Table 3 Univariate and multivariate analyses of variables associated with thrombocytopenia (PLT < 1.3 × 10^5/ul) in HD + HCV patients

Variables	Odds ratio	95% CI	*P* value
Univariate analysis			
Age (years)			
<60	1.0		
≥60	0.616	0.247–1.534	0.298
Sex			
Female	1.0		
Male	1.273	0.518–3.129	0.599
Duration of HD (years)			
<10	1.0		
≥10	1.321	0.555–3.141	0.529
Follow-up period (months)			
<55	1.0		
≥55	1.057	0.445–2.515	0.899
History of diabetes mellitus			
−	1.0		
+	1.426	0.557–3.646	0.459
Serotype			
I	1.0		
II	1.051	0.384–2.871	0.923
AST (IU/L)			
<30	1.0		
≥30	3.4	0.676–17.103	0.138
ALT (IU/L)			
<20	1.0		
≥20	2.686	1.083–6.662	0.033
GGT (IU/L)			
<50	1.0		
≥50	4.333	1.235–15.206	0.022
TC (mg/dl)			
<150	1.0		
≥150	0.727	0.27–1.958	0.528
Multivariate analysis			
ALT (IU/L)			
<20	1.0		
≥20	1.972	0.665–5.847	0.221
GGT (IU/L)			
<50	1.0		
≥50	3.305	0.876–12.467	0.078

Abbreviations as in Table 1

$P = 0.018$) by multivariate analysis using two variables including average ALT levels and GGT at baseline. The average ALT levels were also associated with decreased PLT (OR 4.470; 95% CI, 1.571–12.719; $P = 0.005$) by multivariate analysis using average ALT levels and sex. These results indicate that the clinical course of ALT levels is associated with thrombocytopenia and a decrease in PLT in patients with HCV.

Demographics of HD patients with HCV who were treated with UDCA

We enrolled 16 HD patients with HCV who were treated with 300 mg/day UDCA orally for more than 3 months in August 2008, and compared these patients (UDCA group) to 84 HD patients with HCV who were not treated with UDCA (non-UDCA group). The UDCA group and non-UDCA group showed similar demographics in regard to age, sex, HCV RNA levels, distribution of HCV serotype, GGT and PLT. The UDCA group, however, had a shorter duration of dialysis and higher AST and ALT levels just before UDCA administration compared to those in the non-UDCA group in May 2008 (Table 5).

Efficacy of UDCA in HD patients with HCV

After administering UDCA, percent of ALT and AST significantly decreased after one month and remained constant up to 6 months compared to the non-UDCA group (Fig. 2). Percent of GGT also significantly decreased after 2 months of UDCA treatment compared to the non-UDCA group. In addition, ALT, AST and GGT levels significantly decreased after UDCA treatment compared to levels before treatment, but PLT did not change during the 6 months of UDCA treatment (Fig. 2). In contrast, serum AST, ALT, GGT and PLT in the non-UDCA group did not change during the 6-month period from May 2008 to November 2008.

Discussion

Our study indicated that HD patients persistently infected with HCV are at risk for thrombocytopenia (less than 1.3 × 10^5/μL) and a decrease in PLT (more than 20%), although the exact dates of HCV infection were not clear in our study population. In addition, the basal or clinical course of ALT levels appears to predict decreased PLT or thrombocytopenia in patients with HCV. In this study population, the prevalence [243 anti-HCV positive among 2539 HD patients (9.6%)] and age distribution (average age was 63 years old) of anti-HCV antibody-positive subjects and the frequency of the HCV serotype I (74%) were similar to previous reports on HD patients with HCV in Japan [24–26], suggesting that the clinical course of anti-HCV-positive subjects in this study reflects those in Japan as a whole.

It is known that patients on HD often have thrombocytopenia [27], and there is a negative correlation between the dialysis period and PLTs [27, 28]. It was also reported that megakaryocytes are produced at lower levels in the bone marrow [28], platelets are destroyed due to the

Table 4 Baseline characteristics of four groups of HD patients with HCV according to the clinical course of average ALT levels

Average ALT	A, ALT < 15	B; 15 ≤ ALT < 20	C; 20 ≤ ALT < 30	D; 30 < ALT	*P* value
Number	30	19	18	17	
Age (years)	67.8 ± 10.8	60.8 ± 10.6	64.0 ± 9.7	63.1 ± 8.7	0.105
Sex male/female	11/19	15/4	14/4	14/3	0.001
Duration of dialysis (years)	14.4 ± 10.7	14.2 ± 9.2	12.8 ± 8.8	11.7 ± 9.1	0.945
Follow-up period (months)	53.2 ± 14.3	55.4 ± 16.4	64.2 ± 16.0	57.5 ± 16.3	0.290
HCV-RNA (Log IU/mL)	4.9 ± 1.6	4.8 ± 1.3	5.2 ± 1.2	4.8 ± 1.4	0.774
HCV Serotype (I/II/undetermined)	21/7/2	13/6/0	13/4/1	12/4/1	0.949
History of diabetes mellitus (-)/(+)	23/7	12/7	12/6	10/7	0.592
AST (IU/L)	15.0 ± 4.7	19.8 ± 8.6	22.8 ± 9.8	24.9 ± 8.0	<0.001
ALT (IU/L)	10.4 ± 4.1	19.3 ± 6.8	22.3 ± 8.0	27.8 ± 7.9	<0.001
GGT (IU/L)	21.3 ± 15.2	34.8 ± 22.1	81.2 ± 71.2	48.5 ± 35.2	<0.001
TC (mg/dl)	149.7 ± 31.4	152.3 ± 46.1	154.9 ± 37.0	161.2 ± 57.2	0.970
PLT ($\times 10^5/\mu l$)	1.62 ± 0.55	1.62 ± 0.61	1.46 ± 0.42	1.64 ± 0.51	0.764

Abbreviations as in Table 1

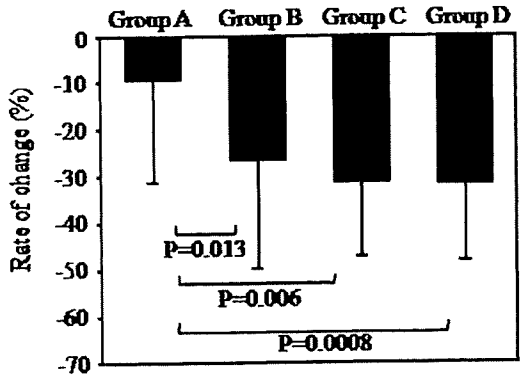

Fig. 1 Comparison of the rate of change in platelet counts by average alanine aminotransferase (ALT) levels during the follow-up period. Group A, average ALT < 15; Group B, 15 ≤ average ALT < 20; Group C, 20 ≤ average ALT < 30; Group D, 30 ≤ average ALT

Table 5 Demographics of HD patients with HCV who were treated with UDCA

	UDCA[a]	Non-UDCA[b]	*P* value
Number	16	84	
Age (years)	66.4 ± 8.6	69.2 ± 10.2	0.261
Sex male/female	9/7	54/30	0.743
Duration of dialysis (years)	6.5 ± 6.6	18.2 ± 9.9	<0.001
HCV-RNA	4.1 ± 2.6	4.9 ± 1.4	0.918
Serotype (I/II/undetermined)	12/4/0	59/21/4	0.669
AST (IU/L)	30.2 ± 24.2	19.2 ± 10.2	0.008
ALT (IU/L)	25.3 ± 16.9	17.1 ± 9.9	0.004
GGT (IU/L)	32.3 ± 23.4	41.4 ± 39.1	0.793
PLT ($\times 10^5/\mu l$)	1.55 ± 0.56	1.39 ± 0.56	0.577

Abbreviations as in Table 1

[a] Data was obtained at just before the treatment period

[b] Data was obtained in May 2008

appearance of the anti-platelet antibodies [28, 29] and uremic materials reduce the effects of hemopoietic cells [30]. In our study, PLT in HD patients without HCV was significantly decreased after 62.2 months (−5.3%). However, PLT decreased even more dramatically in HD patients with HCV after 56.7 months (−22.4%) compared to patients without HCV In addition, persistent HCV infection was independently associated with thrombocytopenia and a decrease in PLT in HD patients by a multivariate analysis, but dialysis period was not associated with those. Although the data regarding liver histology and serum markers of hepatic fibrosis were lacking in our study, it has also been reported that severe hepatic fibrosis is associated with thrombocytopenia in HCV carriers with end-stage renal disease [19]. These results suggest that thrombocytopenia is more associated with HCV viremia than with the HD procedure or dialysis period in HD patients.

Hepatocellular carcinoma (HCC) and hepatic failure are critical complications in HCV patients, even in those undergoing HD [10, 31]. These complications occur more frequently in patients with advanced hepatic fibrosis [32, 33]. It has been reported that hepatic fibrosis can be predicted by thrombocytopenia in chronic hepatitis C patients with or without HD [19, 34]. In addition, hepatitis is usually assessed by ALT levels, and changes in ALT levels have been shown to be the most important factor that affects hepatic fibrosis in chronic hepatitis C patients without HD [11, 12]. In this study, we showed that basal ALT levels are associated with thrombocytopenia by a univariate analysis and with decreased PLT by a multivariate analysis. The clinical

