
F	1	
M	8.282 (1.892–36.259)	0.005
HBV-DNA (log copies/ml)		
≤5.0	1	
>5.0	7.133 (2.699–18.852)	<0.001
Persistently normal ALT[a]		
Presence	1	
Absence	3.939 (1.126–13.776)	0.032
AST (IU/L)		
≤40	1	
>40	4.046 (1.157–14.140)	0.029
Platelets (×10⁴/m³)		
≥15	1	
<15	7.961 (2.922–21.690)	<0.001
Cholinesterase (IU/L)		
≥431	1	
<431	4.865 (1.368–17.298)	0.015
Albumin (g/dl)		
≥3.5	1	
<3.5	8.086 (2.567–25.474)	<0.001
Total cholesterol (mg/dl)		
≥130	1	
<130	9.704 (2.740–34.367)	<0.001
AFP (ng/ml)		
≤10	1	
>10	6.779 (1.445–31.809)	0.015
Cirrhosis[b]		
Absence	1	
Presence	18.033 (6.6055–19.233)	<0.001

W, female; M, male; HBV, hepatitis B virus; AST, aspartate aminotransferase; GTP, glutamyl transpeptidase; AFP, alpha-fetoprotein.
P-values and hazard ratio were calculated by Cox proportional hazard model.
[a]Persistently normal ALT values includes patients with ≤40 IU/L.
[b]Cirrhosis diagnosed by ultrasound.

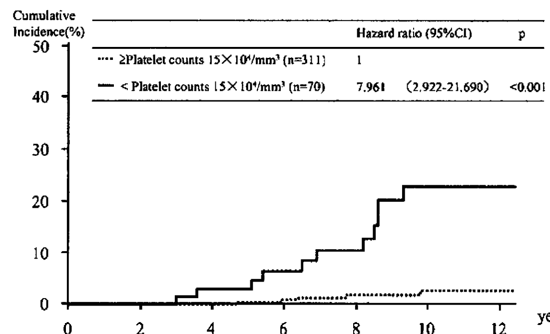

Fig. 4. Incidence of HCC according to platelet counts. The 5- and 10-year cumulative incidences of HCC was 0.4% and 2.6%, respectively, in patients with platelet counts $\geq 15.0 \times 10^4/mm^3$ (n = 311), and 2.9% and 22.9% in patients with platelet counts $<15.0 \times 10^4/mm^3$ (n = 70). The cumulative incidence of HCC was significantly higher in the latter group than in the former.

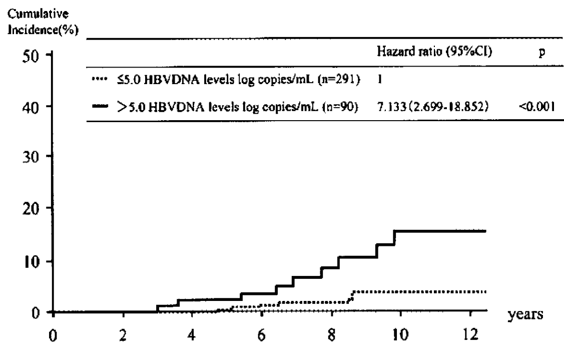

Fig. 5. Incidence of HCC according to serum HBV-DNA levels. The 5- and 10-year cumulative incidences of HCC was 0.4% and 3.7%, respectively, in patients with HBV-DNA levels <5.0 log copies/ml (n = 291) and 2.3% and 15.5%, respectively, in patients with HBV-DNA levels ≥5.0 log copies/ml (n = 90). The cumulative incidence of HCC was significantly higher in the latter group than in the former.

of cirrhosis were highest in Group D (Table IV). Group D showed the highest rate of incidence of HCC, followed by Groups B and C, as compared with Group A (Fig. 6).

DISCUSSION

The current studies revealed that the risk of developing HCC increases with decreasing platelet counts, decreasing total cholesterol levels, and increasing HBV-DNA levels in patients with average ALT integration values ≤40 IU/L.

ALT, AST, gamma-GTP, ALP, and AFP levels fluctuated within individual patients. Therefore, repeated measurements of these tests are important for accurate interpretation of the data. The arithmetic mean value is often used in the measurement of these tests; however, this value can be greatly affected by the period of time between measurements. Therefore, integral calculus was used to determine the value of these markers. Because this determination is strongly affected by the follow-up period, the average integration value was divided by the time of follow-up. The average integration value is more meaningful than the arithmetic mean value [Kumada et al., 2007].

In the present study, there was no difference between patients with average ALT integration values of 0–20 IU/L versus those with 21–40 IU/L. Thus, ALT levels are not good predictors of HCC development in patients with hepatitis B, as opposed to hepatitis C [Yuen et al., 2005; Sherman, 2005]. Furthermore, the change pattern of ALT was evaluated in the persistently normal ALT group and the intermittently normal ALT group. The results of the univariate analysis suggest that intermittently normal ALT levels, high AST levels, low cholinesterase levels, low albumin levels, and high AFP levels are associated significantly with HCC development; however, not all of these factors were significant in the multivariate analysis.

HBV-DNA levels at the start of the follow-up period correlated with the cumulative incidence of HCC. Chen et al. [2006] reported the adjusted hazard ratios for HCC development in HBeAg-seronegative subjects with normal ALT levels. Compared with participants in whom serum HBV-DNA levels were <300 copies/ml, the adjusted hazard ratio for developing HCC was 1.3 (95% confidence interval, 0.5–3.2; $P = 0.05$) for participants with serum HBV-DNA levels of 300–9,999 copies/ml; 2.7 (1.2–6.3; $P = 0.02$) for levels of 10,000–99,999 copies/ml; 7.2 (3.2–16.6; $P < 0.001$) for levels of 100,000–999,999 copies/ml; and 14.3 (6.2–32.8; $P < 0.001$) for levels of 1 million copies/ml and greater. It is emphasized that the cumulative incidence of HCC increases in patients with increased HBV-DNA levels, even if patients have normal ALT levels.

Lok and McMahon [2004] reported that HBV-DNA levels $>10^5$ copies/ml should be considered clinically significant. Their recommendation is supported by a meta-analysis of 26 trials of anti-HBV therapy which evaluated the association between viral load and hepatic inflammatory activity, as determined by hepatic histology and aminotransferase activity [Mommeja-Marin et al., 2003]. Thus, it is important for patients to maintain low HBV-DNA levels (i.e., $\leq 10^5$ copies/ml). These findings suggest that effective control of HBV replication, indicated by a decrease in serum HBV-DNA levels following antiviral therapy, may reduce the ultimate risk of developing HCC. Furthermore, it is believed that treatment with nucleosides or nucleotide analogues will decrease the cumulative incidence of HCC [Liaw et al., 2004; Piao et al., 2005].

The present study reveals that a low platelet count is a predictive factor for the development of HCC. Cirrhosis is an established risk factor for HCC in patients with HBV [Liaw et al., 1989; McMahon et al., 2001; Yu et al., 2002; Murata et al., 2005]. Ultrasonography produces detailed cross-sectional images of the liver and its surrounding structures. To distinguish cirrhosis patients from non-cirrhosis patients was attempted according to typical ultrasound findings [Caturelli et al., 2003; Iacobellis et al., 2005; Shen et al., 2006]. The presence of cirrhosis diagnosed by ultrasonography

TABLE III. Multivariate Analysis of Factors Associated With Development of Hepatocellular Carcinoma

Factor	Hazard ratio (95% CI)	P-value
Sex		
F	1	
M	6.011 (1.353–26.710)	0.018
HBV-DNA (log copies/ml)		
≤5.0	1	
>5.0	5.125 (1.880–13.973)	0.001
Platelets (×10⁴/mm³)		
≥15	1	
<15	4.803 (1.690–13.647)	0.003
Total cholesterol (mg/dl)		
≥130	1	
<130	5.983 (1.558–22.979)	0.009

F, female; M, male; HBV, hepatitis B virus.
P-values and hazard ratios were calculated using the Cox proportional hazard model.

