ium (DMEM; Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Recombinant retrovirus packaging, infection, and selection of FLAG-hTERT expressing stable transformations of Huh7 cells were preformed as described(13).

Preparation of cell lysates and immunoprecipitation

Cells were harvested, washed with phosphate buffered solution (-) and sonicated in CelLytic^{TM} M (SigmaAldrich). Lysates of 5 × 10(6) cells were diluted 10-fold in CelLytic^{TM} M and incubated at 4°C for 3 h with 10 μL of GammaBind G resin containing pre-bound anti-FLAG M2 or anti-hnRNP A2/B1, followed by three washes with CelLytic^{TM} M. The bound proteins were separated by SDS-PAGE and visualized by western blotting.

Antibodies and western blot analysis

For western blot analysis, total cell lysates and their fractions from gel filtration were separated by SDS-PAGE and transferred to a nitrocellulose membrane then probed with anti-hTERT (Rockland Inc., Gilbertsville, PA, USA), anti-Hsp90α/β (Santa Cruz Biotechnology, CA, USA), anti-hnRNP A2/B1 (Abcam), anti-FLAG M2 (Sigma-Aldrich), or anti-β-actin (Sigma-Aldrich) primary antibodies, followed by incubation with horseradish peroxidase conjugated goat anti-mouse immunoglobulin (Ig) G secondary antibody (Amersham Biosciences, Buckinghamshire, England, UK) for anti-Hsp90α/β, anti-hnRNP A2/B1, anti-FLAG M2, and anti-β-actin antibodies or horseradish peroxidase conjugated goat anti-Rabbit IgG secondary antibody (Thermo Scientific, Rockford, IL, USA) for anti-hTERT antibody. Densitometric analysis was conducted directly on the blotted membrane using a CCD camera (LAS-3000 Mini; Fujifilm, Tokyo, Japan) and Scion Image software.

Small interfering RNA synthesis

Small interfering (si) RNA specific to hnRNP A2/B1 (HNRNPA2B1 siGENOME set) and the siGENOME Controls Basic kit were obtained from Thermo Scientific. To each well of a six-well plate, 2 × 10^5 Huh7 cells were seeded 12 h before transfection. Transfection was preformed using TransMessenger^{TM} Transfection Reagent (Qiagen, West Sussex, UK) according to the manufacturer's protocol. A total of 100 pmol/L of siRNA duplex was used for each transfection.

Real-time quantitative reverse-transcription PCR

Real-time quantitative reverse-transcription polymerase chain reaction (RT-PCR) was performed for hnRNP A2/B1 using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, San Francisco, CA, USA). Primers and the TaqMan probe for hnRNP A2/B1were designed using the primer design software Primer Express^{TM} (Applied Biosystems). The probe was labeled with a reporter fluorescent dye (6-carboxy-fluorescein) at the 5′ end and a quencher fluorescent dye (6-carboxy-tetramethyl-rhodamine) at the 3′ end. PCR conditions were 1 cycle at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The level of messenger RNA (mRNA) expression relative to the internal control (β-actin) was calculated.

Telomerase activity assay

Telomerase activity was measured by a PCR-based telomere repeat amplification protocol (TRAP) enzyme-linked immunosorbent assay (ELISA). Telomerase activity was quantitatively measured using a TRAPEZE ELISA telomerase detection kit (MILLIPORE, Billerica, MA, USA) according to the manufacturer's protocol.

Immunohistochemical analysis

Paraffin-embedded sections of tissue blocks were orderly rehydrated to xylene and sequential alcohols, washed, and blocked by incubating slides in 0.6% hydrogen peroxide. The sections were treated with a 1:100 diluted solution of anti-hnRNP A2/B1 antibody for 30 min in a wet incubation box. Detection of the antibody was processed according to the manufacturer's protocol using Envision+ kits (Dako, Carpinteria, CA, USA). Slides were counterstained with hematoxylin for 30 s, dehydrated reversibly using sequential alcohols and xylene, and mounted with a coverslip using Histomount. Photographs for stained tissue section were captured using an Olympus DP70 CCD camera with an Olympus AX80 microscope (Olympus, New York, NY, USA).

Statistical analysis

The student's *t*-test was used to determine the statistical significance of the difference in cell viability between the two groups. The chi-square test was used to evaluate the correlation between clinicopathological characteristics and nuclear and cytoplasmic hnRNP A2/B1 expression. Univariate and multivariate Cox proportional hazards regression analysis was used to evaluate the association of nuclear and cytoplasmic hnRNP A2/B1 expression and clinicopathological parameters with patient outcome. All statistical analysis was performed using SPSS software (SPSS software package; SPSS Inc., Chicago, IL, USA).

Results

Fractionation of protein lysates from hepatocellular carcinoma and cirrhotic liver tissue

Protein lysates from HCC and cirrhotic liver tissue from patients were independently subjected to gel filtration

Liver International (2012)

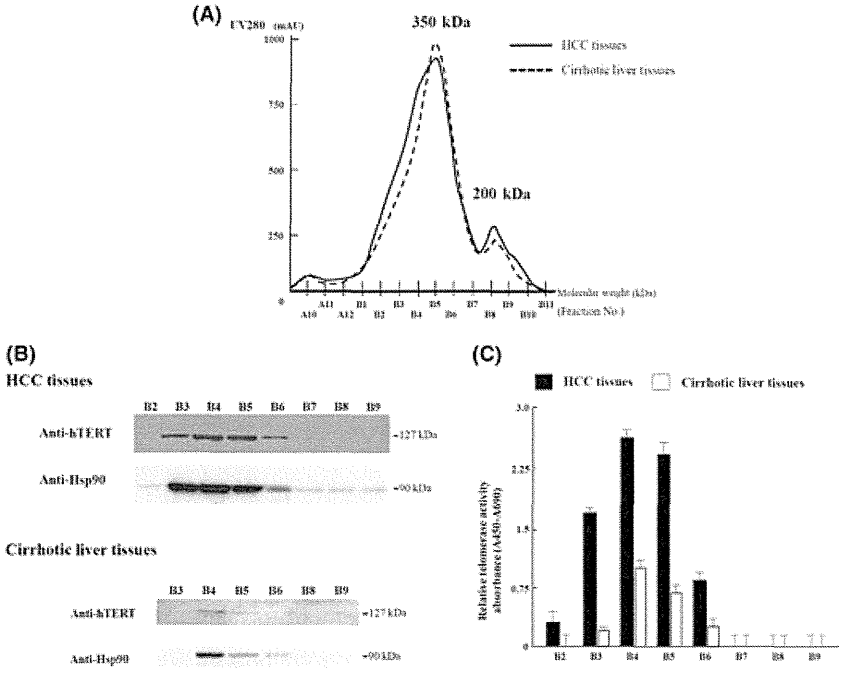

Fig. 1. Fractionation of protein lysates from HCC and cirrhotic liver tissue. (A) Protein lysates from HCC and cirrhotic liver tissues from three patients were fractionated on a 200 pg gel filtration column by high-performance liquid chromatography (HPLC). Mean values were drawn in the same axis. (B) Representative image of quantitative measurement of human telomerase reverse transcriptase (hTERT) protein and Hsp90 protein by western blotting with respective antibodies. (C) Relative telomerase activity of fractions measured by telomere repeat amplification protocol enzyme-linked immunosorbent assay.

by HPLC (Fig. 1A). Two peaks, corresponding to the molecular weights 350 kDa and 200 kDa in the fractionated proteins were found. Interestingly, hTERT was detected around the 350-kDa peak (Fraction Nos. B3–B6) concurrently with telomerase activity (Fig. 1B, 1C). Moreover Hsp90 was broadly distributed in two peaks of around 350 kDa and 200 kDa (Fraction Nos. B2–B9). The expression of hTERT by western blotting was higher (mean, five-fold) in HCC tissue than that in cirrhotic nodule tissue (Fig. 1B), and the telomerase activity quantified by TRAP ELISA was significantly higher (>2.5-fold) in HCC than in cirrhotic nodule tissue (Fig. 1B, 1C).