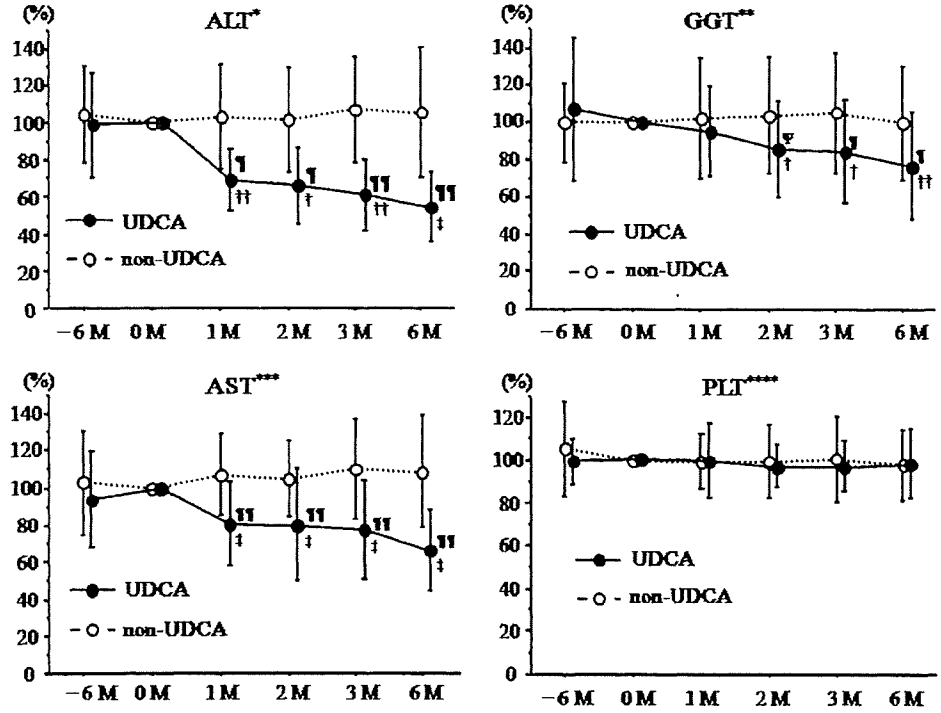

Fig. 2 Efficacy of ursodeoxycholic acid (UDCA) in hemodialysis (HD) patients with hepatitis C virus. Percent of ALT, AST, GGT and PLT in the UDCA group ($n = 16$) 6 months (−6 M) or just (0 M) before and during the treatment period [1, 2, 3 or 6 months (M)] compared to patients in the non-UDCA group ($n = 84$ excluding 6 M) in December 2007 (−6 M), May 2008 (0 M), June (1 M), July (2 M), August (3 M) or November 2008 (6 M, $n = 82$; two patients died before November 2008). Data are expressed as mean ± standard deviation. Closed (*black*) and open circles indicate the UDCA group and non-UDCA group, respectively. The percent of ALT was calculated according to the formula: %ALT = (ALT[−6 M, 0 M, 1 M, 2 M, 3 M or 6 M]/ALT[0 M]) × 100. *ALT* alanine aminotransferase, *AST* asparate aminotransferase, *GGT* gamma-glutamyl transpeptidase, *PLT* platelet count. $^{\Psi}P < 0.05$ (UDCA vs. non-UDCA). $^{\P}P < 0.01$ (UDCA vs. non-UDCA), $^{\P\P}P < 0.001$ (UDCA vs. non-UDCA), $^{\dagger}P < 0.05$ (vs. 0 M), $^{\dagger\dagger}P < 0.01$ (vs. 0 M), $^{\ddagger}P < 0.001$ (vs. 0 M)

course of ALT is also associated with these clinical changes. These results indicate that ALT is an important predictor of thrombocytopenia which should be associated with hepatic fibrosis in HD patients with HCV. In contrast, serum ALT levels are significantly lower in chronic hepatitis C patients on HD than in chronic hepatitis C patients with normal renal function [19]. It was reported that a vitamin B6 deficiency [35], uremic toxins [36], or ultraviolet-absorbing materials [37] are associated with low ALT levels in HD patients. Furthermore, ALT levels have been reported to predict liver disease-related deaths in HD patients, even when ALT levels are in the normal range [38, 39]. Our study also revealed that both patients with abnormal ALT levels (Group D) and normal ALT levels close to the ULN (Groups B and C) had a significant decrease in PLT compared to patients with low ALT levels (Group A). These findings suggest that ALT levels can be used to assess liver damage in HD patients with HCV, although the normal range of ALT should be determined in those patients with HCV in a large cohort study or by liver biopsy.

HCV carriers with persistently normal ALT (PNALT) are more often females than chronic hepatitis C patients with abnormal ALT [40]. This distinction is likely due to lifestyle differences such as alcohol consumption [40], hormonal factors [41] or lower serum iron levels [42]. Although the normal range of ALT in HD patients with HCV may be different compared to the range in HCV carriers with normal renal function, our study demonstrated that females are more likely to have lower ALT levels, even in HD patients (Table 4). This difference in sex may also affect the decrease in PLT. In contrast, the frequency of serotype II, which is reportedly higher in PNALT patients than in chronic hepatitis C patients with abnormal ALT [43], was not different between the four groups in this study (Table 4). A further analysis of the factors associated with elevated ALT levels in those patients with HCV is required.

Interferon therapy has been shown to improve hepatic fibrosis [44] and to reduce the occurrence of HCC in chronic hepatitis C patients with normal renal function. Compared to untreated patients, the risk of HCC after interferon treatment in patients who did not achieve a virological response was shown to be 0.20, 0.36 and 0.91 in chronic hepatitis C patients whose ALT levels were

normal, moderately elevated (less than twice the upper normal limit) and highly elevated, respectively [45]. These results indicate that ALT might predict the mortality of patients with liver-related diseases who have or have not received interferon treatment. Although lower serum ALT levels decreased the risk of HCC, biochemical and virological responses were limited [20, 46] and HD was one of the factors associated with patients who did not respond to interferon and ribavirin treatment [21, 22]. Other therapies that lower serum ALT levels but do not involve interferon-based treatment need to be investigated. Recently, it has been established that UDCA up to 900 mg/day dose-dependently improves biochemical indices such as serum ALT, GGT and bilirubin [23]. Although UDCA seems to lower serum ALT levels, the risk of liver fibrosis, and possibly the incidence of hepatocellular carcinoma, liver histology, serum hepatic fibrosis markers and prognosis (including the incidence of HCC) should also be evaluated over a long time period in HCV carriers with or without HD.

Our study had several limitations; a small number of patients was simply treated with UDCA as routine care, selection of patients depended on each physician and then the data collected retrospectively after a specified duration of therapy. However, this study showed that UDCA effectively had reduced serum ALT, AST and GGT levels in HD patients with HCV Interestingly, UDCA decreased ALT levels even in patients with normal ALT levels less than 30 IU/L (data not shown). Therefore, HCV patients with normal ALT levels should also be considered for the indication of treatment.

Although the patients in this study were treated with 300 mg/day UDCA, it has also been reported that a 600 mg/day dose of UDCA more effectively decreases ALT and AST levels than a 150 mg/day dose in chronic hepatitis C patients with normal renal function [23]. In addition, PLT did not change during UDCA treatment. Future studies need to investigate the dose-dependent effects of UDCA on ALT levels and prospective double-blind UDCA treatment over a long period in HD patients with HCV.

In conclusion, HCV viremia and ALT levels at basal conditions and during the clinical course of disease were associated with thrombocytopenia and decreased PLT in HD patients. We recommend that HCV carriers on HD who have ALT levels greater than 15 IU/mL be considered for treatment. In addition, UDCA should be considered for HD patients who have chronic hepatitis due to HCV infection but cannot receive interferon-based therapy.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

The complement component C3a fragment is a potential biomarker for hepatitis C virus-related hepatocellular carcinoma

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Abstract
Background Hepatocellular carcinoma (HCC) has a high mortality rate, and early detection of HCC improves patient survival. However, the molecular diagnostic markers for early HCC have not been fully elucidated. The aim of this study was to identify novel diagnostic markers for HCC.
Methods Serum protein profiles of 45 hepatitis C virus infection (HCV)-related HCC patients (HCV-HCC) were compared to 42 HCV-related chronic liver disease patients without HCC (HCV-CLD) and 21 healthy volunteers using the ProteinChip SELDI system. One of the identified proteins was evaluated as a diagnostic marker for HCC in patients with HCV
Results Five protein peaks (4067, 4470, 7564, 7929, and 8130 m/z) had p-values less than 1×10^{-7} and were significantly increased in the sera of HCV-HCC patients compared to HCV-CLD patients and healthy volunteers. Among these proteins, an 8130 m/z peak was the most differentially expressed and identified as the complement component 3a (C3a) fragment. For HCV-HCC and HCV-CLD, the relative intensity of this C3a fragment had the best area under the ROC curve [0.70], followed by des-γ-carboxy prothrombin (DCP) [0.68], lectin-bound alpha fetoprotein (AFP-L3) [0.58] and AFP [0.53] for HCC. A combined analysis of the C3a fragment, AFP and DCP led to a 98% positive identification rate. In addition, the measurable C3a fragment in some HCC patients was not only significantly higher in the year of HCC onset compared to the pre-onset year, but also decreased after treatment.
Conclusions The 8130 m/z C3a fragment is a potential marker for the early detection of HCV-related HCC.

Keywords Hepatocellular carcinoma · Complement component C3a · Serum proteomics · Serum biomarkers · Proteinchip SELDI system · Hepatitis C virus

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Introduction

Hepatocellular carcinoma (HCC) is reportedly the third most frequent cause of global cancer-related deaths, and the incidence of HCC is increasing worldwide [1, 2]. The clearly established risk factor for HCC is chronic hepatitis C virus (HCV) infection [3].

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To date, both ultrasonography and serum tumor markers such as the alpha fetoprotein (AFP), and des-γ-carboxy prothrombin (DCP) assay are the principle methods for screening and detecting HCC. Routine screening is the best method to detect early HCC and improve patient survival; however, elevated serum AFP and DCP levels have insufficient sensitivity and specificity, respectively. The sensitivity and specificity of serum elevated AFP levels were reported to range from 39–64% and 76–91%, while those of the serum elevated DCP levels were 41–77% and 72–98%, respectively [4–9]. In addition, it was recently reported that only a small percentage of small HCC tumors were diagnosed based on AFP and DCP [6, 10]. The lens culinaris agglutinin-reactive fraction of AFP (lectin-bound AFP or AFP-L3) has been reported to be elevated in the serum of HCC patients. Although AFP-L3 has a high range of specificity for detecting HCC, the sensitivity is low [11, 12]. The ability to detect early HCC, prior to the onset of clinical symptoms, leads to curative treatment and significantly improves the disease prognosis. Thus, additional biochemical markers are necessary for the specific detection of early HCC.