TABLE IV. Patients Characteristics, According to HBVDNA Levels and Platelet Counts

HBV-DNA (log copies/ml) Platelets ($\times 10^4/m^3$)	Group A ≤5.0 ≥15 × 10^4 (n = 257)	Group B ≤5.0 <15 × 10^4 (n = 45)	Group C >5.0 ≥15 × 10^4 (n = 54)	Group D >5.0 <15 × 10^4 (n = 25)
Age (years)	49 (12–84)	51 (24–75)	47 (15–73)	52 (33–82)
Sex (F/M)	136/121	25/20	29/25	11/14
BMI (kg/m^2)	22.6 (14–36.3)	22.5 (16–28.2)	22.2 (16.7–32.4)	20.9 (16.9–36.4)
HBV genotype (A/B/C/D)	7/20/88/2	0/1/20/0	1/3/26/0	0/0/15/0
HBeAg (positive/negative)***	5/252	3/42	36/18	15/10
ALT (IU/L)***	19.7 (8.7–39.1)	25.3 (11.2–38.2)	29.8 (12.2–39.9)	32.1 (18.3–38.4)
Persistently normal ALT (+/−)[a],***	153/104	14/31	14/40	1/24
Total cholesterol (mg/dl)***	191.5 (114–332.1)	169.1 (102–259.2)	190.1 (147.1–254.4)	165.5 (112–234)
AFP (ng/ml)****	2.2 (0.8–119.8)	2.6 (0.8–20.8)	2.8 (0.8–45.5)	4.7 (1.1–303.6)
Cirrhosis (−/+)[b],***	253/4	27/18	50/4	11/14
Hepatucelluar carcinoma (+/−)***	2/255	5/40	4/50	6/19

F, female; M, male; BMI, body mass index; HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; AFP, alpha-fetoprotein.
P-values were calculated using the Kruskal–Wallis test or the chi-square test. Values are expressed as median (range).
[a]Persistently normal ALT values includes patients with ≤40 IU/L.
[b]Cirrhosis diagnosed by ultrasound findings.
***$P < 0.0001$.
****$P < 0.0005$.

was strongly associated with the increased incidence of HCC by univariate analysis. Anatomical constraints and interobserver variability, however, remain limiting factors. In this study, histological confirmation was obtained in only 20 patients (6.3%). It is thought that this study had limitations because the liver histology was not obtained in many cases. Liver biopsy is still the "gold standard" for assessing liver fibrosis; however, it is not practical to undertake biopsies on all patients because of the potential complications which might arise from this procedure. Furthermore, results often differ depending on the pathologist, and results for liver fibrosis in liver biopsy specimens do not always reflect the grade of fibrosis in the entire liver. In contrast, the platelet count is a useful surrogate marker for the

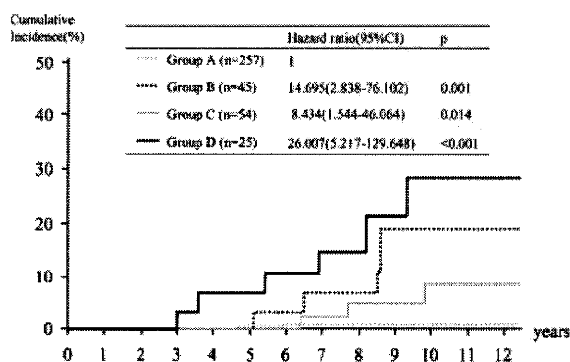

Fig. 6. The cumulative incidence of HCC according to HBV-DNA levels and platelet counts. HBV carriers with normal ALT levels were divided into four groups (A: HBV-DNA levels <5.0 log copies/ml and platelet counts ≥15.0 × 10^4/mm^3 [n = 257]; B: HBV-DNA levels <5.0 log copies/ml and platelet counts <15.0 × 10^4/mm^3 [n = 45]; C: HBV-DNA levels ≥5.0 log copies/ml and platelet counts ≥15.0 × 10^4/mm^3 [n = 54]; and D: HBV-DNA levels ≥5.0 log copies/ml and platelet counts <15.0 × 10^4/mm^3 [n = 25]). Group D had the highest incidence rate of HCC (26.007 [5.217–129.648], $P < 0.001$), followed by Group B (14.695 [2.838–76.102], $P = 0.001$) and Group C (8.434 [1.544–46.064], $P = 0.014$), as compared with Group A.

diagnosis of cirrhosis. Lu et al. [2006] reported that the best cutoff platelet count for a diagnosis of cirrhosis is 15.0 × 10^4/mm^3. The primary aim of this study was to identify serological markers associated with the development of HCC. Because of this, cirrhosis diagnosed by ultrasonography was excluded from the multivariate analysis. On the other hand, a low cholesterol level is associated with hepatocarcinogenesis, too. Hypocholesterolemia is found frequently in advanced liver disease because the liver is the most active site of cholesterol metabolism [D'Arienzo et al., 1998]. Four of 12 patients (33.3%) with <130 mg/dl serum total cholesterol developed HCC during follow-up period. It seemed that low platelet counts and hypocholesterolemia were confounding factors for identifying cirrhosis. Platelet counts were used as a parameter for cirrhosis in this study.

The HBV genotype is also predictive of the development of HCC [Chan et al., 2004; Yu et al., 2005]. In Japan, HBV genotype C is the predominant genotype [Orito et al., 2001]. Genotype C is associated with higher HBV-DNA levels and a greater risk of HCC than genotype B [Chan et al., 2004]. In the present study, 149 of 183 patients (81.4%) were infected with HBV genotype C. All eight patients with HCC in whom HBV genotype was determined were infected with genotype C. It was difficult to evaluate the relationship between HBV genotype and incidence of HCC in this study.

This study has some limitations such as the potential for selection bias due to a retrospective analysis of a cohort of patients. Therefore, an effort was made to minimize the influence of bias by using average integration values of various biochemical markers and a multivariate analysis.

In conclusion, high HBV-DNA levels and low platelet counts are associated with an increased incidence of HCC in patients infected with hepatitis B who have normal ALT values. Therefore, maintenance of low HBV-DNA levels is important for the prevention for

HCC in patients with low platelet counts, even when the ALT values fall within the current normal range.

REFERENCES

Beasley RP. 1988. Hepatitis B virus. The major etiology of hepatocellular carcinoma. Cancer 61:1942–1956.

Beasley RP, Hwang LY, Lin CC, Chien CS. 1981. Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22707 men in Taiwan. Lancet 2:1129–1133.

Caturelli E, Castellano L, Fusilli S, Palmentieri B, Niro GA, del Vecchio-Blanco C, Andriulli A, de Sio I. 2003. Coarse nodular US pattern in hepatic cirrhosis: Risk for hepatocellular carcinoma. Radiology 226:691–697.

Chan HL, Hui AY, Wong ML, Tse AM, Hung LC, Wong VW, Sung JJ. 2004. Genotype C hepatitis B virus infection is associated with an increased risk of hepatocellular carcinoma. Gut 53:1494–1498.

Chen CJ, Yang HI, Su J, Jen CL, You SL, Lu SN, Huang GT, Iloeje UH. 2006. REVEAL-HBV Study Group: Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. JAMA 295:65–73.

D'Arienzo A, Manguso F, Scaglione G, Vicinanza G, Bennato R, Mazzacca G. 1998. Prognostic value of progressive decrease in serum cholesterol in predicting survival in Child-Pugh C viral cirrhosis. Scand J Gastroenterol 33:1213–1218.

EASL Jury. 2003. EASL International Consensus Conference on Hepatitis B. 13–14 September, 2002: Geneva, Switzerland. Consensus statement (short version). J Hepatol 38:533–540.

Iacobellis A, Fusilli S, Mangia A, Clemente R, Festa V, Giacobbe A, Facciorusso D, Niro G, Conoscitore P, Andriulli A. 2005. Ultrasonographic and biochemical parameters in the non-invasive evaluation of liver fibrosis in hepatitis C virus chronic hepatitis. Aliment Pharmacol Ther 22:769–774.

Ikeda K, Arase Y, Kobayashi M, Someya T, Hosaka T, Saitoh S, Sezaki H, Akuta N, Suzuki F, Suzuki Y, Kumada H. 2005. Hepatitis B virus-related hepatocellular carcinogenesis and its prevention. Intervirology 48:29–38.

Kato H, Orito E, Sugauchi F, Ueda R, Gish RG, Usuda S, Miyakawa Y, Mizokami M. 2001. Determination of hepatitis B virus genotype G by polymerase chain reaction with hemi-nested primers. J Virol Methods 98:153–159.

Kiyosawa K, Umemura T, Ichijo T, Matsumoto A, Yoshizawa K, Gad A, Tanaka E. 2004. Hepatocellular carcinoma: Recent trends in Japan. Gastroenterology 127:S17–S26.

Kudo M. 1999. Imaging diagnosis of hepatocellular carcinoma and premalignant/borderline lesions. Semin Liver Dis 19:297–309.

Kumada T, Toyoda H, Kiriyama S, Sone Y, Tanikawa M, Hisanaga Y, Kanamori A, Kondo J, Yamauchi T, Nakano S. 2007. Relation between incidence of hepatic carcinogenesis and integration value of alanine aminotransferase in patients with hepatitis C virus infection. Gut 56:738–739.

Liaw YF, Lin DY, Chen TJ, Chu CM. 1989. Natural course after the development of cirrhosis in patients with chronic type B hepatitis: A prospective study. Liver 9:235–241.

Liaw YF, Sung JJ, Chow WC, Farrell G, Lee CZ, Yuen H, Tanwandee T, Tao QM, Shue K, Keene ON, Dixon JS, Gray DF, Sabbat J. 2004. Cirrhosis Asian Lamivudine Multicentre Study Group. Lamivudine for patients with chronic hepatitis B and advanced liver disease. N Engl J Med 351:1521–1531.