Identification of differentially expressed proteins

To discover hTERT-related proteins, we further analyzed the fractionated lysates of the 350-kDa peak (Fraction No. B4) from HCC and cirrhotic liver tissue by LC-MS/MS. After searching the MASCOT database (http://www.matrixscience.com), 144 proteins were selected according to identification criteria (see Materials and methods), and 24 of all identified proteins displayed more than a two-fold expression difference. Among these 24 proteins, eight were found to be up-regulated in HCC tissue compared with cirrhotic liver tissue, while 16 proteins were found to be down-regulated (Table 1). Of the 24 proteins, nine were already known as HCC-related proteins(14–20). To identify a new prognostic biomarker related to hTERT, we decided to focus on hnRNP A2/B1, which has been reported as a prognostic biomarker for lung cancer(21) and gastric cancer(22).

Validation of heterogeneous nuclear ribonucleoprotein A2/B1 expression and interaction with human telomerase reverse transcriptase subunit

To confirm the altered expression of hnRNP A2/B1 in HCC and non-cancerous liver, western blot analysis was performed with anti-hnRNP A2/B1 antibody. Both of hnRNP A2/B1 expression was detected around 350 kDa (Fraction Nos. B3–B7). The hnRNP A2/B1 protein level was higher in HCC tissue than that in non-cancerous liver tissue (Fig. 2A). To examine whether hnRNP A2/B1 can interact with hTERT, we performed an immunoprecipitation assay. hnRNP A2/B1-immunoprecipitates, derived from fractionated lysates of 350 kDa (Fraction No. B4) contained hTERT (Fig. 2B). However, in a reverse immunoprecipitation experiment, anti-hnRNP A2/B1 antibody was unable to recognize the hTERT protein band in hTERT-immunoprecipitates (data not shown). Therefore, we established Huh7 cells derived from stable cell lines that consistently expressed FLAG-tagged hTERT (see Materials and methods). hnRNP A2/B1-immunoprecipitates contained FLAG-hTERT, and

Table 1. Proteins identified by LC-MS/MS as significantly changed in expression between HCC tissues and cirrhotic nodule tissue[18–24]

Accession no.	Protein name	Molecular function	Protein ratio (HCC/cirrhotioc liver)
Up-regulated proteins in HCC tissue			
P 40925	Malate dehydrogenase	L-malate dehydrogenase activity, malic enzyme activity	2.38
Q 13228	Selenium-binding protein 1	Protein binding, selenium binding	2.07
P 22626	Heterogeneous nuclear ribonucleo protein in A2/B1	RNA binding, nucleotide binding	2.01
Q 13535	Serine/threonine-protein kinase ATR	ATP binding, DNA binding	1.92
Q 5T4S7	Zinc finger UBRl-type protein 1	Ubiquitin-protein in ligase activity, zinc ion binding	1.61
P 07335	Annexin A2	Calcium ion binding, cytoskeletal protein binding	1.53
P 30038	Delta-l-pyrroline-5-carboxylate dehydrogenase	1-pyrroline-5-carboxylate dehydrogenase activity	1.52
P 09651	Heterogeneous nuclear ribonucleo protein A1	RNA binding, nucleotide binding	1.48
Down-regulated proteins in HCC tissue			
Q 8NF91	Nesprin-1	Actin binding, lam in binding	0.67
P 00352	Retinal dehydrogenase 1	Ras GTPase activator activity	0.55
P 24752	Acetyl-coA acetyl transferase	Acetyl-coA acetyl transferase activity	0.51
P 00441	Superoxide dismutase (Cu-Zn)	C haperone binding copper ion binding	0.45
P 02787	Serotransfeirn recursor	Ferric ion binding	0.45
Q 8TE73	Ciliaary dyne in heavy chain 5	ATP binding, ATPase actvity	0.43
P 07327	Alcohol dehydrogenase 1A	Alcohol dehydrogenase activity (Zinc-dependent)	0.41
P 68871	Hemoglobnin subunit beta	Heme binding, hemoglobin binding, oxygen binding	0.39
Q 6PIU 2	Liver carboxyl esterase 1 precursor	Carboxyl esterase activity	0.38
Q 06830	Peroxiredoxin-1	Protein binding,thioredoxin peroxidase activity	0.36
P 69905	Hemoglobin subunit alpha	Heme binding, oxygen binding,	0.34
P 36871	Phosphoglucomutase-1	Magnesium ion binding	0.32
P 05089	Arginase-1	Arginase activity	0.32
P 30041	Peroxiredoxin-6	Glutathione peroxidase activity	0.29
P 00326	Alcohol dehydrogenase 1C	Alcoholdehydrogenase (NAD) activity	0.21
P 08319	Alcohol dehydrogenase 4	NAD binding, NADPH quinone reductase activity	0.15

ATP, adenosine 5'-triphosphate; GTP, guanosine triphosphate; HCC, hepatocellular carcinoma; NAD, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate-oxidase.

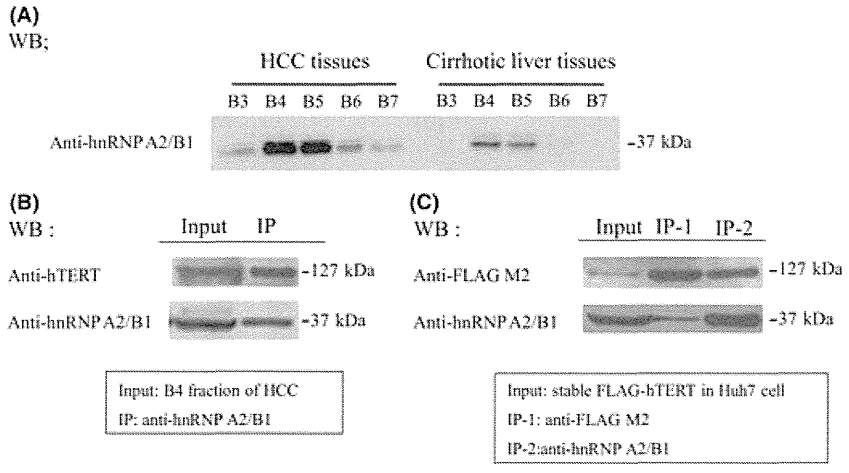

Fig. 2. Validation of hnRNP A1/B2. (A) Representative image of quantitative measurement of hnRNP A2/B1 protein by western blotting with anti-hnRNP A2/B1 antibody. (B) B4 fractionated lysates of HCC were immunoprecipitated with anti-hnRNP A2/B1 antibody. The bound proteins were separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to western blotting with anti-hTERT and anti-hnRNP A2/B1 antibodies as indicated. The B4 fractionated lysates of HCC shown in the input correspond to 5% of the sample. (C) Total cell lysates from Huh7 cells stably expressing FLAG-hTERT were immunoprecipitated with anti-FLAG M2 (IP-1) and anti-hnRNP A2/B1 (IP-2) antibodies. The bound proteins were separated by 8% SDS-PAGE and subjected to western blotting using anti-FLAG M2 and anti-hnRNP A2/B1 antibodies as indicated. Total cell lysates shown in the input correspond to 5% of the sample.

Liver International (2012)

anti-hnRNP A2/B1 antibody recognized the FLAG-hTERT protein band in FLAG-M2 immunoprecipitates (Fig. 2C). These results confirmed that hnRNP A2/B1 can interact with hTERT.

Functional relevance of heterogeneous nuclear ribonucleoprotein A2/B1 expression on telomerase activity

To examine the functional relevance of hnRNP A2/B1 on hTERT activity, we performed knockdown of hnRNP A2/B1. Expression of hnRNP A2/B1 in Huh7 cells was significantly knocked down to 30–40% of the control using hnRNP A2/B1-specific siRNA (siGENOME, Thermo Scientific) (Fig. 3A). Under these conditions, the results of TRAP ELISA showed that telomerase activity was repressed to 43–48% that of the control (Fig. 3B). These results indicate that hnRNP A2/B1 is related to telomerase activity.

Analysis of heterogeneous nuclear ribonucleoprotein A2/B1 expression by immunohistochemistry

To characterize the clinicopathological significance of hnRNP A2/B1 expression in HCC, we performed immunohistochemical staining of hnRNP A2/B1 using paraffin-embedded tumor and non-tumor specimens from 74 HCC patients. We observed hnRNP A2/B1 expression in all HCC specimens, while it was expressed in 16 of 74 (22%) adjacent non-cancerous liver specimens ($P< 0.001$). We did not observe hnRNP A2/B1 expression in normal liver ($n = 5$) or in the early stage (F1–2) of chronic hepatitis ($n = 5$) (data not shown).