Serum profiling using a proteomic approach is thought to be a useful technique to detect or predict early HCC in chronic liver disease patients. Studies using the ProteinChip SELDI system, which is a powerful tool to discover new biomarkers, have shown that this method may be successfully used to diagnose HCC. Zinkin et al. [13], Schwegler et al. [14] and our research group [15] previously detected early HCC using the profile of several protein peaks that were identified by the ProteinChip SELDI system. Paradis et al. [16] reported the highest discriminating peak (8900 Da), which was identified as the V10 fragment of vitronectin. Furthermore, Lee et al. [17] described complement 3a, which had a molecular weight of approximately 8900 Da, as a novel marker of HCC. Therefore, using this proteomic approach to identify specific proteins may not only help establish simple methods to detect HCC, but also further our understanding of the molecular mechanisms of hepatocarcinogenesis and facilitate the development of novel cancer therapies. Therefore, this study assessed and compared the protein expression profiles in the sera of HCC patients in order to identify a more useful biomarker of HCC-associated HCV infection using proteomic approach.

Materials and methods

Samples

Eighty-seven patients [45 HCC patients and 42 patients with chronic liver diseases without HCC (CLD)] with HCV infection were selected to participate in this study (Table 1). These patients provided informed consent. Serum samples were collected by the Faculty of Medicine, University of Miyazaki (Miyazaki, Japan), and some patients were in a hyperendemic HCV area with a cohort study in Miyazaki [18]. The sera of all patients with and without HCC, which was confirmed by abdominal ultrasonography or computed tomography, were obtained prior to treatment. All of the sera samples from HCV-infected patients were analyzed in a previous study [15]. In addition, sera from 10 HCV-HCC patients who were diagnosed with HCC within 1 or 2 years and sera from five patients who had received radiofrequency ablation (RFA), percutaneous ethanol injection therapy (PEIT) and/or transarterial chemoembolization (TACE) for HCC were collected through a cohort study in Miyazaki. We also analyzed the sera of 21 healthy volunteers without HCC as controls. After freezing and thawing once, all samples were separated into 50–100 μl aliquots and refrozen at −80°C. The study protocol was approved by the Ethics Committee of the Faculty of Medicine, University of Miyazaki, Kagoshima University Graduate School of Medical and Dental Sciences, and Harvard School of Public Health and Boston University School of Public Health.

Table 1 Patient characteristics

	HCC[a]	CLD[b]	*p* value
Patients (male/female)	45 (40/5)	42 (40/2)	–
Age	73.6 [63–85]	61.8 [41–83]	<0.0001
PLT[c] (×10⁴/ul)	12.5 ± 5.8	8.4 ± 4.6	0.001
Albumin (g/dl)	3.8 ± 0.8	4.2 ± 1.6	0.8
ALT[d] (IU/l)	57.7 ± 28.3	52.8 ± 37.5	0.7
AFP[e] (ng/ml)	311 ± 1144	51.6 ± 36.1 (38)	0.008
DCP[f] (mAU/ml)	235 ± 605 (44)	37.1 ± 59.8 (39)	<0.0001
HA[g] (ng/ml)	388 ± 446 (40)	280 ± 272 (27)	0.6
Diameter of HCC (mm)	23.2 [10–40]	–	–
TNM stage[h] (I/II/III/IV)	24/18/3/0	–	–

Data are shown as the means ± SD or means [range] (numbers)

[a] Hepatocellular carcinoma

[b] Chronic liver disease

[c] Platelet counts

[d] Alanine aminotransferase

[e] Alpha fetoprotein

[f] Des-γ-carboxy prothrombin

[g] Hyaluronic acid

[h] TNM; primary tumor/lymph node/distant metastasis

SELDI-TOF/MS analysis of sera

Expression difference mapping analysis profiles of the samples were obtained using weak cation-exchange (CM10) ProteinChip Arrays (Bio-Rad Laboratories). Arrays were analyzed by ProteinChip reader as previously reported [15]. In addition, the laser intensity ranged from 220 to 245, with a detector sensitivity of 8, and spectra ranging from 1300 to 150000 m/z were selected for analysis in this study.

Separation of candidate biomarker (8.1 k m/z)

The purification strategy was determined by the ProteinChip Arrays. Two hundred microliters of sera from HCV-HCC patients were diluted 5-fold into 50 mM Na-phosphate buffer, pH 7.0, and loaded onto a CM-Ceramic HyperD F spin column (Bio-Rad Laboratories). After equilibrating with the same buffer, the samples were eluted with a stepwise sodium chloride gradient from 0, 200, 300, and 1000 mM. The elution was desalinated and concentrated using a centrifugal concentrator (VIVA-SPIN, Vivascience, Hannover, Germany), and the purification progress was monitored using NP20 arrays. The flow-through fraction was dialyzed and then separated by 16.5% tricine one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The SDS–PAGE samples were run in tricine sodium dodecyl sulfate buffer according to the manufacturer's instructions and then stained with Coomassie brilliant blue (CBB).

Identification of the candidate biomarker (8.1 k m/z)

Gel pieces containing the target 8.1 k m/z protein were excised. The excised bands were reduced and alkylated for 30 min at room temperature, and then digested with trypsin (Modified Sequence Grade, Roche Diagnostics, Basel, Switzerland) in Tris–HCl, pH 8.0, for 20 h at 35°. The reaction solution was applied to NP20 arrays and allowed to air dry. To identify the protein, the digested peptides were purified by high-performance liquid chromatography (HPLC; MAGIC 2002; Michrom Bioresources Inc., Auburn, CA) and analyzed by Q-Tof2 (Micromass; Waters Ltd., Hertsfordshire, UK). The HPLC solvent consisted of solvent A (2% acetonitlile/0.1% formic acid) and B (90% acetonitlile/0.1% formic acid). The digested peptides were separated with a linear gradient from 10 to 50% solvent B with a flow rate of 400 nl/min using HPLC [19]. Mass spectral data were searched with Mascot (http://www.matrixscience.com) to identify proteins based on the peptide mass [20, 21].

Immunodepletion assay

For immunodepletion, serum samples were prepared as follows. Sera (250 µl) from HCC patients were diluted 5-fold in 50 mM Tris–HCl buffer, pH 8.0, and loaded onto a CM-Sepharose Fast Flow spin column (GE Healthcare Bio-Sciences Corp., NJ). After equilibration with the same buffer, the samples were eluted with a stepwise sodium chloride gradient from 0, 500, and 1000 mM. The elution from each NaCl concentration was monitored using NP20 arrays. To prepare the antibodies for immunodepletion, 6 µl anti-human C3 antibody, which detected C3 and C3a expression, or anti-C4a antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with 20 µl Interaction Discovery Mapping (IDM) affinity beads (Bio-Rad Laboratories) and Protein A (Sigma Chemical Co, St. Louis, MO) over night at 4° with shaking. These beads were centrifuged, and the supernatant was discarded. The beads were washed with 50 mM phosphate buffer (pH 7.0), and 3 µl of the prepared serum sample was incubated with 15 µl IDM affinity beads with shaking for 2 h at 4°. As a negative control, 3 µl sample was incubated with IDM affinity beads and Protein A with an anti-C4a antibody or without antibody. After the incubation, the samples were cleared by centrifugation, and 5 µl of each supernatant was analyzed on NP20 ProteinChip arrays in a PBS II reader.

Cell culture and SELDI-TOF/MS analysis of culture supernatants

The human hepatocarcinoma cell line HuH-7 and human hepatoblastoma cell line HepG2 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin G, and 100 mg/ml streptomycin sulfate (Invitrogen, Carlsbad, CA). Before starting the experiments, the cells were cultured on 96-well microplates in medium without FBS for 24 h. After washing with FBS-free media, the cells were cultured for 24 h with FBS-free media with or without 500 µg/ml of C3a (Calbiochem, San Diego, CA). The supernatants were collected by centrifugation and analyzed for the expression of 8.1 k m/z using the ProteinChip system.

Statistical analysis

Values are shown as the means ± SD. Statistical differences, including laboratory data and individual peaks in SELDI TOF/MS, were determined using the Mann–Whitney U test. Values of $p < 0.05$ were considered statistically significant. The discriminatory power for each putative marker was described via receiver operating characteristics

(ROC) area under the curve (AUC). These statistical analyses were performed using STATVIEW 4.5 software (Abacus Concepts, Berkeley, CA), SPSS software (SPSS Inc., Chicago, IL), JMP software, or Ciphergen ProteinChip Software, version 3.0.2.

Results

Profiling sera from HCC patients and healthy controls

We analyzed the sera of all patients with HCV-HCC or HCV-CLD and healthy controls without HCC using the CM10 ProteinChip array to identity the most differential protein peak. Peaks were automatically detected using the Ciphergen ProteinChip Software 3.0.2. following baseline subtraction as described previously [15, 22]. This analysis identified 178 protein peak clusters, as seen in the spectrum representations from the three groups (HCV-HCC, HCV-CLD, and healthy control) in the 3000- to 15000-m/z range. Peak expressions were increased for 18 proteins and decreased for 14 proteins in sera from HCV-HCC patients compared to HCV-CLD patients. Compared to healthy subjects, 68 protein peaks were increased, and 16 protein peak intensities were decreased in the sera of HCV-HCC patients. Five protein peaks (4067, 4470, 7564, 7929, and 8130 m/z) had a p-value less than 1×10^{-7} and were significantly increased in the sera of HCC patients compared to the sera of HCV-CLD patients and healthy volunteers. In particular, an 8130 m/z peak was the most significantly different peak and had the most differential expression profile between patients with HCV-HCC and with HCV-CLD.

Purification and identification of the 8.1 k m/z peak

We optimized the adsorption and desorption conditions on the arrays using an HCV-HCC patient serum sample and healthy volunteer serum sample in order to determine a procedure to purify the target 8.1 k m/z protein. The optimal pH for retention of the 8.1 k m/z protein was a *pI* value of approximately 7.0 on the CM10 arrays, which indicates that weak cation-exchange sorbents and buffer pH should be fixed for further experiments. The target protein was eluted by increasing the sodium chloride concentrations in a Na-phosphate buffer and was eluted in the 1000 mM sodium chloride fraction. The concentrated serum protein that was eluted with 1000 mM sodium chloride was applied to SDS–PAGE for further separation. The 8.1 k m/z protein was identified and excised by in-gel trypsin digestion for identification. The peptide sequences were analyzed using liquid chromatography (LC)-MS/MS and then examined by a database search with Mascot. The digested peptides matched human complement C3a (Fig. 1).