Lok AS, McMahon BJ. 2004. Practice Guidelines Committee, American Association for the Study of Liver Diseases (AASLD): Chronic hepatitis B: Update of recommendations. Hepatology 39:857–861.

Lu SN, Wang JH, Liu SL, Hung CH, Chen CH, Tung HD, Chen TM, Huang WS, Lee CM, Chen CC, Changchien CS. 2006. Thrombocytopenia as a surrogate for cirrhosis and a marker for the identification of patients at high-risk for hepatocellular carcinoma. Cancer 107:2212–2222.

McMahon BJ, Holck P, Bulkow L, Snowball M. 2001. Serologic and clinical outcomes of 1536 Alaska Natives chronically infected with hepatitis B virus. Ann Intern Med 135:759–768.

Mommeja-Marin H, Mondou E, Blum MR, Rousseau F. 2003. Serum HBV DNA as a marker of efficacy during therapy for chronic HBV infection: Analysis and review of the literature. Hepatology 37:1309–1319.

Murata K, Sugimoto K, Shiraki K, Nakano T. 2005. Relative predictive factors for hepatocellular carcinoma after HBeAg seroconversion in HBV infection. World J Gastroenterol 11:6848–6852.

Orito E, Ichida T, Sakugawa H, Sata M, Horiike N, Hino K, Okita K, Okanoue T, Iino S, Tanaka E, Suzuki K, Watanabe H, Hige S, Mizokami M. 2001. Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. Hepatology 34:590–594.

Piao CY, Fujioka S, Iwasaki Y, Fujio K, Kaneyoshi T, Araki Y, Hashimoto K, Senoh T, Terada R, Nishida T, Kobashi H, Sakaguchi K, Shiratori Y. 2005. Lamivudine treatment in patients with HBV-related hepatocellular carcinoma–using an untreated, matched control cohort. Acta Med Okayama 59:217–224.

Prati D, Taioli E, Zanella A, Della Torre E, Butelli S, Del Vecchio E, Vianello L, Zanuso F, Mozzi F, Milani S, Conte D, Colombo M, Sirchia G. 2002. Updated definitions of healthy ranges for serum alanine aminotransferase levels. Ann Intern Med 137:1–10.

Rustgi VK. 1987. Epidemiology of hepatocellular carcinoma. Gastroenterol Clin North Am 16:545–551.

Shen L, Li JQ, Zeng MD, Lu LG, Fan ST, Bao H. 2006. Correlation between ultrasonographic and pathologic diagnosis of liver fibrosis due to chronic virus hepatitis. World J Gastroenterol 28:1292–1295.

Sherman M. 2005. Predicting survival in hepatitis B. Gut 54:1521–1523.

Szmuness W. 1978. Hepatocellular carcinoma and the hepatitis B virus: Evidence for a causal association. Prog Med Virol 24:40–69.

Torzilli G, Minagawa M, Takayama T, Inoue K, Hui AM, Kubota K, Ohtomo K, Makuuchi M. 1999. Accurate preoperative evaluation of liver mass lesions without fine-needle biopsy. Hepatology 30:889–893.

Wong CH, Chan SK, Chan HL, Tsui SK, Feitelson M. 2006. The molecular diagnosis of hepatitis B virus-associated hepatocellular carcinoma. Crit Rev Clin Lab Sci 43:69–101.

Yang HI, Lu SN, Liaw YF, You SL, Sun CA, Wang LY, Hsiao CK, Chen PJ, Chen DS, Chen CJ. 2002. Taiwan Community-Based Cancer Screening Project Group. Hepatitis B e antigen and the risk of hepatocellular carcinoma. N Engl J Med 347:168–174.

Yu MW, Chen CJ. 1994. Hepatitis B and C viruses in the development of hepatocellular carcinoma. Crit Rev Oncol Hematol 17:71–91.

Yu MW, Chang HC, Chen PJ, Liu CJ, Liaw YF, Lin SM, Lee SD, Lin SC, Lin CL, Chen CJ. 2002. Increased risk for hepatitis B-related liver cirrhosis in relatives of patients with hepatocellular carcinoma in northern Taiwan. Int J Epidemiol 31:1008–1015.

Yu MW, Yeh SH, Chen PJ, Liaw YF, Lin CL, Liu CJ, Shih WL, Kao JH, Chen DS, Chen CJ. 2005. Hepatitis B virus genotype and DNA level and hepatocellular carcinoma: A prospective study in men. J Natl Cancer Inst 97:265–272.

Yuen MF, Yuan HJ, Wong DK, Yuen JC, Wong WM, Chan AO, Wong BC, Lai KC, Lai CL. 2005. Prognostic determinants for chronic hepatitis B in Asians: Therapeutic implications. Gut 54:1610–1614.

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Original Article

Evaluation for clinical utility of GPC3, measured by a commercially available ELISA kit with Glypican-3 (GPC3) antibody, as a serological and histological marker for hepatocellular carcinoma

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Aims: We evaluated the clinical utility of glypican-3 (GPC3), which has been proposed as a potential novel tumor marker for hepatocellular carcinoma (HCC), as a serological and histological marker for HCC.

Methods: The serum GPC3 level was compared between 200 patients with HCC and 200 patients with chronic liver disease (CLD). In addition, the expression of GPC3 was examined with immunohistochemistry on 38 resected specimens from patients with HCC. A commercially available GPC3 antibody was used for these analyses.

Results: The median values of serum GPC3 in patients with HCC and with CLD were 924.8 pg/mL and 1161.6 pg/mL, respectively. We found no elevation of serum GPC3 level in patients with HCC in comparison with those with CLD; rather the level was higher in patients with CLD ($P < 0.0001$). In immunohistochemical analysis, 14 of 38 (36.9%) HCC tissues were positive for GPC3, whereas no corresponding non-cancerous tissue was positive. The positivity for GPC3 tended to increase with pathologic decreased differentiation of HCC.

Conclusions: We did not find serum GPC3 level, measured by a commercially available ELISA kit with GPC3 antibody, to be useful in the diagnosis of HCC. However, we did observe increased GPC3 staining in HCC tissue with moderate or poor differentiation, suggesting that GPC3 is produced by HCC tumors. This lack of utility could have been due to the measuring procedure used in the present study. Further evaluation of GPC3 in HCC with other measuring procedures is needed.

Key words: ELISA, glypican-3, hepatocellular carcinoma, immunohistochemistry, tumor marker

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is one of the most prevalent malignancies worldwide. It is the sixth most common cancer, and the third most common cause of cancer-related death, in the world.[1] In Japan, HCC is the third most common cause of death from cancer in men, and the fifth most common in women.[2] The most important risk factor for the development of HCC is liver cirrhosis, regardless of etiology.[3] In addition, chronic infection with hepatitis viruses such as hepatitis B virus (HBV) and hepatitis C virus (HCV), as well as high alcohol intake, increase the risk of HCC.[4–7]

Alpha-fetoprotein (AFP),[8–11] Lens culinaris agglutinin-reactive fraction of alpha-fetoprotein (AFP-L3),[12–14] and des-gamma-carboxy prothrombin (DCP)[15–17] have been reported to be useful as serological tumor marker for HCC in cases of HCC surveillance and diagnosis, and in the evaluation of patient prognosis.[18] Nevertheless, all tumor markers have limitations and therefore the identification of additional tumor markers for HCC with high sensitivity and specificity is necessary.

Glypican-3 (GPC3) is a member of the glypican family of glycosyl-phosphatidylinositol-anchored cell-

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surface heparan sulfate proteoglycans.[19–21] It has been suggested that GPC3 might be a useful histological[22–24] and serological[25–27] marker for HCC. However, there has not been sufficient agreement on its clinical utility, and the relationship between the expression of GPC3 in tissue and GPC3 level in the serum of patients with HCC has not been fully characterized.

In the present study, we evaluate the clinical utility of GPC3 as a serological and histological marker for HCC, and compare histological results with serological ones. In addition, we compare the utility of GPC3 with other serological markers for HCC, such as AFP, AFP-L3, and DCP.

METHODS

Patients and controls

A TOTAL OF 434 consecutive patients with HCC visited the Department of Gastroenterology at Ogaki Municipal Hospital during the period from January 2000 to December 2004. Two hundred and three patients underwent hepatic resection or radiofrequency ablation (RFA) as treatment for HCC. Stored serum samples that had been obtained before the therapy were available for 200 of these 203 patients; these constituted the subjects of the present study. Written informed consent was obtained from all patients for the analyses of their serum or tissue samples.