Interestingly, we noticed that anti-hnRNP A2/B1 antibody reacted to nuclear and cytoplasmic isoforms of hnRNP A2/B1. We defined HCC cells in which only nuclear hnRNP A2/B1 was expressed in tumor cells as nuclear hnRNP A2/B1-positive HCC (Fig. 4A). Similarly, both nuclear and cytoplasmic hnRNP A2/B1 was expressed in tumor cells as nuclear and cytoplasmic hnRNP A2/B1-positive HCC, respectively (Fig. 4B). Western blotting analysis showed that hnRNP A2/B1 was expressed significantly more, about 2.5-fold, in the nuclear and cytoplasmic hnRNP A2/B1-positive HCC than in the nuclear hnRNP A2/B1-positive HCC Fig. 5A, B).

The expression pattern of hnRNP A2/B1 in the nucleus and the cytoplasm was different in each HCC tissue type. Nuclear hnRNP A2/B1-positive HCC was observed in 40.5% (30 of 74) of HCC patients, whereas nuclear and cytoplasmic hnRNP A2/B1-positive HCC was observed in 59.5% (44 of 74) of HCC patents (Table 2). We then compared the clinicopathological features of nuclear hnRNP A2/B1-positive HCC and nuclear and cytoplasmic hnRNP A2/B1-positive HCC (Table 2). Nuclear and cytoplasmic expression of hnRNP A2/B1 was frequently observed in patients with a progressive histological grading (Edmondson-Steiner grades; $P = 0.002$) and microvascular invasion ($P = 0.013$) (Table 2). No relationship was apparent between the expression pattern of hnRNP A2/B1 and age, gender, type of infected virus, Child-Pugh score, AFP value, PIVKA-II value, tumor size, tumor morphology, TNM stages, or recurrence rate of HCC (Table 2). Importantly, survival analysis using the Kaplan-Meier method revealed that HCC patients with nuclear and cytoplasmic expression of hnRNP A2/B1 showed a significant lower survival rate than those with nuclear expression of hnRNP A2/B1 (log-rank test, $P = 0.0027$; Fig. 6). Furthermore, univariate Cox regression analysis showed that nuclear and cytoplasmic expression of hnRNP A2/B1 was significantly associated with low patient survival (HR, 2.37; 95% CI, 1.33–4.23;

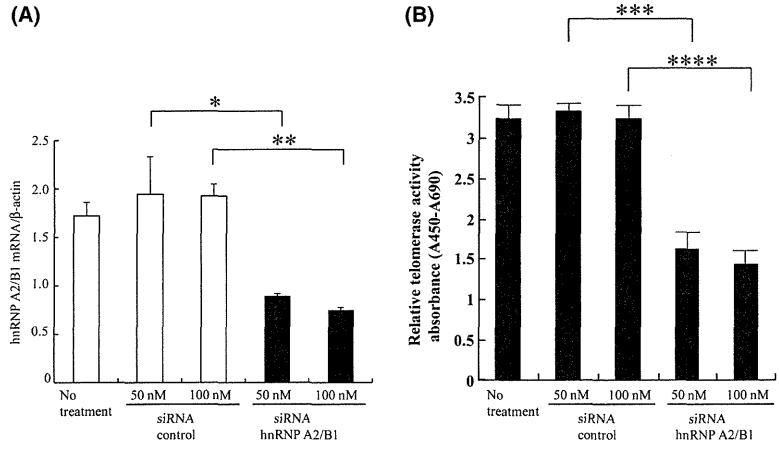

Fig. 3. siRNA against hnRNP A2/B1 suppresses telomerase activity. (A) mRNA expression of hnRNP A2/B1 after small interfering (si)RNA transfection in Huh7 cells transfected with sihnRNP A2/B1 and that of control siRNAs. Levels of mRNA were determined by real time polymerase chain reaction (PCR). (B) The activity of each lysate was determined by TRAP ELISA (*,**,***,****$P < 0.05$).

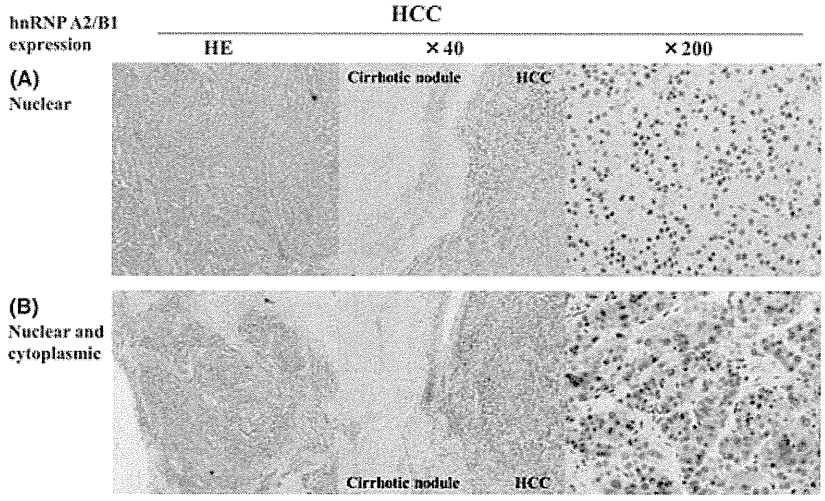

Fig. 4. Immunohistochemical analysis of hnRNP A2/B1 expression in HCC and adjacent cirrhotic liver tissue. A representative photomicrograph of hematoxylin and eosin staining and hnRNP A2/B1 staining in HCC tissue (× 40 and × 200, respectively) and adjacent cirrhotic liver tissue (× 40). (A) Nuclear hnRNP A2/B1-positive HCC. (B) Nuclear and cytoplasmic hnRNP A2/B1-positive HCC.

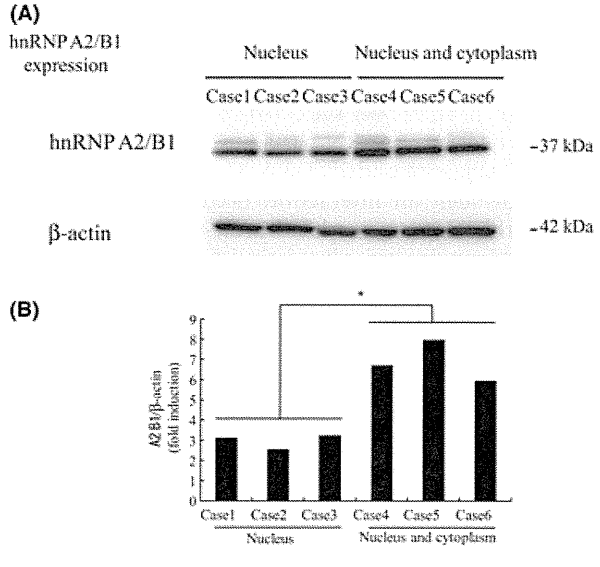

Fig. 5. The protein expression of hnRNP A1/B2 in HCC tissue. (A) Equal amounts of HCC tissue from Nuclear hnRNP A2/B1-positive HCC (cases 1–3) and Nuclear and cytoplasmic hnRNP A2/B1-positive HCC (cases 4–6) were loaded into the SDS-PAGE gel and normalized by comparing with β-actin. (B) The value in the graph is presented as mean ± SD (*$P < 0.05$).

Table 2. Clinicopathological characteristics and hnRNP A2/B1 expression of nucleus (&) cytoplasm in HCC ($n = 74$)

hnRNP A2/B1 expression	nuclear (n=30)	nuclear and cytoplasm ic (n=44)	P-value
Age (<60 years/ ≥ 60 years)	12/18	18/26	0.938
Gender (male/female)	22/8	18/26	0.531
Virus (HBV/HCV/NBNC)	5/21/4	14/21/9	0.160
Child-Pugh (5/6/7)	28/2/0	37/5/2	0.377
AFP (<100 ng/ml/ ≥ 100 ng/ml	20/10	26/18	0.509
PIVKA-II (<100 mAU/ ml/ ≥ 100 mAU/ml	18/12	22/22	0.397
Histological grading (well/moderately/poorly)	12/18/0	0/32/12	0.002
Tumor size (<3 cm/ ≥ 3 cm)	16/14	22/22	0.778
Tumor morphology (uni/multi)	26/4	35/9	0.429
TMN classification (I/II/III/IVa)	6/17/6/1	2/27/10/5	0.139
Microvascular invasion (Yes/No)	11/19	29/15	0.013
HCC recurrence (Yes/No)	11/19	20/24	0.452

AFP, α-fetoprotein; HBV, Hepatitis B virus; HCC, hepatocellular carcinoma; HCV, Hepatitis C virus.