After reacting the HCC sera with anti-complement C3a or anti-C4 antibodies or without antibody, the supernatants were analyzed by the SELDI ProteinChip system for immunodepletion. Analysis of the supernatant showed that only the 8.1 k m/z peak corresponding to complement C3a

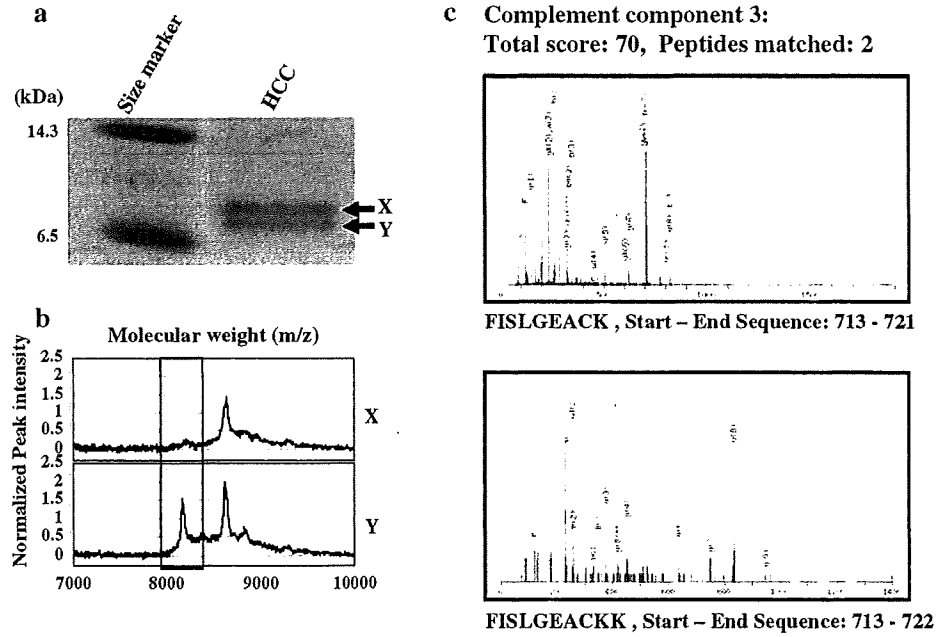

Fig. 1 **a** Partially purified proteins were separated by SDS–PAGE using serum samples from HCV-HCC patients. The Coomassie-stained SDS-PAGE gel shows two clear bands at approximately 8 kDa (*X* and *Y*). **b** After each band (*X* and *Y*) was excised from the gel, the proteins were extracted and analyzed using the ProteinChip system. The target protein in the excised band was detected, and the 8.1 k m/z peak corresponded only to the "*Y*" band contained in gel. **c** The excised "*Y*" band was alkylated and digested using trypsin. The peptides were collected and subjected to LC–MS/MS analysis. The proteins, which were derived from complement C3a, were identified using a database search

was reduced. On the other hand, immunodepletion with a control anti-C4 antibody or without antibody did not reduce the 8.1 k m/z peak (Fig. 2).

Profiling the C3a of sera from patients with HCC and without HCC

The 8.1 k m/z peak was confirmed as the complement C3a fragment using an immunodepletion assay. However, C3a was stabilized as C3adesArg with a molecular weight of approximately 8.9 k m/z. Figure 3a, b compares the expression of the 8.1 k m/z peak in the sera of HCV-HCC or HCV-CLD patients and healthy controls. The intensities

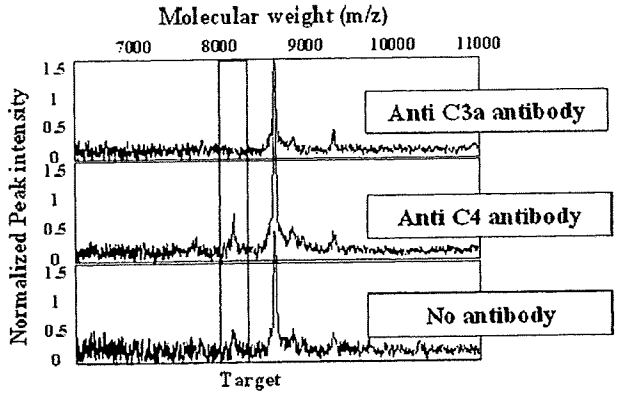

Fig. 2 Immunodepletion assay of the C3a fragment. Analysis of supernatant that had been immunodepleted with an anti-C3a antibody showed that only the 8.1 k m/z peak corresponding to complement C3a was reduced. Supernatants that had been immunodepleted with either a control anti-C4 antibody or without antibody did not have reduced 8.1 k m/z peaks by the ProteinChip system

in HCC patient sera were significantly higher than those in the HCV-CLD patients or healthy controls. The expression of the 8.9 k m/z peak in HCV-HCC patients was also higher than that in HCV-CLD patients or healthy controls (Fig. 3c, d). Although the 8.9 k m/z peak was not identified as C3adesArg, it is possible that both the 8.1 and 8.9 k m/z peaks were specific tumor markers for HCC. Furthermore, we analyzed sera from 10 HCV-HCC patients who were diagnosed with HCC within 1 or 2 years and sera from five patients who had received curative treatments using RFA, PEIT, and TACE for HCC. The 8.1 k m/z C3a fragment in the HCV-HCC patients was significantly increased in the year of disease onset compared to the pre-onset year. After treatment, expression of the C3a fragment significantly decreased in all five of the patients who had measurable samples after treatment (Fig. 4a). In contrast, the 8.9 k m/z peak did not change regardless of the occurrence of HCC over time (Fig. 4b). Thus, the 8.1 k m/z C3a fragment appears to be the most discriminatory tumor marker for HCV-HCC.

Relationship between the C3a fragment and other tumor markers

AFP and DCP levels were measured in sera from 83 of 87 patients with HCV-associated liver disease. The recommended cutoff levels for these tumor markers, AFP and DCP, are 20 ng/ml and 40 mAU/ml, respectively. AFP-L3 in 26 patients with HCV-associated liver disease was also investigated among measurable samples in which AFP in a total 35 patients was higher than 20 ng/ml. The cutoff level of AFP-L3 was set at 10%. When samples from patients

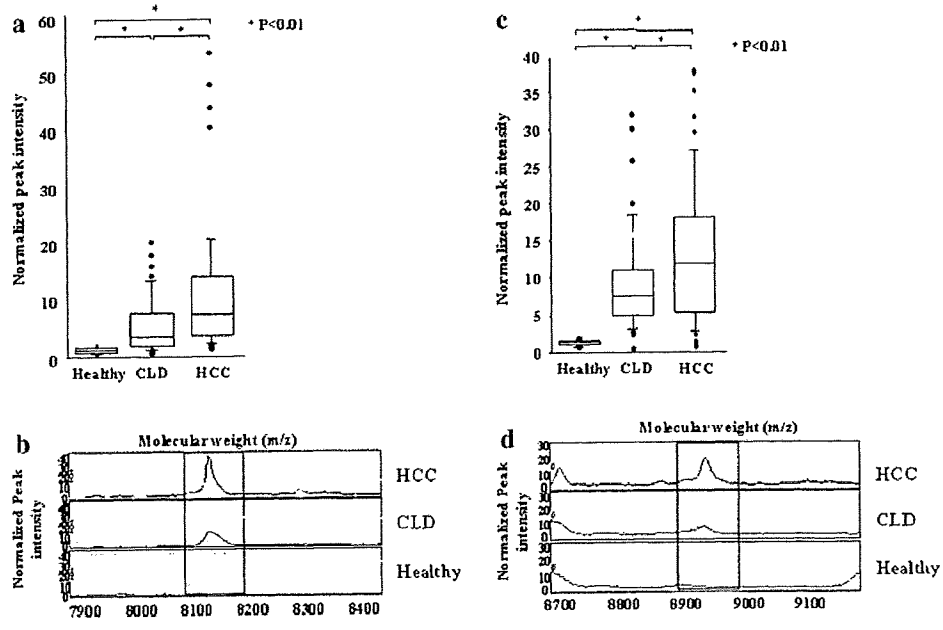

Fig. 3 **a** and **c** Comparisons of the expression profiles of the 8.1 and 8.9 k m/z peaks in HCV-HCC, HCV-CLD, and healthy sera. *Boxes* indicate the median ± 25th percentile. The *lower* and *upper bars* represent the 10th and 90th percentiles, respectively. **b** and **d** Representative spectra of the 8.1 and 8.9 k m/z peaks from patients in each group. The *horizontal axis* indicates the protein molecular weight, while the *vertical axis* designates the relative intensity

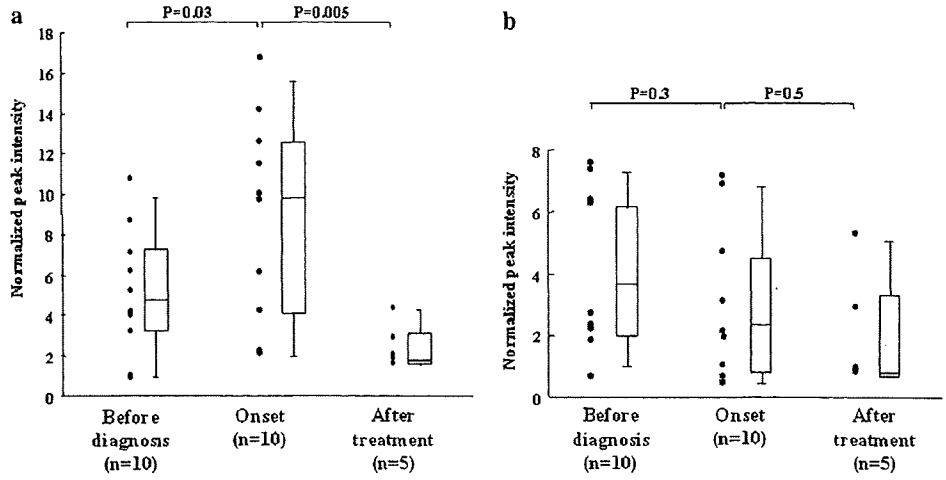

Fig. 4 Comparisons of the expression profiles of the 8.1 k m/z (**a**) and 8.9 k m/z (**b**) peaks in sera from HCV-HCC patients before diagnosis, during disease onset, and after treatment. The samples in the before diagnosis group included sera collected 1 or 2 years before the onset of HCC. *Boxes* indicate the median ± 25th percentile, the *lower bar* indicates the 10th percentile and the *upper bar* indicates the 90th percentile