Diagnosis of HCC was based on histologic examination of tumor tissue taken from resected specimens in 120 patients who underwent hepatectomy, 29 of the 80 patients (36.3%) treated by RFA were diagnosed with HCC based on specimens by fine-needle biopsy. The remaining 51 patients were diagnosed based on clinical criteria:[28,29] a pertinent clinical background (association with liver cirrhosis or viral hepatitis) and typical imaging findings. Typical imaging features of HCC include a mosaic pattern with a halo observed with B-mode ultrasonography; hypervascularity on angiographic images; and a high-density mass on arterial phase dynamic computed tomography (CT) images together with a low-density mass on portal phase dynamic CT images obtained with a helical or multidetector row CT scanner. When findings typical of HCC were not obtained by means of dynamic CT or angiography, CT during hepatic arteriography and CT during arterial portography or T1- and T2-weighted imaging associated with superparamagnetic iron oxide-enhanced magnetic resonance imaging (MRI) were performed.

Serum samples from 200 HCC patients were obtained at the diagnosis of HCC and before therapy. As controls, serum samples from patients with CLD but without HCC that had been obtained during the same period as the serum samples from HCC patients were selected. We selected samples from patients in whom the lack of HCC development had been confirmed by ultrasonography, CT or MRI at serum sampling and for 3 years after the date of sampling. This was to avoid the inclusion in the control group of patients with occult HCC that could not be detected by imaging modalities at the time of serum sampling. Among them, we made random selection and finally selected 200 samples as controls.

Measurement of GPC3, AFP, AFP-L3 and DCP

GPC3, AFP, AFP-L3, and DCP were measured from the same serum samples. GPC3 was measured using a commercially available ELISA kit (BioMosaics, Burlington VT) according to the manufacturer's instructions. Total AFP and percentage of AFP-L3 were measured by a liquid-phase binding assay with the Wako LiBASys Autoanalyzer (Wako Pure Chemical Industries, Osaka).[30,31] DCP level was determined by sensitive enzyme immunoassay (Eitest PIVKA-II kit; Eisai Laboratory, Tokyo) according to the manufacturer's instructions.[32]

Immunohistochemical staining

Immunohistochemical staining for GPC3 was performed on 38 resected HCC tissue specimens using a commercially available kit (BioMosaics) according to the manufacturer's instructions. Briefly, 4-μm sections from formalin-fixed, paraffin-embedded tissue blocks were deparaffinized, rehydrated and treated with 3% hydrogen peroxide for 15 min to inhibit endogenous peroxidase. Following water bath-based heat-induced epitope retrieval in 0.1 M citrate buffer at 95 °C centidegree and pH 6.0 for 40 min, slides were incubated with blocking solution for 20 min at room temperature. After blocking, slides were incubated with a mouse monoclonal antibody specific for GPC3 (1:200 dilution, clone 1G12; BioMosaics) for 6 hours at room temperature. After washing, detection was performed with biotin-free horseradish peroxidase-labeled polymers using the ChemMate EnVision System (Dako Real EnVision: Dako, Carpinteria CA). Staining was visualized using 3,3′-diaminobenzidine substrate-chromogen solution and a hematoxylin counterstain.

The intensity of staining was graded according to the percentage of the stained area and the intensity of staining as: 0, no staining or partial staining of cytoplasm in <25% of cells; 1+, weak/barely perceptible cytoplasm stain in >25% of cells; 2+, moderate stain of the complete cytoplasm in >25% of cells; or 3+, strong stain of

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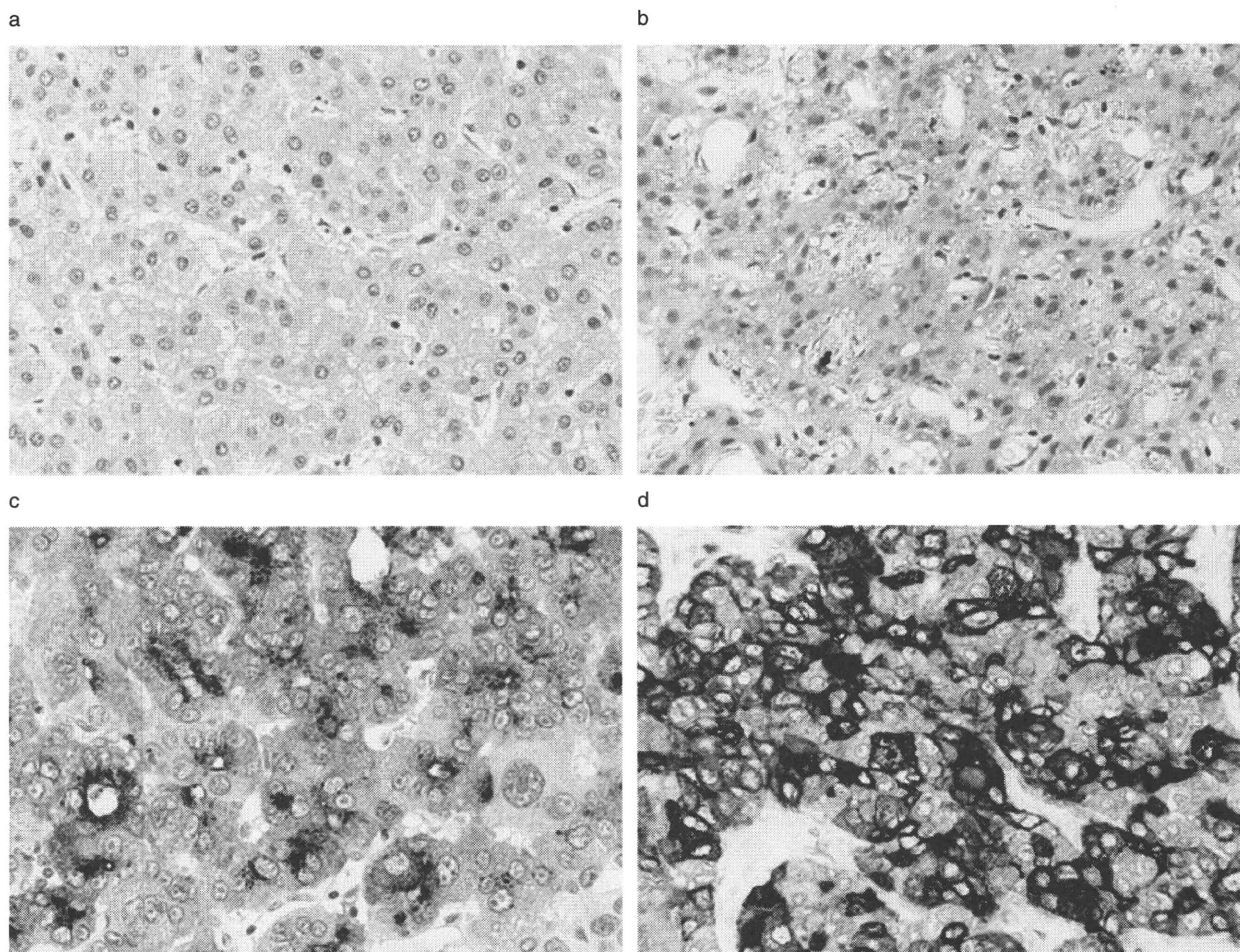

Figure 1 The degree of immunohistochemical staining for glypican-3. (a) No staining, (b) light staining, (c) moderate staining, (d) heavy staining.

the complete cytoplasm in >25% of cells (Fig. 1). HCC with 2+ or 3+ staining was considered to be positive for GPC3. Microscopic findings were evaluated by two authors independently, in comparison with negative and positive controls from the same immunohistochemistry series. Final evaluations of ambiguous cases (fewer than 20% of the samples) were made on a conference microscope with other authors.

Statistical analysis

Data are expressed as the mean ± SD or median and range. Differences in the proportions of patients between groups were analyzed by chi-square test. Differences in quantitative values were analyzed by Mann–Whitney *U*-test and Kruskal–Wallis test. All *P*-values were derived from two-tailed tests, and $P < 0.05$ was accepted as statistically significant. All analyses were performed using JMP6 statistical software (SAS Institute Japan, Tokyo).

RESULTS

THE DEMOGRAPHIC CHARACTERISTICS of the patients included in the analysis are summarized in Table 1. Patient with HCC comprised 153 males (76.5%) and 47 females (23.5%), with a mean age of 67.2 ± 8.5 years. Control patient comprised 112 males (56.0%) and 88 females (44.0%), with a mean age of 61.5 ± 11.8 years. The percentage of patients without cirrhosis, which was clinically evaluated according to typical US findings (e.g. superficial nodularity, a coarse parenchymal echo pattern, and signs of portal

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Table 1 Clinical characteristics of the study patients ($n = 400$)

	HCC patients ($n = 200$)	Control ($n = 200$)
Age (years)	67.2 ± 8.5	61.5 ± 11.8
Sex		
Male	153 (76.5)	112 (56.0)
Female	47 (23.5)	88 (44.0)
Etiology of underlying liver disease		
HBV	32 (16.0)	65 (32.5)
HCV	155 (77.5)	132 (66.0)
HBV + HCV	3 (1.5)	3 (1.5)
non-HBV, non-HCV	10 (5.0)	0
Patients without cirrhosis	81 (40.5)	141 (70.5)
Child–Pugh class (in patients with cirrhosis)		
A	86 (72.3)	36 (61.0)
B	33 (27.7)	18 (30.5)
C	0	5 (8.5)
Platelet count (/mm^3)	122 150 ± 57 830	176 830 ± 69 730
Alanine aminotransferase (IU/L)	58.8 ± 39.5	47.4 ± 56.6
Albumin (g/dL)	3.72 ± 0.50	3.87 ± 0.56
Total-bilirubin (mg/dL)	0.84 ± 0.94	0.85 ± 0.92

HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus.
Percentages are shown in parentheses.