$P = 0.004$) (Table 3). Multivariate Cox regression analysis showed that nuclear and cytoplasmic expression of hnRNP A2/B1 was an independent prognostic factor associated with low patient survival (HR, 3.86; 95% CI, 1.80–8.28; $P = 0.001$). Other clinicopathological features did not add independent prognostic information in this study (Table 3). These results demonstrate that the expression of hnRNP A2/B1 correlates with the severity and progression of HCC and that nuclear and cytoplasmic hnRNP A2/B1 expression could be a useful biomarker for predicting the survival of HCC patients after surgical resection.

Discussion

The development of useful biomarkers for the early detection and prediction of HCC is urgently required to improve prognosis of patients with HCC. Moreover,

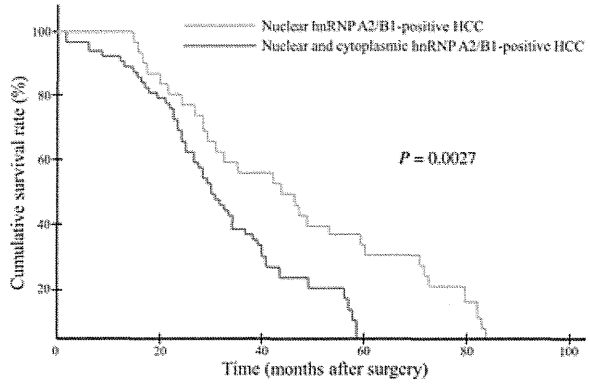

Fig. 6. Kaplan-Meier survival analysis of nuclear (and) cytoplasmic hnRNP A2/B1-positive HCC (log rank test).

biomarkers reflecting malignant features of HCC might be useful for the following patients and HCC therapy selection since HCC relapse frequently occurs in residual liver where HCC has been curatively removed by surgical treatment. Although AFP and PIVKA II are reliable tumor markers for the detection of recurrence of HCC, more than 50% of patients with small HCC (<2 cm) test negative for these markers(23).

A two-dimensional electrophoresis and mass spectrum-based proteomic strategy provide high-throughput simultaneous identification of hundreds of proteins; such a strategy is considered very valuable for screening tumor biomarkers(24, 25). Here, we applied a different approach for identifying telomerase-associated proteins in this study. According to previously reported methods (12), protein lysates of HCC tissue and non-cancerous tissue were subjected to HPLC gel filtration. A previous study using a soluble fraction of Huh7 cells identified two peak of endogenous hTERT at around 680 kDa and 350 kDa (data not shown). It was speculated that a dimer form of hTERT existed in the 680 kDa peak and a monomer in the 350 kDa peak. Hsp90 was exclusively distributed in the 350 kDa peak but not in the 680 kDa peak (data not shown). Unlike previous studies using Huh7 cells, we could not detect the 680 kDa peak fraction in HCC tissue. We could however detect the 350 kDa peak and the 200 kDa peak fraction in HCC tissue. The telomerase complex existed in the 350 kDa peak fraction. Moreover, Hsp90 existed in 350 kDa peak fraction, while many of the metabolic-related proteins were identified in the 200 kDa peak fraction. These data suggest that hTERT-associated proteins might exist in the 350 kDa peak fraction in HCC tissue.

Further analysis of the fractionated lysates of 350 kDa by LC-MS/MS revealed that 24 proteins were differentially expressed in HCC tissue when compared with nontumor tissue. In addition, 9 of the 24 proteins were already known as HCC-related proteins(14–20). We therefore focused on hnRNP A2/B1, one of the most abundant and important nuclear RNA-binding proteins involved in packaging nascent mRNA, alternative splicing(26, 27) and cytoplasmic RNA trafficking(28), translation(29), and stabilization(30).

To our knowledge, this is the first demonstration of hnRNP A2/B1 interaction with hTERT by immunoprecipitation *in vitro* and *in vivo*. hnRNP A2/B1 acts as a molecular adapter between single-stranded telomeric repeats or telomerase RNA (hTERC), and another segment of single-stranded DNA(31, 32). However the details for such an interaction need further investigation. Of the telomerase-related proteins, Hsp90 has been shown to be a functionally critical factor for telomerase activity *in vivo* and *in vitro*(33). The telomerase complex with Hsp90 within the 350 kDa complex was also detected in this study, confirming the biological functionality of telomerase activity (Fig. 1B,C). Hsp90 inhibitors reduce the amount of hTERT as well as telomerase activity(12). Despite the concentration of Hsp90

Table 3. Cox regression analysis of cumulative survival rate relative to nuclear and cytoplasmic hnRNP A2/B1 expression and clhicopathobgical parameters of primary HCC patients (*n*=74)

	Univariate		Mulivarate	
Variables	HR (95%CI)	*P*-value	HR (95%CI)	*P*-value
Age ≥ 60 years	1.25 (0.74–2.11)	0.453		
Male gender	1.16 (0.69–2.05)	0.606		
Child-Pugh ≥ 6	1.01 (0.36–2.84)	0.979		
AFP ≥ 100 ng/ml	1.42 (0.84–2.41)	0.188	1.25 (0.72–2.18)	0.429
PIVKA-II ≥ 100 mAU/ml	1.44 (0.85–2.45)	0.171	1.05 (0.59–1.90)	0.864
Histological grade (poorly)	1.33 (0.60–2.96)	0.485		
Tumorisize ≥ 3 cm	1.37 (0.83–2.25)	0.225		
Tumor morphology (multi)	1.09 (0.57–2.06)	0.797		
TMN cfessificaton (III, IVa)	1.63 (0.89–2.96)	0.112	1.24 (0.65–2.36)	0.51
Microvascular invasion (Yes)	1.08 (0.65–1.79)	0.765		
HCC recurrence (Yes)	0.73 (0.44–1.23)	0.238		
Nuclear and cytoplasmic hnRNP A2/B1	2.37 (1.33–4.23)	0.004	2.18 (1.19–4.00)	0.012

AFP, α-fetoprotein; HCC, hepatocellular carcinoma.

inhibitors, no telomere shortening of Hsp90 inhibitor-treated cells was observed (data not shown). The present study also demonstrated that the suppression of hnRNP A2/B1 by siRNA inhibited telomerase activity *in vitro*. However, unlike Hsp90, the suppression of hnRNP A2/B1 could potentially shorten telomere length and inhibit cell proliferation, although such an hypothesis should be confirmed by further experiments.

To further examine the clinicopathological significance of hnRNP A2/B1, immunohistochemical staining in clinical HCC samples was performed. Both nuclear and cytoplasmic expression of hnRNP A2/B1 in HCC was significantly related to tumor differentiation and microvascular invasion of HCC (Table 2). Furthermore survival analysis showed a significant correlation between the high nuclear and cytoplasmic expression of hnRNP A2/B1 in HCC and the low survival rate of patients (Table 3, Fig. 6). Although high nuclear and cytoplasmic expression of hnRNP A2/B1 in HCC was not associated with the recurrence rate of HCC (Tables 2, 3), it was associated with the recurrence pattern of HCC. Tumor morphology (multiple HCCs; $P = 0.100$) and vascular invasion ($P = 0.070$) in recurrence was observed more frequently in patients with nuclear and cytoplasmic hnRNP A2/B1-positive HCC than in those with nuclear hnRNP A2/B1-positive HCC, although the difference was not statistically significant (data not shown). The metastatic and invasive features of HCC with nuclear and cytoplasmic expression of hnRNP A2/B1 may contribute to the poor prognosis of affected patients.