Table 2 Diagnostic rates for hepatocellular carcinoma in the HCV infected patients

Markers	Sensitivity (%)	Specificity (%)	ROC AUC
AFP[a] (>20 ng/ml)	38 (17/45)	47 (18/38)	0.53
DCP[b] (>40 mAU/ml)	45 (20/44)	74 (29/39)	0.68
AFP-L3[c] (>10%)	58 (8/14)	50 (6/12)	0.58
C3a fragment (>3.5)	78 (37/45)	52 (22/42)	0.70
C3a fragment + AFP	91 (41/45)	26 (10/38)	0.72
C3a fragment + DCP	93 (41/44)	33 (13/39)	0.77
AFP + DCP	64 (28/44)	34 (12/35)	0.70
C3a fragment + AFP + DCP	98 (43/44)	20 (7/35)	0.80

[a] Alpha fetoprotein

[b] Des-γ-carboxy prothrombin

[c] Alpha fetoprotein, lectin lens culinaris agglutin-bound fraction

with HCV-HCC and HCV-CLD without HCC were compared, the sensitivity and specificity of AFP were 38 and 47%, whereas those of DCP were 45 and 74% and those of AFP-L3 were 58 and 50%, respectively. When the cutoff level for the relative intensity of the C3a fragment was set at 3.5, the sensitivity and specificity were 78 and 52%, respectively; the C3a fragment had the most sensitivity for the diagnosis of HCC. Furthermore, the ROC AUC of the C3a fragment, AFP, DCP, and AFP-L3 was 0.70, 0.53, 0.68, and 0.58, respectively (Table 2). There was no relationship between the C3a fragment and several other tumor and inflammation markers [AFP, DCP, AFP-L3, alanine aminotransferase (ALT), and high-sensitivity C-reactive protein (hs-CRP)], and each of these markers was independent of the diameter and number of tumors. The ROC AUC using AFP and DCP was highly similar to the ROC AUC with the C3a fragment alone. In addition, we investigated a combination assay that included the C3a fragment, AFP and DCP. This combination test, in which at least AFP, DCP, or the C3a fragment was positive, had a positive identification rate of 98%, although the specificity of this assay was too low at 20%. The ROC AUC of the combination test using AFP, DCP, and the C3a fragment was higher than those of any other markers. This result indicates that this combination assay using three markers is more useful than the combination assay using AFP ± DCP, which are measured worldwide to detect HCC (Table 2).

Profiling C3a expression in culture medium

C3a reacted with HCC cell lines, and the C3a peak in the culture medium was monitored by the ProteinChip system. The C3a fragment (approximately 8.1 k m/z) was not detected in the supernatants of HuH-7 and HepG2 cell cultures. However, the 8.9 k m/z peak was detected in the culture medium. This 8.9 k m/z peak was considered to be a stabilized form of C3a. This result indicated that the stabilized form of C3a (8.9 k m/z) was not undergoing proteasome-mediated degradation to yield the C3a fragment (8.1 k m/z) in these HCC cell lines.

Discussion

Because the HCC disease-associated mortality rate remains high, it is highly important to develop early diagnostic tools and treatments for HCC. Our study indicates that an 8.1 k m/z peak, which was identified as the C3a fragment by both peptide sequencing and an immunoassay, is up-regulated in the serum of HCC patients, 93% (42/45) of whom were TNM stage I or II. The C3a fragment in some HCC cases was also significantly higher in the year of HCC onset compared to the pre-onset year and decreased after curative treatment. Therefore, the C3a fragment appears to

be a promising simple tumor marker for the diagnosis of early HCC. In addition, a combination serum HCC diagnostic test that included AFP, DCP, and the C3a fragment had higher sensitivity than each individual marker. These results suggest that this combination test may be a useful HCC screening method, although the low specificity may pose challenges. Further examinations are needed to determine whether the C3a fragment or a combination test can be used to detect early HCC.

The results of our study demonstrated that the C3a fragment (8.1 k m/z) is a highly expressed novel tumor marker that is abundant in the sera of early HCC patients but not in the sera of healthy volunteers or HCV-CLD patients. A similar study by Lee et al. [17] used the ProteinChip SELDI system to show that C3a is a potential candidate biomarker for HCV-HCC. However, Lee et al. found that the molecular weight of C3a was represented by an approximately 8.9 k m/z peak. C3a has a very short half-life and is immediately cleaved into the more stable C3adesArg (8.9 k m/z), which is the anaphylatoxin C3a that lacks the C-terminal arginine and is stable state in the serum [23]. In our study, the 8.9 k m/z peak was also significantly different among HCV-HCC patients, HCV-CLD patients, and healthy volunteers (Fig. 3c, d). However, the discriminatory power of the 8.9 k m/z peak (ROC AUC was 0.60) was lower than the 8.1 k m/z peak (ROC AUC was 0.70) to distinguish between HCV-HCC and HCV-CLD. In addition, unlike the 8.1 k m/z peak, the levels of the 8.9 k m/z peak did not significantly increase with time as HCC progressed in 10 HCV-HCC cases (Fig. 4b). In contrast, Li et al. identified two proteins (8926 m/z and 8116 m/z) as complement component C3adesArg and a C-terminal truncated form of C3adesArg; the latter was a C-terminal truncation of C3adesArg that lacked the C-terminal sequence RASHLGLA (referred to as C3adesArgΔ8) in breast cancer patients [24]. However, these two biomarkers cannot be used to discriminate between breast cancers and benign tumors, and there were minimal differences in the peak intensities between breast cancer patients and healthy controls. Therefore, the C3a fragment with a molecular weight of 8.1 k m/z appears to be a potential diagnostic marker for HCC, although we cannot explain why the 8.1 k m/z fragment of C3a is overexpressed in HCC patients and did not confirm whether our C3a fragment (8.1 k m/z) is C3adesArgΔ8.

C3a, including C3adesArg, was also previously identified as a tumor marker for lymphoid malignancies, breast and colorectal cancers using the ProteinChip SELDI system [24–26]. Complement activation and subsequent deposition of complement components on tumor tissues has been demonstrated in cancer patients [27]. Malignant ovarian cells isolated from ascitic fluid samples had C3 activation products deposited on their cell surface [28]. Complement components are important mediators of inflammation and help regulate the immune response. C3a is biologically active and binds to mast cells and basophils, triggering the release of their vasoactive contents [29]. We investigated C3a expression by immunochemical examination of HCC tissues and Western blot analysis of proteins extracted from human HCC cell lines, including HepG2 and HuH-7 However, specific C3a expression, including the C3a fragment (8.1 k m/z), was not detected.

The complement system can be activated after exposure to tumor antigens [30]. It is speculated that small tumors can trigger a systematic reaction. Therefore, elevated C3a (8.9 k m/z) levels in the serum of HCV-HCC patients may reflect both a systematic immune response to HCV infection and non-specific tumor antigens rather than a specific immune response to HCC [24–26, 31]. In contrast, it is possible that overexpression of the C3a fragment (8.1 k m/z) is specific for HCC in addition to non-specific C3 activation.

In contrast to our results, Steel et al. [32] searched for HCC biomarkers using HCC-associated HBV-infected patient sera and found that the C-terminal fragment of complement C3 was down-regulated. Kawakami et al. [33] searched for characteristic alterations in the sera of HBV- and HCV-HCC-infected patients who had undergone curative radiofrequency ablation treatment and showed that C3 was up-regulated after treatment. In these studies, C3 was separated and identified using 2-DE of a mixture of proteins from a small number of patient sera samples, and this process identified various molecular weights for C3. In addition, we analyzed the sera of 25 patients with HCC-associated HBV infections, and the profile of several proteins was different between HCV- and HBV-infected patients. Although 35 protein peaks, including the C3a fragment, were overexpressed in the sera of both HCV-HCC and HBV-HCC patients compared to sera from healthy volunteers, the C3a fragment (8.1 k m/z) was particularly overexpressed in the sera of HCV-HCC patients and was not significantly different between HBV-HCC patients and HCV-CLD patients without HCC (data not shown). The biologic and pathogenic activities of HCV and HBV are different, and the molecular mechanisms underlying the development of hepatitis and hepatocarcinogenesis may differ between HBV and HCV infections [34–36]. Although the number of samples, cause of liver disease, and method of protein identification may affect these results, we speculate that the C3a fragment with a molecular weight of 8.1 k m/z is a candidate tumor marker for HCV-HCC but not HBV-HCC.

AFP, which is a commonly used HCC tumor marker, is elevated not only during HCC, but also during hepatocyte regeneration following liver damage. Previous reports revealed that AFP was abnormally elevated in the sera of patients with acute hepatitis, chronic hepatitis, and liver

cirrhosis. This lack of specificity for HCC means that AFP has a comparatively high false-positive rate [37]. The C3a fragment may also be elevated during hepatocyte regeneration following liver damage [38], and early diagnosis of small HCC tumors may be difficult with one marker alone. Therefore, the false-positive rates for HCC must be carefully considered [39–41]. Also, a combination of markers, including AFP, DCP, and the C3a fragment, in the serum should be verified to improve the diagnostic rate.