Table 2 Characteristics of hepatocellular carcinoma ($n = 200$)

Size of largest tumor (cm)	2.76 ± 2.49
<2	99 (49.5)
≥2 to <3	88 (44.0)
≥3	13 (6.5)
Number of tumors	1.37 ± 1.00
Single	158 (79.0)
Multiple	42 (21.0)
Portal vein thrombosis	
Absent	192 (96.0)
Present	8 (4.0)
Tumor stage	
I	86 (43.0)
II	80 (40.0)
III	32 (16.0)
IV	2 (1.0)

hypertension – splenomegaly >120 mm, dilated portal vein diameter >12 mm, patent collateral veins, or ascites), was 27.5% of patients with HCC and 29.5% of control patients. The Child–Pugh class of patients with HCC was class A in 72.3% and class B in 27.7%. The characteristics and the progression of HCC tumor were summerized in Table 2. The percentage of patients at stages I, II, III, and IV were 43.0%, 40.0%, 16.0%, and 1.0%, respectively, according to the TNM Classification of Malignant Tumours of the Liver Cancer Study Group of Japan.[33]

Serum concentration of GPC3, AFP, AFP-L3, and DCP

Serum concentrations of GPC3, AFP, AFP-L3, and DCP are summarized in Table 3. The median GPC3 values

Table 3 Median and quartiles of serological markers for hepatocellular carcinoma ($n = 400$)

	HCC patients ($n = 200$)	Control ($n = 200$)	*P* value
Glypican-3 (pg/mL)	924.8 (495.2, 1335.6)	1161.6 (762.0, 1784.0)	<0.0001
Alpha-fetoprotein (ng/ml)	15.3 (6.3, 78.5)	4.0 (1.6, 7.3)	<0.0001
Lens culinaris agglutinin fraction of AFP	0.5 (0.0, 2.9)	0.0 (0.0, 0.0)	<0.0001
Des-gamma caroxy prothrombin (mAU/mL)	32.5 (18.0, 178.3)	21.0 (16.0, 27.0)	<0.0001

AFP, alpha-fetoprotein; HCC, hepatocellular carcinoma. Median (25%, 75% quarile) are shown.

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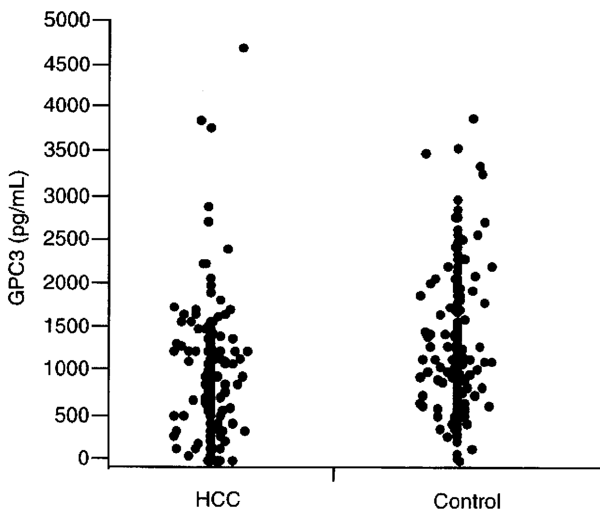

Figure 2 Serum glypican-3 (GPC3) level in patients with hepatocellular carcinoma (HCC) and in patients with chronic liver disease (CLD, control). Serum GPC3 level was higher in patients with CLD (1161.6 pg/mL) than those with HCC (924.8 pg/mL; $P < 0.0001$).

in patients with HCC and those with CLD were 924.8 pg/mL and 1161.6 pg/mL, respectively; patients with CLD showed significantly higher GPC3 concentration than those with HCC (Fig. 2). In contrast, serum concentrations of AFP, AFP-L3, and DCP in patients with HCC were significantly higher than those in patients with CLD (Fig. 3). We found no difference in serum GPC3 level according to the size of the maximal HCC tumor, the number of HCC tumors, or the stage of HCC in 200 patients with HCC (data not shown). Also, we found no difference according to the presence of cirrhosis in 200 control patients (data not shown).

The area under the receiver-operating curve (AUROC) was calculated to compare the clinical utilities of GPC3, AFP, AFP-L3 and DCP (Fig. 4). AUROC values for GPC3, AFP, AFP-L3 and DCP were 0.64, 0.80, 0.77, and 0.66, respectively. The AUROC value for GPC3 was significantly lower than those for AFP and AFP-L3 (both, $P < 0.05$). In addition, patients with HCC were identified by the decreased GPC3 under cut-off level in this ROC analysis; the serum value of GPC3 in patients with HCC was significantly lower than that in patients with

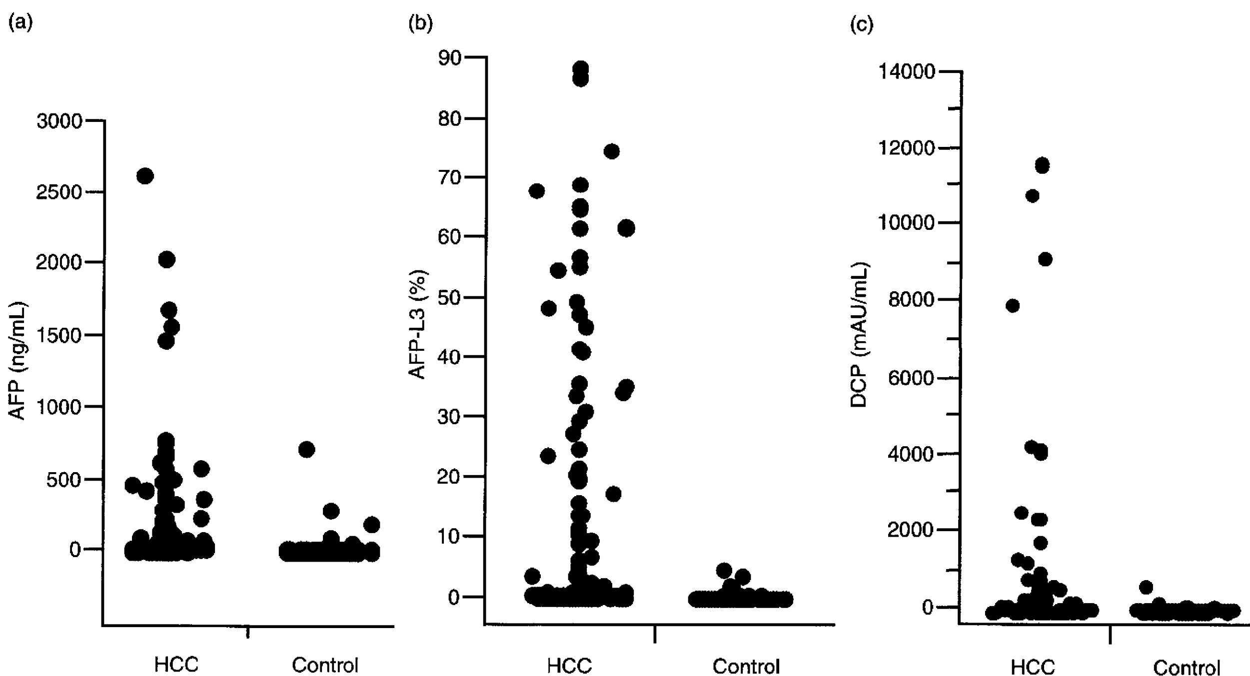

Figure 3 Serum alpha-fetoprotein (AFP), Lens culinaris agglutinin-reactive fraction of AFP (AFP-L3), and des-gamma carboxy prothrombin (DCP) levels in patients with hepatocellular carcinoma (HCC) and in patients with chronic liver disease (CLD, control). Serum AFP, AFP-L3, and DCP levels were significantly higher in patients with HCC (15.3 ng/mL vs. 4.0 ng/mL for AFP; 0.5% vs. 0.0% for AFP-L3; 32.5 mAU/mL vs. 21.0 mAU/mL for DCP; all $P < 0.0001$).