Because hnRNP A2/B1 is a RNA shuttling factor(34), nuclear and cytoplasmic expression patterns might reflect increased hnRNP A2/B1 in cells; however, the expression of hTERT is presumably increased and colocalized with hnRNP A2/B1. It was reported that nucleolin, a molecule shuttling into and out of the nucleus, mediates hTERT and localization of hTERT shifted from the nucleus to the cytoplasm depending on its sublocalization(35). Increased hTERT might be associated with tumor differentiation, microvascular invasion and survival of HCC.

While preparing this study, Cui H. *et al.*(36) reported similar findings. They showed increased localization of hnRNP A2/B1 in the cytoplasm of HCC cells during dedifferentiation of HCC. Our study, however, showed the functional relevance of hnRNP A2/B1 on telomerase activity and demonstrated the clinical importance of hnRNP A2/B1 for patient survival.

In conclusion, employing a proteomic screening and molecular biology verification approach revealed a potential HCC biomarker, hnRNP A2/B1, and confirmed its usefulness in the diagnosis of and prediction of prognosis of HCC. Although the present data suggest that hnRNP A2/B1 is clinically significant, the understanding of its underlying mechanisms falls short of that required for the development of practical applications. Further approaches are thus needed to improve the diagnostic performance of hnRNP A2/B1 for biological and clinical detection of HCC.

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CLINICAL STUDIES

Expression of chondroitin-glucuronate C5-epimerase and cellular immune responses in patients with hepatocellular carcinoma

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Keywords
cancer – CTL – epitope – immunotherapy – peptide vaccine – tumour-associated antigen

Abbreviations
ELISPOT, enzyme-linked immunospot; HCV, hepatitis C virus; HLA, human leucocyte antigen; IFN, interferon; PBMC, peripheral blood mononuclear cells; SART, squamous cell carcinoma antigen recognized by T cells; TIL, tumour infiltrating lymphocytes.

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Abstract

Background & Aims: Chondroitin-glucuronate C5-epimerase is an enzyme that converts D-glucuronic acid to L-iduronic acid residues in dermatan sulphate biosynthesis. It is also identified to be a tumour-associated antigen recognized by cytotoxic T cells (CTLs) and its enhanced expression in many cancers has been reported. In the present study, we investigated the usefulness of this molecule as an immunotherapeutic target in hepatocellular carcinoma (HCC). *Methods:* The expression of chondroitin-glucuronate C5-epimerase in hepatoma cell lines and HCC tissues was confirmed by immunofluorescence and immunohistochemical analysis. CTL responses were investigated by several immunological techniques using peripheral blood mononuclear cells (PBMCs) or tumour-infiltrating lymphocytes. To determine the safety of immunotherapy using chondroitin-glucuronate C5-epimerase-derived peptide, 12 patients with HCC were administered s.c. vaccinations of the peptides and analysed. *Results:* Chondroitin-glucuronate C5-epimerase was expressed in HCC cell lines and human tissues including alpha-foetoprotein (AFP)-negative individuals. Chondroitin-glucuronate C5-epimerase-specific CTLs could be generated by stimulating PBMCs of HCC patients with peptides and they showed cytotoxicity against HCC cells expressing the protein. The frequency of CTL precursors investigated by enzyme-linked immunospot (ELISPOT) assay was 0–34 cells/3 $\times$ 10^5 PBMCs and the infiltration of interferon-gamma-producing CTLs into the tumour site was confirmed. In the vaccination study, no severe adverse events were observed and the peptide-specific CTLs were induced in 4 of 12 patients tested. *Conclusions:* Chondroitin-glucuronate C5-epimerase is a potential candidate for tumour antigen with immunogenicity and the peptides derived from this antigen could be useful in HCC immunotherapy.

Hepatocellular carcinoma (HCC) is the most frequent primary malignancy of the liver and has gained much clinical interest because of its increasing incidence (1). It is treatable by hepatectomy or percutaneous ablation when the lesion is localized to some extent, and radical therapeutic effects can be obtained when resection or cauterization with a safety margin can be performed (2). However, the recurrence rate is very high (3), because active hepatitis and cirrhosis in surrounding non-tumour liver tissues have the potential to generate HCC de novo.

To protect against recurrence, tumour antigen-specific immunotherapy is an attractive option. Many tumour-associated antigens and their epitopes recognized by cytotoxic T cells (CTLs) have been identified during the last two decades. However, only a few HCC-specific tumour antigens and their antigenic epitopes have been used for human trials (4, 5).

Chondroitin-glucuronate C5-epimerase is an enzyme that converts D-glucuronic acid to L-iduronic acid residues in dermatan sulphate biosynthesis and identical to squamous cell carcinoma antigen recognized by T cells 2 (SART2) (6). It is expressed in many malignant tumour cell lines and various histological types of cancer tissues and function as tumour rejection antigens (7). In addition, peptides containing chondroitin-glucuronate C5-epimerase epitopes are capable of generating CTLs, and therefore, have been used for immunotherapy to treat several kinds of cancers (8, 9). These reports suggest chondroitin-glucuronate C5-epimerase to be useful as a target antigen in HCC immunotherapy. Furthermore, in previous study, we compared T-cell immune responses against the various tumour-associated antigen (TAA)-derived peptides (10). The results of the study showed that CTLs of HCC patients were frequently responsive against a single

chondroitin-glucuronate C5-epimerase-derived peptide. Regarding tumour immunotherapy, it has recently been reported that strong immune responses can be induced at an earlier post-vaccination time using, as peptide vaccines, epitopes that frequently occur in peripheral blood CTL precursors (11). These results also suggest that chondroitin-glucuronate C5-epimerase is useful as a target for HCC immunotherapy.

In the present study, we examined chondroitin-glucuronate C5-epimerase expression in various hepatoma cell lines and HCC tissues of patients, and analysed immune responses to the antigen using peripheral blood mononuclear cells (PBMCs) and tumour-infiltrating lymphocytes (TILs). Furthermore, to investigate the usefulness of HCC immunotherapy targeting chondroitin-glucuronate C5-epimerase, we analysed the safety and cellular immune responses in the patients vaccinated with chondroitin-glucuronate C5-epimerase-derived peptide.

Materials and methods

Patients

Forty-four HLA-A24-positive HCC patients were examined for the expression of chondroitin-glucuronate C5-epimerase and cellular immune responses. Twelve HCC patients were enrolled in vaccination study. Informed consent was obtained from each patient included in the present study and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the regional ethics committee.

The diagnosis of HCC was histologically confirmed by taking US-guided needle biopsy specimens in 17 cases, surgical resection in nine cases, and autopsy in five cases. For the remaining 13 patients, the diagnosis was based on typical hypervascular tumour staining on angiography in addition to typical findings, which showed hyperattenuated areas in the early phase and hypoattenuation in the late phase on dynamic CT (12). The pathological grading of tumour-cell differentiation was assessed according to the general rules for the clinical and pathological study of primary liver cancer (13). The severity of liver disease was evaluated according to the criteria of Desmet *et al.* using biopsy specimens of liver tissue (14). Eleven normal blood donors and 23 chronic hepatitis C patients (11 cirrhosis) with HLA-A24, who were diagnosed by liver biopsy, served as controls.

Cell lines

Four human hepatoma cell lines (HLF, Hep3B, HLE and Huh7) and Paca-2, which is a pancreatic cancer cell line, were cultured in DMEM (Gibco, Grand Island, NY, USA) with 10% fetal calf serum (FCS) (Gibco). The HLA-A*2402 gene-transfected C1R cell line (C1R-A24) was cultured in RPMI 1640 medium containing 10% FCS and 500 μg/ml of hygromycin B (Sigma, St Louis, MO, USA), and K562 was cultured in RPMI 1640 medium containing 10% FCS (15).

Immunofluorescence and immunohistochemical analysis

The expression of chondroitin-glucuronate C5-epimerase was examined in four different Hepatoma cell lines. A pancreatic cancer cell line (Paca 2) was used as a positive control. They were fixed in acetone with methanol for 5 min and incubated with rabbit anti-human chondroitin-glucuronate C5-epimerase (ProteinTech Group, Inc., Chicago, IL, USA; diluted 1:50) or mouse anti-human AFP (Nichirei Bioscience, Tokyo, Japan) antibody overnight at 4°C. For immunofluorescence analyses, Alexa Fluor 488-conjugated anti-rabbit and anti-mouse IgG (Invitrogen, Tokyo, Japan) were used for chondroitin-glucuronate C5-epimerase and AFP detection respectively.