The ProteinChip SELDI system can separate and partially characterize multiple proteins in tissue and serum samples. Our previous report used a panel of proteins to diagnose early HCC with the ProteinChip SELDI system [15]. This panel diagnosis of seven protein peaks included a discriminant peak of 4060 m/z. This 4060 m/z peak may be a double-charged 8130 m/z peak, although the C3a fragment (8130 m/z) was not used to develop this diagnostic method. These results suggest that the C3a fragment is a useful HCC biomarker, regardless of whether this fragment carries a single or double charge. In addition, the panel diagnosis method is more useful than measuring the C3a fragment alone to diagnose and predict the occurrence of HCC. However, this method must be performed using the ProteinChip SELDI system, which is expensive and does not detect putative interactions between various proteins. Identifying a specific HCC protein such as the C3a fragment will also further our understanding of the molecular mechanisms of hepatocarcinogenesis. Therefore, the C3a fragment should not only be considered a simple HCC tumor marker, but should also be evaluated for its contribution to HCC carcinogenesis.

In conclusions, serum profiling with the ProteinChip SELDI system may be used to distinguish HCC from chronic liver disease without HCC and to detect early HCC in HCV-infected patients. Because we identified the C3a fragment (8.1 k m/z) in serum samples from HCC patients, the C3a fragment is a promising marker that can be used to screen for HCV-HCC and to develop new therapeutic targets.

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Combined therapy of transcatheter hepatic arterial embolization with intratumoral dendritic cell infusion for hepatocellular carcinoma: clinical safety

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Summary

The curative treatments for hepatocellular carcinoma (HCC), including surgical resection and radiofrequency ablation (RFA), do not prevent tumour recurrence effectively. Dendritic cell (DC)-based immunotherapies are believed to contribute to the eradication of the residual and recurrent tumour cells. The current study was designed to assess the safety and bioactivity of DC infusion into tumour tissues following transcatheter hepatic arterial embolization (TAE) for patients with cirrhosis and HCC. Peripheral blood mononuclear cells (PBMCs) were differentiated into phenotypically confirmed DCs. Ten patients were administered autologous DCs through an arterial catheter during TAE treatment. Shortly thereafter, some HCC nodules were treated additionally to achieve the curative local therapeutic effects. There was no clinical or serological evidence of adverse events, including hepatic failure or autoimmune responses in any patients, in addition to those due to TAE. Following the infusion of ^{111}Indium-labelled DCs, DCs were detectable inside and around the HCC nodules for up to 17 days, and were associated with lymphocyte and monocyte infiltration. Interestingly, T lymphocyte responses were induced against peptides derived from the tumour antigens, Her-2/neu, MRP3, hTERT and AFP, 4 weeks after the infusion in some patients. The cumulative survival rates were not significantly changed by this strategy. These results demonstrate that transcatheter arterial DC infusion into tumour tissues following TAE treatment is feasible and safe for patients with cirrhosis and HCC. Furthermore, the antigen-non-specific, immature DC infusion may induce immune responses to unprimed tumour antigens, providing a plausible strategy to enhance tumour immunity.

Keywords: clinical safety, dendritic cells, hepatocellular carcinoma, immunotherapy, transcatheter hepatic arterial embolization

Introduction

Hepatocellular carcinoma (HCC) occurs primarily in individuals with cirrhosis related to either hepatitis C virus (HCV) or hepatitis B virus (HBV) infections [1–3]. The curative treatments for HCC, including surgical resection and percutaneous radiofrequency ablation (RFA), do not prevent tumour recurrence efficiently because active hepatitis and cirrhosis in the surrounding nontumour liver tissues exhibit high carcinogenetic potentials to develop *de novo* HCC [4–7]. In addition, their reduced hepatic reserve due to cirrhosis decreases the tolerance to these local treatments and reduces drug metabolism, including that of anti-cancer agents, and therefore limits their usefulness. Among many novel strategies targeting HCC recurrence, immune-based therapies are believed to enhance the sensitivity, specificity and self-regulation of the immune system to find and eradicate tumour cells wherever they reside [8].

Dendritic cells (DCs) are the most potent type of antigen-presenting cells in the human body, and are involved in the regulation of both innate and adaptive immune responses [9–11]. During DC development, immature DCs exhibit the unique ability to take up and process antigens in the peripheral blood and tissues [12,13]. Subsequently, they migrate to draining lymph nodes, where they must mature to fully activated DCs to present the antigens to resting lymphocytes and elicit T cell responses [14–16]. During the maturation processes, they express high levels of cell-surface major

histocompatibility complex (MHC) antigen complexes and co-stimulatory molecules [17]. So far, most of the DC-based immunotherapies have been performed using intravenous (i.v.), intradermal (i.d.) and subcutaneous (s.c.) routes and lymph node injection following the predictive tumour antigen stimulation *ex vivo* [18,19]. Yet the clinical efficacy remained controversial because consistent tumour destruction or extended life span has not been observed in most treated cancer patients [20–22]. Accordingly, antigen stimulation may not be suitable for cancer treatments, or the proper tumour antigens may not be present to be taken up by DCs after the infusion.

In addition, DCs are reported to induce immune responses to target antigens by a cross-priming mechanism that is greatly enhanced when the target cells are apoptotic [23,24]. Apoptosis of tumour cells is induced by the standard treatments for HCC, i.e. transcatheter hepatic arterial embolization (TAE), percutaneous ethanol injection (PEI), RFA and intra-arterial chemotherapy [25,26]. Importantly, we have observed recently that immune responses specific for tumour antigens and peptides were enhanced during the course of the therapies, while anti-tumour responses were not enough to prevent HCC recurrence [27].

Based on these observations, we suggested a novel DC-based therapy in which immature DCs were injected through an arterial catheter into apoptotic tumour tissues following TAE. Immature DCs were delivered to HCC tumour tissues, by which we hypothesized that the physiological maturation steps including antigen ingestion, migration and presentation might proceed within the patient's body. In the current study, clinical safety was evaluated in patients with HCV-related cirrhosis complicated with HCC. The results suggest that immature DCs were infused successfully and safely to tumour tissues, and immune responses were induced to the tumour antigen peptides with human leucocyte antigen (HLA)-A24 binding motif, which are shared by most Asian individuals.

Patients and methods

Patients

Inclusion criteria were a radiological diagnosis of primary HCC by CT angiography, HCV-related HCC, a Karnofsky score of $\geq$ 70%, an age of $\geq$ 20 years, informed consent, the following normal baseline haematological parameters (within 1 week before DC administration): haemoglobin $\geq$ 8·5 g/dl; white cell count $\geq$ 2000/ml; platelet count $\geq$ 50 000/ml; creatinine < 1·5 mg/dl, and liver damage A or B [28].

Exclusion criteria included severe cardiac, renal, pulmonary, haematological or other systemic disease associated with a discontinuation risk; human immunodeficiency virus (HIV) infection; prior history of other malignancies; history of surgery, chemotherapy or radiation therapy within 4 weeks; immunological disorders including splenectomy and radiation to the spleen; corticosteroid or anti-histamine therapy; current lactation; pregnancy; history of organ transplantation; or difficulty in follow-up.

There were 10 patients enrolled in the study (one woman and nine men), with an age range of 45–79 years (Table 1). Patients with verified radiological diagnoses of HCC stage II or more were eligible and enrolled in this study. Similarly, a group of 11 patients treated with TAE without DC administration was also enrolled in this study as a control. The Institutional Review Board reviewed and approved the study protocol. This study complied with ethical standards outlined in the Declaration of Helsinki. Adverse events were monitored for 1 month after the DC infusion in terms of fever, vomiting, abdominal pain, encephalopathy, myalgia, ascites, gastrointestinal disorder, bleeding, hepatic abscess and autoimmune diseases.

Preparation and injection of autologous DCs

DCs were generated from blood monocyte precursors as reported previously [29,30]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation in Lymphoprep™ Tubes (Nycomed, Roskilde, Denmark). For generating DCs, PBMCs were plated in six-well tissue culture dishes (Costar, Cambridge, MA, USA) at $1{\cdot}4 \times 10^7$ cells in 2-ml per well and allowed to adhere to plastic for 2 h. Adherent cells were cultured in RPMI-1640 supplemented with 1% heat-inactivated autologous plasma, 100 U/ml penicillin G (GMP grade; Meiji, Tokyo, Japan), 100 μg/ml streptomycin sulphate (GMP grade; Meiji), 1000 U/ml recombinant human interleukin (IL)-4 (GMP grade; Cell Genix, Freiburg, Germany) and 100 ng/ml recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF) (GMP grade; Novartis, Basel, Switzerland) for 7 days. In the selected cases, the cells were pulsed with 10 μg/ml keyhole limpet haemocyanin (KLH) [depyrogenated, lipopolysaccharide (LPS) free; Calbiochem-Novabiochem Corp., San Diego, CA, USA] overnight 1 day before injection. On day 7, the cells were harvested for injection, 5×10^6 cells were reconstituted in 5 ml normal saline containing 1% autologous plasma, mixed with absorbable gelatin sponge (Gelfoam; Pharmacia & Upjohn, Peapack, NJ, USA) and infused through an arterial catheter following Lipiodol (iodized oil) (Lipiodol Ultrafluide, Laboratoire Guerbet, Aulnay-Sous-Bois, France) injection during selective TAE therapy. Release criteria for DCs were viability > 80%, purity > 30%, negative Gram stain and endotoxin polymerase chain reaction (PCR) and negative in process cultures from samples sent 48 h before release. All products met all release criteria, and the DCs had a typical phenotype of $CD14^-$ and $HLA\text{-}DR^+$.

Flow cytometry analysis

The DC preparation was assessed by staining with the following monoclonal antibodies (MoAb) for 30 min on ice:

Table 1. Patient characteristcs and treatments.