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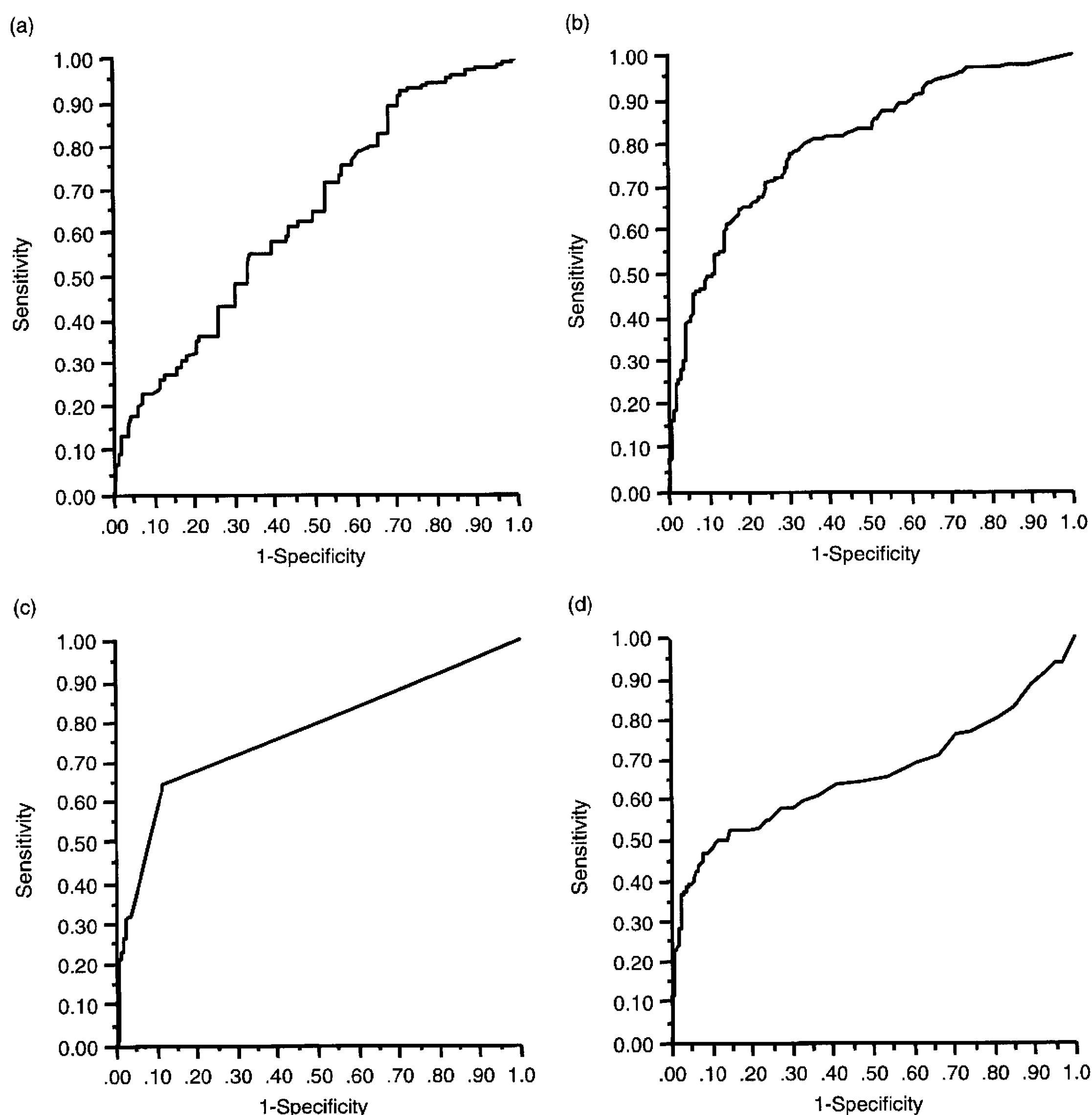

Figure 4 Area under the receiver-operating curve (AUROC) of (a) serum glypican-3 (GPC3), (b) alpha-fetoprotein (AFP), (c) Lens culinaris agglutinin-reactive fraction of AFP (AFP-L3), and (d) des-gamma carboxy prothrombin (DCP) for the diagnosis of hepatocellular carcinoma. AUROC was 0.64 for GPC3, 0.80 for AFP, 0.77 for AFP-L3, and 0.66 for DCP, respectively. AUROC was lowest for GPC3, significantly lower than both AFP and AFP-L3 (both, $P < 0.05$).

CLD. Serum GPC3 level for the diagnosis of HCC in the present analysis therefore was used inversely to the previous report.

GPC3 expression in HCC tissue

Thirty-eight resected liver tissues from patients with HCC were examined by immunohistochemistry for GPC3 expression. Table 4 shows the positivity of GPC3 staining in cancerous and non-cancerous parts of the resected liver tissue. The positivity of GPC3 staining in cancerous parts was 36.8% (14 cases), and that in non-cancerous parts was 0%. When light GPC3 staining was taken to be positive, these values increased to 81.6% (31 cases) and 23.7% (9 cases) for the cancerous and non-cancerous parts, respectively. We found no difference in serum GPC3 concentration according to the degree of staining for GPC3 by immunohistochemistry in these 38 patients (Fig. 5).

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Table 4 Immunohistochemical staining of cancerous and non-cancerous parts of hepatocellular carcinoma tissues for glypican-3 ($n = 38$)

	No staining	Light staining	Moderate staining	Heavy staining
Cancerous part	7 (18.4)	17 (44.7)	11 (29.0)	3 (7.9)
Non-cancerous part	29 (76.3)	9 (23.7)	0	0

Percentages are shown in parentheses.

Table 5 shows GPC3 expression in HCC tissue according to the differentiation of HCC. All poorly differentiated HCC showed GPC3 expression, and GPC3 immunoreactivity tended to increase with decreasing differentiation of HCC.

DISCUSSION

RECENT REPORTS HAVE shown significant elevation of GPC3 in the serum of patients with HCC, enabling early detection of HCC with high specificity.[25–27]

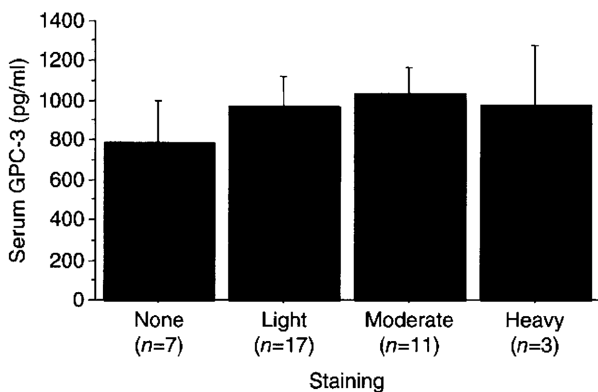

Figure 5 Serum glypican-3 (GPC3) level in 38 patients with hepatocellular carcinoma (HCC) who underwent hepatectomy according to the immunohistochemical staining of GPC3 on the resected HCC specimens. No association was found between serum GPC3 level and immunohistochemical staining of GPC3 on HCC tissues.

Therefore, in the present study we evaluated the usefulness of GPC3 for the diagnosis in comparison with the three standard tumor markers (AFP, AFP-L3, DCP). However, we observed that serum GPC3 concentration showed no increase in patients with HCC; rather, it was higher in patients without HCC. In addition, serum GPC3 did not correlate the stage of HCC, suggesting that the level did not reflect the progression of HCC tumor.

We also evaluated the expression of GPC3 in HCC tissue by immunohistochemistry, on the basis of reports that the clinical utility of GPC3 is higher when as a histological tumor marker.[22–25] In our study, the sensitivity of GPC3 in 38 HCC tissues was 36.8% when light staining was considered to be negative, whereas all noncancerous tissue was negative for GPC3. When light staining was included to be positive, sensitivity was 81.6% in HCC tissue and 23.7% in non-cancerous tissue. Most HCC specimens (13/14, 92.9%) with positive staining were moderately or poorly differentiated HCC. GPC3 staining tended to increase with decreasing differentiation, suggesting that GPC3 production might increase with the progression of HCC. In contrast to the report by Wang *et al.*[34], who suggested that GPC3 was useful in the differential diagnosis of liver cell adenomas and well-differentiated HCC, we found positive staining for GPC3 in only one of seven (14.3%) well-differentiated HCCs. Shirakawa *et al.* recently reported the low rate of staining of GPC3 in well-differentiated HCC in a larger study population.[35] Our results were in accordance with their report. The immunohistochemical staining, not serum level, of GPC3 might be an

Table 5 Association between differentiation and immunohistochemical staining for glypican-3 in hepatocellular carcinoma tissuses ($n = 38$)

	No staining ($n = 7$)	Weak staining ($n = 17$)	Moderate staining ($n = 11$)	Heavy staining ($n = 3$)
Well-differentiated ($n = 7$)	2 (28.6)	4 (57.1)	1 (14.3)	0
Moderately differentiated ($n = 27$)	5 (18.5)	13 (48.1)	7 (25.9)	2 (7.4)
Poorly differentiated ($n = 4$)	0	0	3 (75.0)	1 (25.0)

Percentages are shown in parentheses.