The expression in HCC tissue was examined in 26 patients. Non-cancerous tissues were also obtained by a paired liver biopsy or surgical resection from the non-neoplastic liver tissue. The 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 sections were deparaffinized, treated in a pressure cooker for 1–4 min, and incubated with rabbit anti-human chondroitin-glucuronate C5-epimerase or AFP (DakoCytomation, Inc, Carpinteria, CA, USA) antibody overnight at 4°C. The tissue sections were visualized using the DAKO EnVision^{TM}+ System (DakoCytomation, Inc.). The expression levels were semi-quantitatively classified into four categories (negative to low, moderate and high; negative: no staining, low: <20% of the area stained, moderate: 20%-80% of the area stained, high: >80% of the area stained).

ELISPOT assay

The PBMCs and TILs were isolated as described previously (16). ELISPOT assays were performed as reported previously with the following modifications (16). Three different peptides (Peptide 1; DYSARWNEI, Peptide 2; AYDFLYNYL, Peptide 3; SYTRLFLIL) derived from chondroitin-glucuronate C5-epimerase were used for the detection of CTLs. Negative controls consisted of a HIV envelope-derived peptide ($HIVenv_{584}$) (17). Positive controls consisted of 10 ng/ml of phorbol 12-myristate 13-acetate (PMA, Sigma) or a CMV pp65-derived peptide ($CMVpp65_{328}$) (18). The peptides were synthesized at Sumitomo Pharmaceuticals (Osaka, Japan). The coloured spots were counted with a KS ELISPOT Reader (Zeiss, Tokyo, Japan). The number of specific spots was determined by subtracting the number of spots in the absence of an antigen from the number in its presence. Responses to peptides derived form chondroitin-glucuronate C5-epimerase in HCC

patients were considered positive if the number of specific spots was more than the mean + 3SD of that in normal donors and if the number of spots in the presence of an antigen was at least two-fold greater than the number in its absence. Responses to peptides HIVenv$_{584}$ and CMVpp65$_{328}$ were considered positive if more than 10 specific spots were detected and if the number of spots in the presence of an antigen was at least two-fold that in its absence. ELISPOT assays were also performed in 12 patients whose PBMCs were available for analysis at 2–4 weeks after RFA.

CTL induction and Cytotoxicity assay

Peptide 3 (SYTRLFLIL), which corresponds to HLA-A24-restricted CTL epitope (7, 19), was used to produce chondroitin-glucuronate C5-epimerase-specific T cells. CTLs were expanded from PBMCs as detailed previously (16). C1R-A24 cells and human hepatoma cell lines were used as targets. Cytotoxicity assay was performed by chromium-release assay. Percent cytotoxicity was calculated as previously described (16). For the assay using hepatoma cell lines, cytotoxicity was considered positive when it was higher than that of CTLs against K562 which shows non-specific lysis.

Vaccination study

Twelve HLA-A24-positive HCC patients who were treated by radiofrequency ablation (RFA) and obtained complete necrosis of tumour with safety margin were enrolled in this vaccine study (Trial registration: UMIN000004540). They were vaccinated with peptide 3 (SYTRLFLIL) into the subcutaneous tissue of the armpit 4 weeks after RFA. The peptide utilized in the present study was prepared under conditions of Good Manufacturing Practice (NeoMPS, San Diego, CA, USA). One millilitre of the peptide, which was supplied in vials containing 0.04–4 mg/ml sterile solution, was mixed with an equal volume of incomplete Freund's adjuvant (Montanide ISA-51; Seppic, Paris, France) and emulsified in 5-ml syringes. 1.5 ml of the preparing peptide was injected and the patients received three biweekly vaccinations. Toxicity was assessed every 2 weeks using the National Cancer Institute's Common Toxicity Criteria. To evaluate the immunological effect, ELISPOT assay was performed before and 4 weeks after the final vaccination. Responses to vaccination were considered positive if more than 10 specific spots were detected and if the number of spots after vaccination was at least two-fold than before vaccination. After final vaccination, HCC recurrence was evaluated by dynamic CT or MRI every 3 months.

Statistical analysis

Data are expressed as the mean ± SD. The Mann–Whitney's *U*-test was used for statistical analyses of chondroitin-glucuronate C5-epimerase expression in HCC and non-cancerous liver tissues. The χ^2 test with Yates' correction and the unpaired *t*-test were used for univariate analysis of the effect of variables on the T-cell response against chondroitin-glucuronate C5-epimerase. A level of $P < 0.05$ was considered significant.

Results

Expression of chondroitin-glucuronate C5-epimerase in hepatoma cell lines and HCC tissues

Chondroitin-glucuronate C5-epimerase was expressed in all hepatoma cells (Fig. 1a) and its cellular distribution was cytoplasmic, similar to that in Paca-2, a pancreatic cancer cell line reported to express the protein (7). The expression was observed even in the hepatoma cell lines not expressing AFP, namely HLF and HLE.

The expression of chondroitin-glucuronate C5-epimerase in HCC tissues was examined in 26 HCC patients. A representative result for one HCC patient is shown in Fig. 1b. In this case, the expression of chondroitin-glucuronate C5-epimerase was observed in HCC tissue but not in non-cancerous areas. In addition, AFP was not detected in HCC tissue. To compare the expression levels of this protein between cancerous and non-cancerous tissues, the expression was semi-quantitatively classified into four categories as described in materials and methods, and analysed. The expression levels were higher in HCC tissue than in the non-cancerous tissue ($P < 0.0001$) (Fig. 1c). The expression in liver tissue was also observed in the patients with chronic hepatitis and cirrhosis (Control), however, the expression levels were lower than those in HCC tissue ($P = 0.0137$). The expression of chondroitin-glucuronate C5-epimerase and AFP in HCC tissue was observed in 26 (100%) and 12 (46%) of 26 patients respectively (Fig. 1d). The expression of chondroitin-glucuronate C5-epimerase was observed even in the HCC tissues without AFP expression.

Detection of chondroitin-glucuronate C5-epimerase-specific T cells by IFN-γ ELISPOT analysis

The clinical profiles of the 11 healthy normal donors, 12 patients with chronic hepatitis C, 11 patients with cirrhosis and 44 patients with HCC analysed in the present study are shown in Table 1.

To determine whether a significant number of T cells specifically reacted with the chondroitin-glucuronate C5-epimerase-derived peptides (peptide 1, 2 and 3) in HCC patients, ELISPOT assays were performed using PBMCs from 11 healthy donors (Fig. 2a). The number of specific spots was 1.0 ± 1.3, 1.5 ± 1.3 and 1.0 ± 1.4/3×10^5 PBMCs respectively. Similarly, cells that specifically reacted with the peptides were counted among chronic hepatitis C and cirrhosis patient-derived PBMCs. Regarding a value larger than the mean + 3SD

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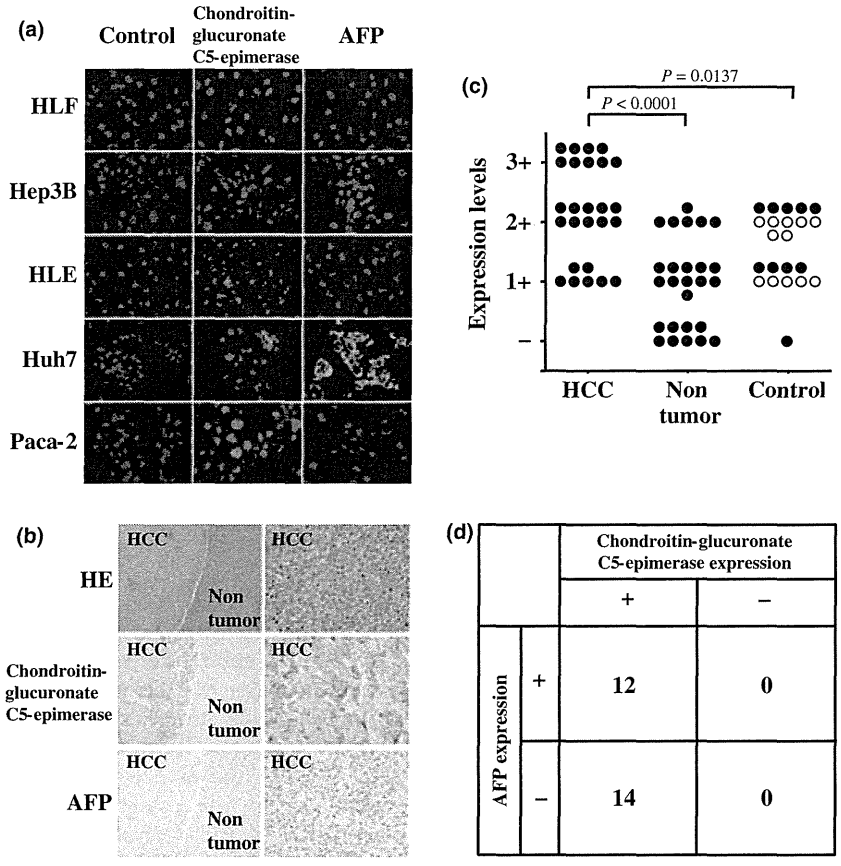