Patient no.	Gender	Age (years)	HLA	TNM stages	No. of tumours	Largest tumour (mm)	Child-Pugh	KPS	Post-TAE Rx	Image complete Rx
1	M	45	A2 A11	IVB	Multiple	50	A	100	No	Complete
2	M	60	A26 A33	II	3	15	A	100	RFA	Incomplete
3	F	70	A3 A24	II	2	15	A	100	RFA	Complete
4	M	77	A2 A24	II	3	10	A	100	RFA	Complete
5	M	73	A24	III	Multiple	70	B	80	Chemo	Incomplete
6	M	62	A24 A26	III	Multiple	32	A	100	RFA	Complete
7	M	67	A11 A24	III	Multiple	35	A	100	Chemo	Incomplete
8	M	60	A2 A24	II	2	40	B	100	MCT	Complete
9	M	79	A2 A33	III	5	60	A	100	RFA	Complete
10	M	76	A11 A24	II	1	45	A	100	Ope	Complete
11	M	71	n.d.	II	1	35	B	100	No	Complete
12	M	68	A24	II	4	20	A	100	RFA	Complete
13	F	66	A2 A24	IVA	3	30	A	100	No	Complete
14	F	68	A11 A33	IVA	Multiple	50	B	100	Chemo	Incomplete
15	F	74	n.d.	II	2	20	B	100	RFA	Complete
16	F	67	A2 A24	III	3	25	A	100	RFA	Incomplete
17	F	70	A2 A11	I	1	20	B	100	No	Complete
18	M	59	n.d.	II	3	20	A	100	RFA	Complete
19	M	68	n.d.	III	Multiple	25	B	100	RFA	Complete
20	M	70	A2 A26	II	5	15	B	100	No	Complete
21	M	80	A2 A24	III	3	38	B	100	No	Complete

Chemo: chemotherapy; Child-Pugh: Child-Pugh classification; HCC: hepatocellular carcinoma; KPS: Karnovsky performance scores; n.d.: not determined; Ope: partial hepatectomy; RFS: percutaneous radiofrequency ablation; Rx: treatment; TAE: transcatheter arterial embolization; TNM: tumour-node metastasis.

anti-CD14-allophycocyanin (APC) (MÖP9), anti-HLA-DR-fluorescein isothiocyanate (FITC) (L243), anti-CD11c-APC (S-HCL-3), anti-CD123-phycoerythrin (PE) (9F5) (BD PharMingen, San Diego, CA, USA), anti-CD80-PE (MAB104), anti-CD83-PE (HB15a) and anti-CD86-PE (HA5·2B7) (Beckman Coulter, Fullerton, CA, USA). Cells were analysed on a FACSCalibur™ flow cytometer. Data analysis was performed with CELLQuest™ software (Becton Dickinson, San Jose, CA, USA).

^{111}Indium oxinate labeling and autoradiography

DCs were labelled with ^{111}Indium (In) oxinate at a specific activity of 32·5 μCi/10^6 cells according to the protocols supplied by the manufacturer (Nihon Medi-Physics, Hyogo, Japan). Scintigraphic images of the depot were acquired with a gamma camera 6, 24, 48 h and 7 days after injection. In one case, the treated HCC nodule was accidentally resected surgically 17 days after DC infusion. Autoradiography was conducted and analysed on a BAS 1000 image analyser (Fuji Photo Film, Tokyo, Japan).

Immunohistochemical analysis

The liver tissues were fixed in buffered zinc formalin (Anatech Ltd, Battle Creek, MI, USA), embedded in paraffin, sectioned (at 3 μm), and stained with haematoxylin and eosin. The paraffin sections were deparaffinized, treated in a pressure cooker for 1–40 min, and incubated with mouse anti-human CD1a (MTB1; 1 : 20 diluted), CD4 (1F6; 1 : 20), CD8 (1A5; 1 : 20), CD20 (7D1; 1 : 100), CD56 (CD564; 1 : 50), CD83 (1H4b; 1 : 20) (Novocastra, Newcastle, UK), CD14 (7; 1 : 20) or HLA-DR (LN3; 1 : 100) (LabVision, Fremont, CA, USA) antibody overnight at 4°C. The cells were then visualized using a Vectastain ABC Standard Kit (Vector Laboratories, Burlingame, CA, USA), and the tissue sections were counterstained with haematoxylin before mounting.

Interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assay

The prevalence of activated, tumour antigen peptide-specific PBMCs was determined by IFN-γ ELISPOT analysis (Mabtech, Nacka, Sweden). Briefly, 96-well mixed cellulose ester membrane-backed plates (MAHA S4510; Millipore, Bedford, MA, USA) were coated with 100 μl of an anti-IFN-γ MoAb 1-D1K (15 μg/ml; Mabtech) overnight at 4°C. Peptides were added directly to the wells at a final concentration of 10 μg/ml. PBMCs were added to the wells at 3×10^5 cells/well. The plates were incubated at 37°C, 5% CO_2 overnight (14–16 h) and then processed as described [31,32]. ELISPOT assays were conducted in duplicate wells. IFN-γ producing cells were counted by direct visualization and are expressed

as mean number of spots per 3×10^5 cells. The number of specific spots was determined by subtracting the number of spots in the absence of antigen. Responses were considered positive if more than 10 specific spots were detected and if the number of spots in the presence of antigen was at least twofold greater than the number of spots in the absence of antigen. The negative controls consisted of incubation of PBMCs with a peptide representing an HLA-A24 restricted epitope derived from HIV envelope protein ($HIVenv_{584}$) [33] and were always < five spots per 3×10^5 cells. The positive controls consisted of 10 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO, USA) and 500 ng/ml ionomycin (Sigma) or a cytomegalovirus (CMV) pp65-derived peptide ($CMVpp65_{328}$) [34].

HLA-A24 restricted peptide epitopes used in this study, AFP_{403} (KYIQESQAL), AFP_{424} (EYYLQNAFL), AFP_{434} (AYTKKAPQL), AFP_{357} (EYSRRHPQL) [27], $hTERT_{1088}$ (TYVPLLGSL), $hTERT_{845}$ (CYGDMENKL), $hTERT_{167}$ (AYQVCGPPL) (unpublished), $hTERT_{461}$ (VYGFVRACL), $hTERT_{324}$ (VYAETKHFL) [35], Her-2/neu_8 (RWGLLLALL) [36], $MRP3_{765}$ (VYSDADIFL) and $MRP3_{692}$ (AYVPQQAWI) [37], were synthesized at Mimotope (Melbourne, Australia) and Sumitomo Pharmaceuticals (Osaka, Japan). They were identified using mass spectrometry, and their purities were determined to be > 80% by analytical high performance liquid chromatography (HPLC). Regarding the immunological properties of the epitopes in HCC patients, we found that AFP- and hTERT-derived epitopes were recognized by cytotoxic T lymphocytes in 7·9–21·1% and 6·9–12·5% of 38 and 72 patients, respectively [27,38]. On the other hand, immune responses to Her-2/neu- and MRP3-derived epitopes have not been reported; however, overexpression of Her-2/neu protein was reported to be 2·42% in HCC patients [39], and in our unpublished data the immune responses to Her-2/neu-derived epitope were observed in 5·1% HCC patients (tested 38 patients). In addition, expression of MRP3 protein was reported to be 100% in HCC patients [40]. In our unpublished data, the immune responses to MRP3-derived epitope were observed in 20·5–25·6% HCC patients (38 tested patients).

Statistical analysis

Results are expressed as mean ± s.d. Differences between groups were analysed for statistical significance by the Mann–Whittney *U*-test. The estimated probability of tumour recurrence-free survival was determined using the Kaplan–Meier method. The Mantel–Cox log-rank test was used to compare curves between groups. Any *P*-values less than 0·05 were considered statistically significant.

Results

Isolation and characterization of DCs

Adherent cells isolated from PBMCs were differentiated into DCs in the presence of IL-4 and granulocyte–macrophage colony-stimulating factor (GM-CSF). DCs from each study patient were shown to develop high levels of MHC class II (HLA-DR) and co-stimulatory molecule B7-2 (CD86) and showed the absence of markers for mature monocytes (CD14). In addition, DCs obtained were phenotypically immature ($CD80^{low}CD83^{low}$) and classified to myeloid ($CD11c^+$ $CD123^-$) and plasmacytoid ($CD11c^-CD123^+$) subsets (Table 2). Furthermore, sufficient numbers (at least 1×10^7) of functional DCs were isolated from 200 ml of peripheral blood in all patients in this clinical trial.

Safety of autologous DC administration

DC administration was performed during TAE therapy, in which DCs were mixed together with Gelfoam and infused through an arterial catheter following Lipiodol injection. Adverse events were monitored clinically and biochemically after DC infusion (Table 3). There were no grades III or IV National Cancer Institute common toxicity criteria adverse

Table 2. Properties of infused dendritic cells.

		lin$^-$ HLA-DR$^+$				
Patient no.	%CD14$^-$ HLA-DR$^+$	%CD11c$^+$ CD123$^-$	%CD11c$^-$ CD123$^+$	%CD80$^+$	%CD83$^+$	%CD86$^+$
1	34·5	5·8	8·4	1·6	1·9	11·5
2	56·2	49·7	39·3	4·1	3·2	92·4
3	35·6	63·6	6·1	0	5·7	96·0
4	32·7	22·6	56·5	1·9	1·2	61·6
5	40·4	24·3	57·3	34·9	27·7	94·4
6	60·4	35·8	47·0	11·4	1·5	84·4
7	46·4	54·7	10·7	12·9	12·5	83·2
8	62·8	7·6	32·3	19·0	20·5	49·1
9	55·2	35·2	35·5	28·8	18·3	73·0
10	34·0	9·5	7·0	12·1	8·4	31·8
Mean	45·8	30·9	30·0	12·7	10·1	67·7
s.d.	11·9	20·5	20·5	11·9	9·3	28·9

Table 3. General clinical outcome.