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indicator of the progression of HCC tumor and predictor of patient prognosis.[35]

GPC3 is a member of the heparan sulfate proteoglycans and its C-terminal region binds to the cell membrane via glycosilphosphatidylinositol anchors. Therefore, the existence of a soluble form of GPC3 is predicted, which would allow detection of GPC3 in the serum of HCC patients. The cleavage sites of GPC3 were between amino acids 358 and 359, and between amino acids 482 and 483. Hippo *et al.*[27] demonstrated that soluble GPC3 was present in the serum (51% of patients with HCC), and the antibody they used for the measurement of serum GPC3 was the NH_2-terminal portion of GPC3 cleaved at Arg358 (amino acids 25–358). Nakatsura *et al.*[26] reported the elevation of serum GPC3 in 40% of patients with HCC, and they used the antibody with amino acids 303–464. The commercially available kit (BioMosaics) used for the measurement of serum GPC3 in the present study uses the anti-GPC3 monoclonal antibody "clone 1G12" that recognizes the last 70 amino acids of the C-terminal of the core protein (amino acids 491–560).[25] This C-terminal region of GPC3 binds to the cell membrane and might not be released into the serum, although the original study by Capurro *et al.* reported the increase in serum GPC3 using the antibody clone 1G12′ in 53% of patients with HCC.[25] This could explain why we did not observe an increase in the level of soluble GPC3 between patients with HCC in comparison to those without it, or within patients with HCC according to the progression of HCC, despite the staining of GPC3 in many moderately or poorly differentiated HCC specimens. This discrepancy is the reason we found no clinical utility of serum GCP3 for the diagnosis of HCC in the present study. We might have observed an increase in serum GPC3 level in patients with HCC in case of the use of antibody other than monoclonal antibody clone 1G12, such as antibodies by Hippo *et al.*[27] or Nakatsura *et al.*,[26] which recognize another part of GPC3. A recent study by Beale *et al.*,[36] comparing AFP, AFP-L3%, DCP, GPC3 and SCCA-I between patients with HCC and those with cirrhosis, also did not find clinical utility for GPC3 in HCC detection, in agreement with the present study. According to a report by Capurro *et al.*,[37] however, the NH_2-terminal region and C-terminal region of GPC3 are linked despite the cleavage of GPC3 by convertase at Arg358, due to the presence of one or more disulfide bonds in the molecule. This would allow the "clone 1G12" antibody to detect GPC3 in the serum. It seems that further evaluation is needed for GPC3 as a serological marker of HCC, with the most important question being the form of the GPC3 protein in circulating blood.

In conclusion, we found no clinical utility of GPC3 as a serologic marker for detection of HCC in comparison to AFP, AFP-L3, and DCP. Further, high clinical utility of GPC3 as a histological marker was not observed in our study population, although we did observe an increase in GPC3 expression in HCC tissue in association with the progression of HCC. The lack of utility of the measurement of serum GPC3 may be due to the measuring procedure used in the present study. Further evaluation with other measuring procedures will be needed in the future; the clinical utility of GPC3 as a serological marker for HCC will remain unclear until further evaluation with other measuring procedures is undertaken. In addition, identification of a soluble form for GPC3, which could be useful as a serological marker for HCC, will require further study.

REFERENCES

1 Parkin D, Bray F, Ferlay J, Pisani P. Global Cancer Statistics, 2002. *CA Cancer J Clin* 2002; **55:** 74–108.
2 Umemura T, Kiyosawa K. Epidemiology of hepatocellular carcinoma in Japan. *Hepatol Res* 2007; **37:** S95–100.
3 Zaman SN, Melia WM, Johnson RD, Portmann BC, Johnson PJ, Williams R. Risk factors in development of hepatocellular carcinoma in cirrhosis: prospective study of 613 patients. *Lancet* 1985; **1:** 1357–60.
4 Poynard T, Aubert A, Lazizi Y *et al.* Independent risk factors for hepatocellular carcinoma in French drinkers. *Hepatology* 1991; **13:** 896–901.
5 Colombo M, de Franchis R, Ninno ED *et al.* Hepatocellular carcinoma in Italian patients with cirrhosis. *N Engl J Med* 1991; **325:** 675–80.
6 Tsukuma H, Hiyama T, Tanaka S *et al.* The factors for hepatocellular carcinoma among patients with chronic liver disease. *N Engl J Med* 1993; **328:** 1797–801.
7 Chevret S, Trinchet JC, Mathieu D, Rached AA, Beaugrand M, Chastang C. A new prognostic classification for predicting survival in patients with hepatocellular carcinoma. *J Hepatol* 1999; **31:** 133–41.
8 Abelev GI. Production of embryonal serum alpha-globulin by hepatomas: review of experimental and clinical data. *Cancer Res* 1968; **28:** 1344–50.
9 O'Connor GI, Tatarinov YS, Abelev GI, Uriel J. A collaborative study for the evaluation of a serologic test for primary liver cancer. *Cancer* 1970; **25:** 1091–8.
10 Di Bisceglie AM, Hoofnagle JH. Elevations in serum alpha-fetoprotein levels in patients with chronic hepatitis B. *Cancer* 1989; **64:** 2117–20.
11 Di Bisceglie AM, Sterling RK, Chung RT *et al.* Serum alpha-fetoprotein levels in patients with advanced hepatitis C: results from the HALT-C Trial. *J Hepatol* 2005; **43:** 434–41.

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12 Taketa K, Sekiya C, Namiki M *et al.* Lectin-reactive profiles of alpha-fetoprotein characterizing hepatocellular carcinoma and related conditions. *Gastroenterology* 1990; **99:** 508–18.
13 Taketa K, Endo Y, Sekiya C *et al.* A collaborative study for the evaluation of lectin-reactive a-fetoproteins in early detection of hepatocellular carcinoma. *Cancer Res* 1993; **53:** 5419–23.
14 Oka H, Saito A, Ito K *et al.* Multicenter prospective analysis of newly diagnosed hepatocellular carcinoma with respect to the percentage of Lens culinaris agglutinin-reactive a-fetoprotein. *J Gastroenterol Hepatol* 2001; **16:** 1378–83.
15 Liebman HA. Isolation and characterization of a hepatoma-associated abnormal (des-gamma carboxy) prothrombin. *Cancer Res* 1989; **49:** 6493–7.
16 Okuda H, Obata H, Nakanishi T, Furukawa R, Hashimoto E. Production of abnormal prothrombin (des-gamma-carboxy prothrombin) by hepatocellular carcinoma. A clinical and experimental study. *J Hepatol* 1987; **4:** 357–63.
17 Yano Y, Yamashita F, Kuwaki K *et al.* Clinical features of hepatitis C virus-related hepatocellular carcinoma and their association with alpha-fetoprotein and protein induced by vitamin K absence or antagonist-II. *Liver Int* 2006; **26:** 789–95.
18 Toyoda H, Kumada T, Kiriyama S *et al.* Prognostic significance of simultaneous measurement of three tumor markers in patients with hepatocellular carcinoma. *Clin Gastroenterol Hepatol* 2006; **4:** 111–17.
19 Filmus J, Church J, Buick R. Isolation of a cDNA corresponding to a developmentally regulated transcript in rat intestine. *Mol Cell Biol* 1988; **8:** 4243–9.
20 Filmus J, Selleck S. Glypicans: proteoglycans with a suprise. *J Clin Invest* 2001; **108:** 497–501.
21 Bernfield M, Gotte M, Park P *et al.* Function of cell surface heparan sulfate proteoglycans. *Annu Rev Biochem* 1999; **68:** 729–77.
22 Libbrecht L, Severi T, Cassiman D *et al.* Glypican-3 expression distinguishes small hepatocellular carcinomas from cirrhosis, dysplastic nodules, and focal nodular hyperplasia-like nodules. *Am J Surg Pathol* 2006; **30:** 1405–11.
23 Tommaso LD, Franchi G, Park Y *et al.* Diagnostic value of HSP70, glypican 3, and glutamine synthetase in hepatocellular nodules in cirrhosis. *Hepatology* 2007; **45:** 725–34.
24 Wang H, Anatelli F, Zhai Q, Adley B, Chuang S, Yang X. Glypican-3 as a useful diagnostic marker that distinguishes hepatocellular carcinoma from benign hepatocellular mass lesions. *Arch Pathol Lab Med* 2008; **132:** 1723–8.
25 Capurro M, Wanless I, Sherman M *et al.* Glypican-3: A novel serum and histochemical marker for hepatocellular carcinoma. *Gastroenterology* 2003; **125:** 89–97.
26 Nakatsura T, Yoshitake Y, Senju S *et al.* Glypican-3, overexpressed specifically in human hepatocellular carcinoma, is a novel tumor marker. *Biochem Biophys Res Commun* 2003; **306:** 16–25.
27 Hippo Y, Watanabe K, Watanabe A *et al.* Identification of soluble NH_2-terminal fragment of glypican-3 as a serological marker for early-stage hepatocellular carcinoma. *Cancer Res* 2004; **64:** 2418–23.
28 Torzilli G, Minagawa M, Takayama T *et al.* Accurate preoperative evaluation of liver mass lesions without fine-needle biopsy. *Hepatology* 1999; **30:** 889–93.
29 Kudo M. Imaging diagnosis of hepatocellular carcinoma and premalignant/ borderline lesions. *Semin Liver Dis* 1999; **19:** 297–309.
30 Katoh H, Nakamura K, Tanaka T, Satomura S, Matsuura S. Automatic and simultaneous analysis of Lens culinaris agglutinin-reactive alpha-fetoprotein ratio and total alpha-fetoprotein concentration. *Anal Chem* 1998; **70:** 2110–14.
31 Yamagata Y, Katoh H, Nakamura K, Tanaka T, Satomura S, Matsuura S. Determination of alpha-fetoprotein concentration based on liquid-phase binding assay using anion exchange chromatography and sulfated peptide introduced antibody. *J Immunol Methods* 1998; **212:** 161–8.
32 Okuda H, Nakanishi T, Takatsu K *et al.* Measurement of serum levels of des-gamma-carboxy prothrombin in patients with hepatocellular carcinoma by a revised enzyme immunoassay kit with increased sensitivity. *Cancer* 1999; **85:** 812–18.
33 Liver cancer Study Group of Japan. *The General Rules for the Clinical and Pathological Study of Primary Liver Cancer.* English edn. Tokyo: Kanehara & Co, 2003.
34 Wang X, Degos F, Dubois S *et al.* Glypican-3 expression in hepatocellular tumors: diagnostic value for preneoplastic lesions and hepatocellular carcinomas. *Hum Pathol* 2006; **37:** 1435–41.
35 Shirakawa H, Suzuki H, Shimomura M *et al.* Glypican-3 expression is correlated with poor prognosis in hepatocellular carcinoma. *Cancer Sci* 2009; **100:** 1403–7.
36 Beale G, Chattopadhyay D, Gray J *et al.* AFP, PIVKAII, GP3, SCCA-1 and follisatin as surveillance biomarkers for hepatocellular cancer in non-alcoholic and alcoholic fatty liver disease. *BMC Cancer* 2008; **8:** 200.
37 Capurro M, Filmus J. Glypican-3 as a serum marker for hepatocellular carcinoma. *Cancer Res* 2005; **65:** 372–3.