Fig. 1. Expression of chondroitin-glucuronate C5-epimerase. (a) immunofluorescence analysis for the expression of chondroitin-glucuronate C5-epimerase in hepatoma cell lines. Original magnification, ×400. (b) Immunohistochemical analysis for the expression of chondroitin-glucuronate C5-epimerase and AFP in sequential non-cancerous and HCC tissue sections. Original magnification, ×200 (left) and ×400 (right). (c) Analysis of chondroitin-glucuronate C5-epimerase expression levels among the three groups (HCC; tumour tissue in HCC patients, Non-tumour; non-tumour tissue in HCC patients, Control; liver tissue in disease control groups). Closed and open circles show the level of *chondroitin-glucuronate C5-epimerase expression in the patients with cirrhosis and chronic hepatitis respectively. (d) The expression of* chondroitin-glucuronate C5-epimerase was also compared with AFP expression in HCC tissues.

of the number of T cells that specifically reacted with the peptide in healthy donor-derived PBMCs as a significant response, 1 of 23 (4.3%) patients showed a significant response to each of the chondroitin-glucuronate C5-epimerase-derived peptides (Fig. 2a).

In the same analysis of HCC patients, 10.8, 16.2 and 27.0% of the patients showed significant responses to peptide 1, 2 and 3 respectively (Fig. 2b). A significant response specific to $CMVpp65_{328}$ was detected in 36.4%, 34.8% and 45.9% of healthy donors, disease control groups and HCC patients, respectively, with no significant difference among the three groups. On the other hand, no significant response to $HIVenv_{584}$ was observed in all groups.

To clarify the clinical characteristics of chondroitin-glucuronate C5-epimerase-specific T-cell responses in HCC patients, the clinical background was compared between patients who showed positive responses to chondroitin-glucuronate C5-epimerase-derived peptides and those who did not. The clinical features of both groups were not statistically different in terms of age, gender, serum AFP levels, differentiation of HCC, tumour multiplicity, vascular invasion, TNM factors and stages, histology of the non-tumour liver, liver function and the type of viral infection (Table 2). Chondroitin-glucuronate C5-epimerase-specific T cells had been generated even in the early stages of HCC.

Next, to examine the existence of chondroitin-glucuronate C5-epimerase-specific T cells among TILs, we performed a similar analysis in another seven patients from whom samples of both PBMCs and TILs could be obtained. In the assay using PBMCs and TILs, four of seven (57.1%) and five of seven (71.4%) patients, respectively, showed significant responses to chondroitin-glucuronate C5-epimerase-derived peptide (peptide 3) (Fig. 3a). A positive T-cell response in TILs was observed even in one patient without a positive T-cell response in PBMCs (patient 39).

Table 1. Characteristics of the patients studied

Clinical diagnosis	No. of patients	gender M/F	Age (yr) Mean ± SD	ALT (IU/L) Mean ± SD	AFP (ng/ml) Mean ± SD	Aetiology (B/C/Others)	Child-Pugh (A/B/C)	Diff. degree[a] (wel/mod /por/ND)	Tumour size[b] (large/small)	Tumour multiplicity (multiple/solitary)	Vascular Invasion (+/−)	TNM stage (I/II/IIIA/IIIB/ IIIC/IV)
Normal donors	11	8/3	35 ± 2	ND	ND	ND	ND	ND	ND	ND	ND	ND
Chronic hepatitis	12	7/5	54 ± 11	104 ± 119	12 ± 4	0/12/0	12/0/0	ND	ND	ND	ND	ND
Liver cirrhosis	11	5/6	60 ± 11	83 ± 73	79 ± 140	1/7/3	6/5/0	ND	ND	ND	ND	ND
HCC	44	35/9	66 ± 8	67 ± 32	1629 ± 7874	8/34/2	28/14/2	11/17/3/13	29/15	25/19	12/32	13/17/5/ 1/2/6

[a]Histological degree of HCC; wel: well-differentiated, mod: moderately differentiated, por: poorly differentiated, ND: not determined.
[b]Tumour size was divided into either 'small' ($\leq$2 cm) or 'large' (>2 cm).

Cytotoxic activity of chondroitin-glucuronate C5-epimerase-specific CTLs against hepatoma cell lines

Whether the chondroitin-glucuronate C5-epimerase-derived peptides used were capable of generating peptide-specific CTLs from PBMCs was investigated in 18 HCC patients. The CTLs specific to chondroitin-glucuronate C5-epimerase could be induced in 8 of 18 (44.4%) patients (Fig. 3b and c). They exhibited cytotoxicity against hepatoma cell lines with the HLA-A24 molecule and expression of chondroitin-glucuronate C5-epimerase, that correspond to HLF and HLE, but not against Hep3B and Huh7 cells without HLA-A24 (Fig. 3d).

Clinical safety of chondroitin-glucuronate C5-epimerase-derived peptide and its immunological effects

The clinical profiles of the 12 HCC patients with vaccination are shown in Table 3. The treatment was well-tolerated and there were no treatment-related serious adverse events. The most common adverse event was grade 1 injection-site reaction manifesting as pain, pruritus, skin induration and rubor. The worsening of hepatitis or liver function was not observed in any of the vaccinated patients.

In the analysis of ELISPOT assay using PBMCs of patients with vaccination, 4 patients demonstrated an immune response (Fig. 4a and Table 3). All of the patients that responded were immunized with 3.0 mg of peptide. None of the patients immunized with 0.03 or 0.3 mg of peptide showed an enhancement of peptide-specific immune response. The enhancement of immunological response to $HIVenv_{584}$ and $CMVpp65_{328}$ was not observed in any patients except patient A2.

To examine whether similar occurs for the immune response in HCC patients with only RFA, we analysed chondroitin-glucuronate C5-epimerase-derived peptide-specific T-cell responses in 12 HCC patients without vaccination, whose PBMCs were available for analysis at 2–4 weeks after RFA. In this analysis, we observed an increase of the frequency of chondroitin-glucuronate C5-epimerase-derived peptide-specific T cells in 2 of 12 patients (Fig. 4b). The frequency of the patients who showed an increase in the number of chondroitin-glucuronate C5-epimerase-derived peptide-specific T cells was higher in the patients with vaccination of 3 mg of peptide (66.7%) than in those without vaccination (16.7%).