Patient no.	Adverse events: Fever (days)	Vomiting	Abdominal pain	Encephalopathy	Others	DTH to KLH	Tumour recurrence	Survival w/o HCC (months)	Survival (months)	Death/alive
1	1	No	No	No	No	n.d.	Yes	6	17	Death
2	2	No	No	No	No	n.d.	Yes	13	17	Alive
3	No	No	No	No	No	n.d.	Yes	8	30	Alive
4	4	Yes	No	No	No	n.d.	Yes	11	36	Alive
5	No	No	No	No	No	n.d.	n.d.	2	2	Death
6	No	No	No	No	No	n.d.	Yes	5	34	Alive
7	8	No	No	No	No	n.d.	Yes	14	22	Death
8	No	No	No	No	No	Positive	No	9	9	Death
9	5	Yes	No	No	No	n.d.	Yes	6	30	Alive
10	No	No	No	No	No	Positive	Yes	22	24	Alive
Mean								9·6	22·1	
s.d.								5·7	11·0	
11	No	No	No	No	No	n.d.	Yes	4	6	Death
12	No	No	No	No	No	n.d.	Yes	9	24	Death
13	No	No	Yes	No	No	n.d.	Yes	2	8	Alive
14	No	Yes	Yes	No	No	n.d.	Yes	3	9	Death
15	3	No	No	No	No	n.d.	Yes	18	20	Death
16	1	No	No	No	No	n.d.	Yes	8	36	Alive
17	No	No	No	No	No	n.d.	Yes	6	12	Death
18	No	No	No	No	No	n.d.	Yes	11	36	Alive
19	5	No	Yes	Yes	No	n.d.	Yes	7	30	Alive
20	No	No	No	No	No	n.d.	Yes	5	11	Death
21	No	No	No	No	No	n.d..	n.d.	4	4	Alive
Mean								7·0	17·8	
s.d.								4·5	12·0	

Adverse events/others: myalgia, gastrointestinal disorder, bleeding, hepatic abcess and autoimmune diseases; DTH: delayed-type hypersensitivity skin test; KLH: keyhole limpet haemocyanin; n.d.: not determined.

events associated with DC infusion and TAE in this study. Furthermore, the adverse events of the patients treated with DC infusion and TAE were compared with the control patients treated with TAE alone in terms of fever, vomiting, abdominal pain, encephalopathy, myalgia, ascites, gastrointestinal disorders, bleeding, hepatic abscess and autoimmune diseases. Although the clinical courses of five of the patients infused with DCs were complicated with high fever, there were no significant differences in the frequency or severity of adverse events associated with DC infusion. There was also no clinical or serological evidence of hepatic failure or autoimmune response in any patients. Thus, the current treatment of DC infusion was performed safely at the same time as TAE in patients with cirrhosis and HCC.

Kinetics and *in situ* effects of DCs following infusion into HCC tissues

DCs were labelled with ^{111}In-oxine and infused into tumour tissues through an arterial catheter following TAE in two patients (numbers 9 and 10). The kinetics of ^{111}In-labelled DCs were monitored by a gamma camera after the infusion (Fig. 1a). Radioactivity at the tumour site decreased after the infusion, but was still detectable up to 14 days after the infusion, indicating that the DCs stayed alive for more than 2 weeks. However, tracking of labelled DCs to regional lymph nodes was not seen in the current imaging, due possibly to insufficient numbers of migrated DCs, or otherwise due to DC paralysis in tumour tissues [41].

One of the patients (number 10) proceeded unexpectedly to curative surgery 17 days after the DC infusion. The tissue radioactivity was investigated using autoradiography and analysed on an image analyser (Fig. 1b,c,d). Surprisingly, radioactivity was still detectable in the tumour tissue and surrounding liver parenchyma. In addition, immunohistochemistry of the liver tissue was performed using MoAbs specific for cell surface markers CD8, CD14 (monocyte), CD20 (B cell), CD1a (DC) HLA-DR (antigen-presenting cell) and CD83 (DC maturation/activation) (Fig. 2). Immunohistochemical analysis revealed many CD1a-positive DCs in the area surrounding HCC nodules treated with autologous DCs and TAE 17 days previously. In addition, CD83-positive cells and HLA-DR-positive antigen-presenting cells were seen in the same areas of liver tissue. Interestingly, CD83-positive cells were rarely detected in liver tissues of HCC patients, as reported previously [42]. Many $CD8^+$ T cells, $CD14^+$ monocytes and $CD20^+$ B cells were recruited in the same area. In conjunction with the detection of

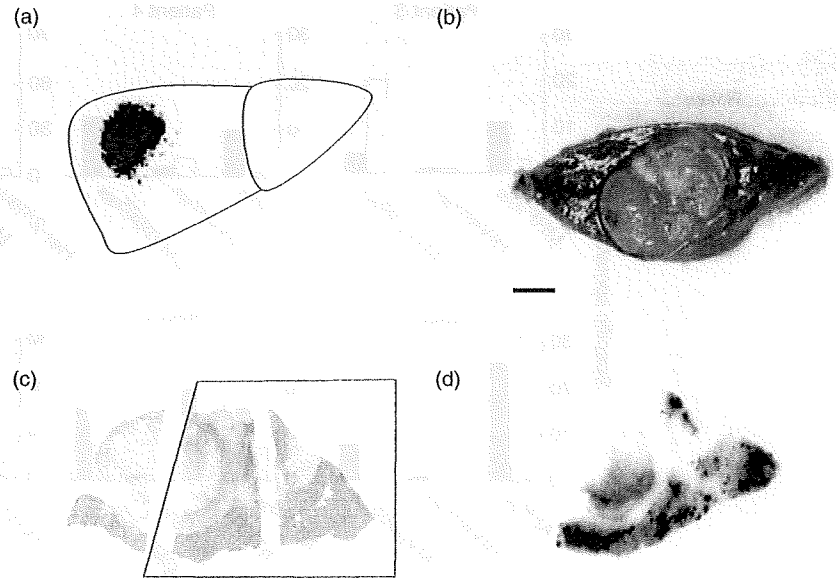

Fig. 1. Kinetics of autologous dendritic cells (DCs) following infusion into tumour tissues in patients with hepatocellular carcinoma (HCC). DCs were labelled with ^{111}In-oxine and infused into tumour tissues through an arterial catheter following transcatheter hepatic arterial embolization (TAE). (a) Scintigraphic images of the depot were acquired with a gamma camera 7 days after injection. (b) In one case, the treated HCC nodule was accidentally resected surgically 17 days after DC infusion. Bar represents 1 cm. Resected tissues were stained with haematoxylin and eosin (c) and, using the tissue comparable to the inset in (c), autoradiography was conducted and analysed on an image analyser (d).

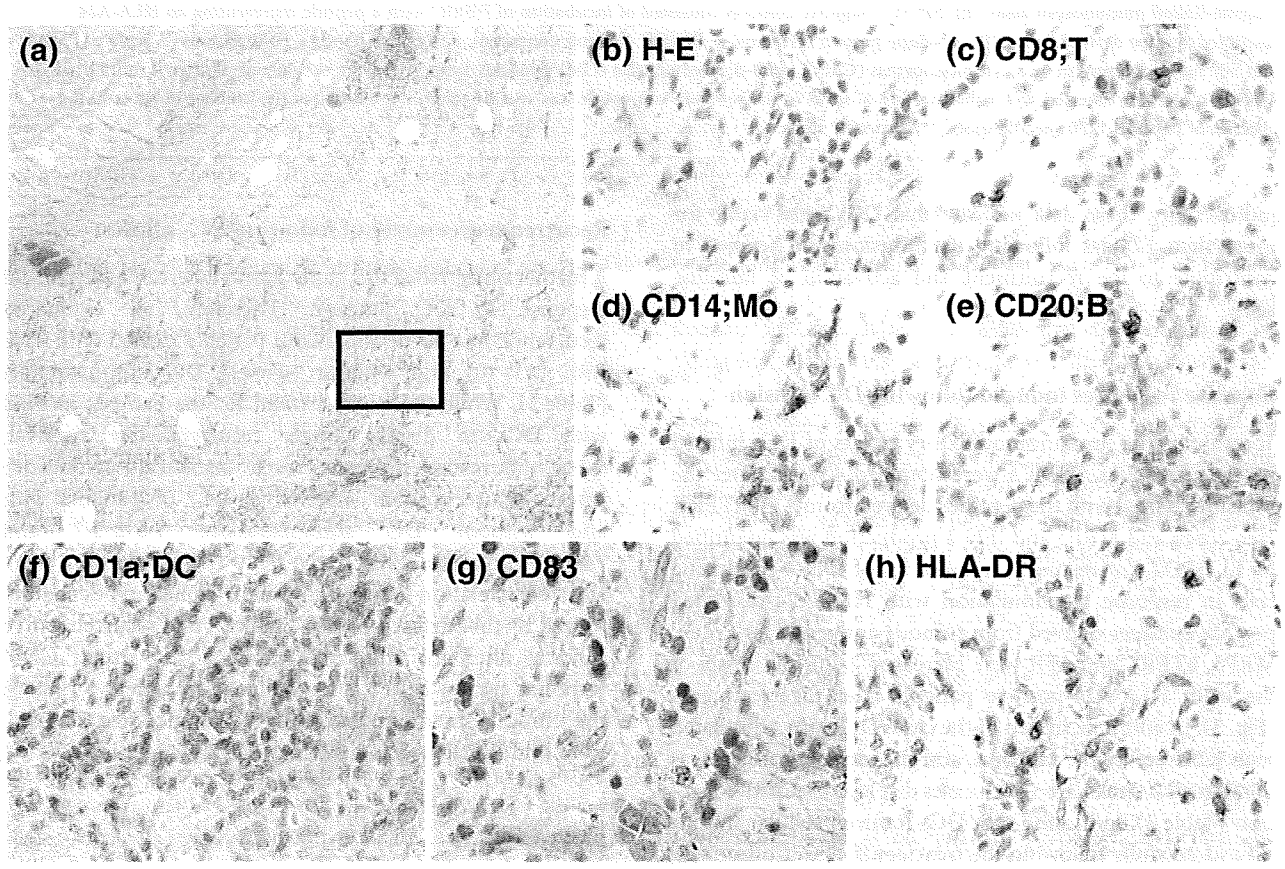

Fig. 2. Immunohistochemical analysis of a surgically resected liver tissue containing a nodule of hepatocellular carcinoma in a patient infused with autologous dendritic cells (DCs) 17 days previously, described in the legend to Fig. 1. The liver tissue was stained with haematoxylin and eosin (a and b) and for CD8 (c), CD14 (d), CD20 (e), CD1a (f), CD83 (g) and human leucocyte antigen D-related (HLA-DR) (h). (b) Close-up of the inset in (a). Areas comparable to (b) are indicated in (c–h). Cells were stained positively in brown. Original magnifications, ×40 (a) and ×400 (b–h).