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ペプチドワクチン 国内で臨床試験の行われているペプチドワクチン療法 GPC3

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☞Point

- 肝細胞がん特異的ながん胎児性抗原としてGPC3を同定し、GPC3が肝細胞がんの腫瘍マーカーとしても有用であることも示した。
- 進行肝細胞がん患者を対象としたGPC3由来ペプチドワクチンの臨床第Ⅰ相試験の結果、免疫学的効果や臨床的な効果が認められた。無増悪期間中央値は4ヵ月、全生存期間中央値は9ヵ月であった。
- GPC3ペプチドワクチン3回投与で終了した後は末梢血中ペプチド特異的CTLの頻度は持続せず、4回目以降のワクチンは意味があると考えられた。
- 初回肝細胞がん根治的治療後の再発予防効果を検証する第Ⅱ相試験を実施中であり、腫瘍量が少なく免疫抑制のない患者での検証が行われている。卵巣明細胞腺がんを対象とした第Ⅱ相臨床試験も行われている。
- わが国でのがんワクチンの医師主導の臨床試験は、資金面、体制面とも不足しており、国家プロジェクトでしっかりとエビデンスを構築できるような支援が必要とされる。

がんの免疫療法の概念はすでに19世紀からあった。医師たちはがん患者が細菌に感染すると、がんが小さくなる場合があることに気づいていた。そこから生まれたColey's vaccine (toxin)は、時にはがんの完全退縮を得たが、広くは受け入れられなかった。また、がんにはまれではあるが、自然退縮が

起こる。これにはおそらく免疫も関与している。1967年ごろには、がん細胞の自家移植の報告がなされている。手術で得られたがん組織からがん細胞をばらばらにして、1万個、10万個、100万個、1,000万個、1億個とその患者の皮下に移植した結果、進行がんの患者でも1万個は完全に拒絶され、10万個では時に移植が成立し、1億個ではほとんど移植が成立することがわかった。これらの研究結果は、がんに対する免疫の確かな存在と、一方ではその限界も示しているといえよう。

1991年にBoonらにより、ヒトの免疫系ががんを異物として認識し、排除しうることに科学的な根拠が与えられた[1]ことによって、またがん免疫研究は勢いを盛り返し現在に至っている。現在までに、さまざまながん拒絶抗原およびペプチドが同定され、世界中で臨床試験が進められている。最近では、前立腺がんに対して「Provenge®」という樹状細胞療法がFDAに承認され話題になったが、その他にもいくつかの第Ⅲ相臨床試験での有効性も報告されている。日本国内でもさまざまな施設からがんに対するペプチドワクチンの有効例の報告が散見される。一方では、最近、子宮頸がんの予防ワクチンが話題であるが、免疫療法がより有効であるのは、がんの再発予防や予防であると考えられ、免疫療法を用いた根治治療後の再発予防法やがん発症予防法の開発も必要である。本稿では、われわれが研究しているglypican-3(GPC3)ペプチドを用いたワクチン療法について述べたいと思う。

がん特異的抗原glypican-3(GPC3)の同定

われわれは、東大医科研・ヒトゲノムセンターの中村祐輔教授(現国立がん研究センター研究所長)との共同研究により、cDNAマイクロアレイを利用した2万種類を超える遺伝子の肝細胞がんと正常組織における発現解析データを用いて、肝細胞がん特異的ながん胎児性抗原としてGPC3を同定した。GPC3遺伝子および蛋白質は、ほとんどの肝細胞がん組織ならびに細胞株で高発現するが、正常組織においては、胎生期の肝臓あるいは免疫学的に隔離された胎盤でしか発現がみられない。GPC3は肝細胞がんの腫瘍マーカーとしても有用であることも示した[2]。われわれは、GPC3が理想的な腫瘍拒絶抗原になりうるかどうかを検討した。日本人の約60％が陽性であるHLA-A24とBALB/cマウスのMHCクラスⅠ分子のK^dにはほぼ同じペプチドが結合することがわかっている。さらに、ヒトとマウスのGPC3では93％のホモロジーを認めることから、HLA-A24、K^dのいずれにも結合しうるGPC3由来のペプチドを合成した。これらをBALB/cマウスに免疫して解析し、K^d拘束性のHLA-A24結合性CTLエピトープペプチド(EYILSLEEL)を同定した[3]。同様にこれらをHLA-A24 transgenic mouse(Tgm)に免疫して解析した結果もやはり、同じCTLエピトープペプチド(EYILSLEEL)が同定された。このHLA-A24結合性GPC3由来ペプチド(EYILSLEEL)を用いて、ヒトのHLA-A24陽性の肝細胞がん患者の末梢血リンパ球を刺激することで、約半数からGPC3特異的CTLを誘導することができた[4]。GPC3特異的CTL株を、GPC3高発現ヒト肝細胞がん細胞株を移植したヌードマウスに移入して、その治療効果を証明した[4]。また、日本人の40％が陽性で、欧米白人のメジャータイプであるHLA-A2拘束性のCTLエピトープペプチドを同定するために、HLA-A2に結合しうるペプチドを合成し、これらをHLA-A2 Tgmに免疫して解析した。同定したHLA-A2結合性GPC3由来ペプチド(FVGEFFTDV)を用いてヒトのHLA-A2陽性の肝細胞がん患者の末梢血リンパ球を刺激することで、約半数からGPC3特異的CTLを誘導することができた[4]。また、非常に重要なことであるが、以上のいずれのマウスの実験においても、GPC3抗原の免疫によって、ペプチド特異的CTLが誘導され、抗腫瘍効果は認められたが、自己免疫現象は決して誘導されなかった[3,4]。

進行肝細胞がん患者を対象としたGPC3由来ペプチドワクチンの臨床第Ⅰ相試験の結果

平成19年2月、臨床第Ⅰ相試験をスタートし、平成21年11月に完了した。

1回の投与量を0.3、1、3、10、30mgの5段階とし、2週間おきに3回、左右の腋窩部、腹部および鼠径部の皮内に不完全フロイントアジュバント(incomplete Freund's adjuvant；IFA)とともに、安全性を確認しながら