Finally, we examined the HCC recurrence rate after RFA between the patients with and without the peptide-specific CTL response to examine the clinical effect of an increase of chondroitin-glucuronate C5-epimerase-derived peptide-specific CTLs after vaccination. In the analysis, the recurrence rate in the patients with an increase in the peptide-specific CTLs after vaccination (two of four patients, 50%) was lower than that in the patients without immune response (six of eight patients, 75%) at 300 days after RFA, although there was no sta-

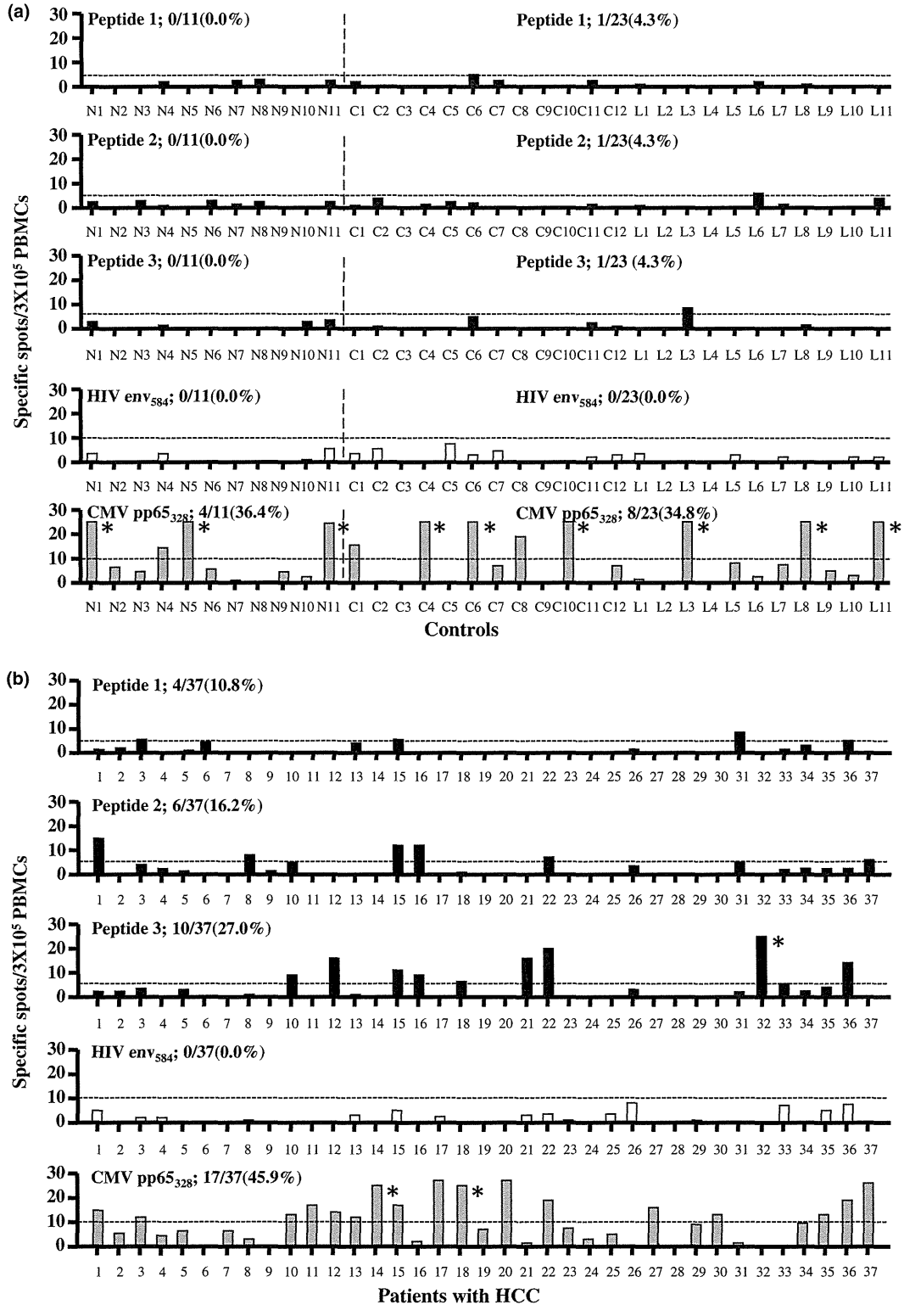

Fig. 2. Immune responses of chondroitin-glucuronate C5-epimerase-specific T cells. (a) IFN-γ ELISPOT assay of PBMCs to chondroitin-glucuronate C5-epimerase-derived peptides (peptides 1, 2 and 3: solid bars) or control peptides (peptides HIVenv$_{584}$ and CMVpp65$_{328}$: open and grey bars respectively) in normal donors and disease control groups. "N" denotes normal donors. "C" denotes the patients with chronic hepatitis. "L" denotes the patients with cirrhosis.% shows the ratio of the patients who showed positive responses. *denotes more than 30 specific spots. (b) IFN-γ ELISPOT assay in HCC patients. *denotes more than 30 specific spots.

Table 2. Univariate analysis of the effect of variables on the T-cell response against chondroitin-glucuronate C5-epimerase

	Patients with positive T-cell response	Patients without positive T-cell response	*P*-value[a]
No. of patients	15	22	
Age (years)[b]	64.6 ± 9.8	68.7 ± 5.9	NS
gender(M/F)	14/1	15/7	NS
AFP (ng/ml)	3569.7 ± 13070.0	580.7 ± 2394.2	NS
Diff. degree of HCC (well/moderate or poor/ND)[c]	3/7/5	8/6/8	NS
Tumour multiplicity (multiple/solitary)	10/5	13/9	NS
Vascular invasion (+/-)	5/10	6/16	NS
TNM factor			
(T1/T2–4)	4/11	8/14	NS
(N0/N1)	14/1	22/0	NS
(M0/M1)	13/2	20/2	NS
TNM stage (I/II–IV)	4/11	8/14	NS
Histology of non-tumour liver (LC/Chronic hepatitis)	12/3	20/2	NS
Liver function (Child A/B/C)	11/4/0	13/7/2	NS
Aetiology (HCV/HBV/Others)	11/3/1	20/1/1	NS
T-cell response against to CMV $pp65_{328}$ (+/-)	9/6	9/13	NS

[a]NS: not significant.
[b]Data are expressed as the mean ± SD.
[c]ND: not determined.

tistical significance owing to the small number of patients.

Discussion

Many tumour-associated antigens and their epitopes capable of inducing HLA-class I-restricted CTLs have been identified in various cancers. Some of the epitopes have been under investigation for the treatment of cancer, with major clinical responses in some trials (11, 20–22).

With regard to immunotherapy for HCC, AFP is considered a useful tumour-associated antigen and AFP-derived peptides have actually been used in clinical trials (5, 23–25). However, in general, the production of AFP depends on the size of the tumour, with AFP expressed in only 0–40% of HCCs less than 30 mm in size (26). Therefore, for immunotherapy for HCC in cases where AFP is not expressed in tumour tissue, it is necessary to identify other tumour-associated antigens.

In the present study, the expression of chondroitin-glucuronate C5-epimerase was observed in all of the HCC tissues examined and independent of differential degree, size, TNM stage and the expression of AFP in the tumour. These results suggest the advantage of this antigen as a target for immunotherapy of HCC.

On the other hand, the expression of this protein was also observed in non-cancerous tissue of HCC patients, although less frequently and at lower levels than in HCC tissue. Our results are consistent with the recent finding that chondroitin-glucuronate C5-epimerase is expressed in some normal tissues including liver tissue (6). Such results imply that immunotherapy targeting chondroitin-glucuronate C5-epimerase may have adverse effects on liver tissue expressing the protein. Therefore, we next examined the existence and specificity of chondroitin-glucuronate C5-epimerase-specific CTLs in HCC patients.

The presence of chondroitin-glucuronate C5-epimerase-recognizing CTLs has been reported as SART2-specific CTLs in lung, gastric and pancreatic cancer patients (7, 27, 28). However, to our knowledge, there has been no report of the presence of chondroitin-glucuronate C5-epimerase-specific CTLs in HCC patients except our recent study using only one SART2-derived peptide (10). In this study, we used three different HLA-A24 restricted peptides which were previously identified and derived from naturally processed squamous cell carcinoma antigen. The HLA-A24 allele is found in 60% of Japanese (29), and therefore, to use HLA-A24-restricted peptides has the advantage of analysing CTL responses to tumour-associated antigens in Japanese patients.

We showed that chondroitin-glucuronate C5-epimerase-specific CTLs could be generated by stimulating PBMCs with peptides, and the CTLs were cytotoxic to hepatoma cell lines. Chondroitin-glucuronate C5-epimerase-specific immune responses were observed frequently only in HCC patients and the frequency of CTLs was higher in HCC patients than control groups, indicating that the immune responses are specific to HCC. Furthermore, the CTLs were also detected among TILs, suggesting that they infiltrate the tumour. Based on these findings, we confirmed that chondroitin-glucuronate C5-epimerase-specific CTL precursors exist in HCC patients and the immune responses are specific for HCC.

In previous study, we reported that the frequency of TAA-derived peptide-specific CTLs in HCC patients was 0–92 cells/3 × 10^5 PBMCs and the frequency of the

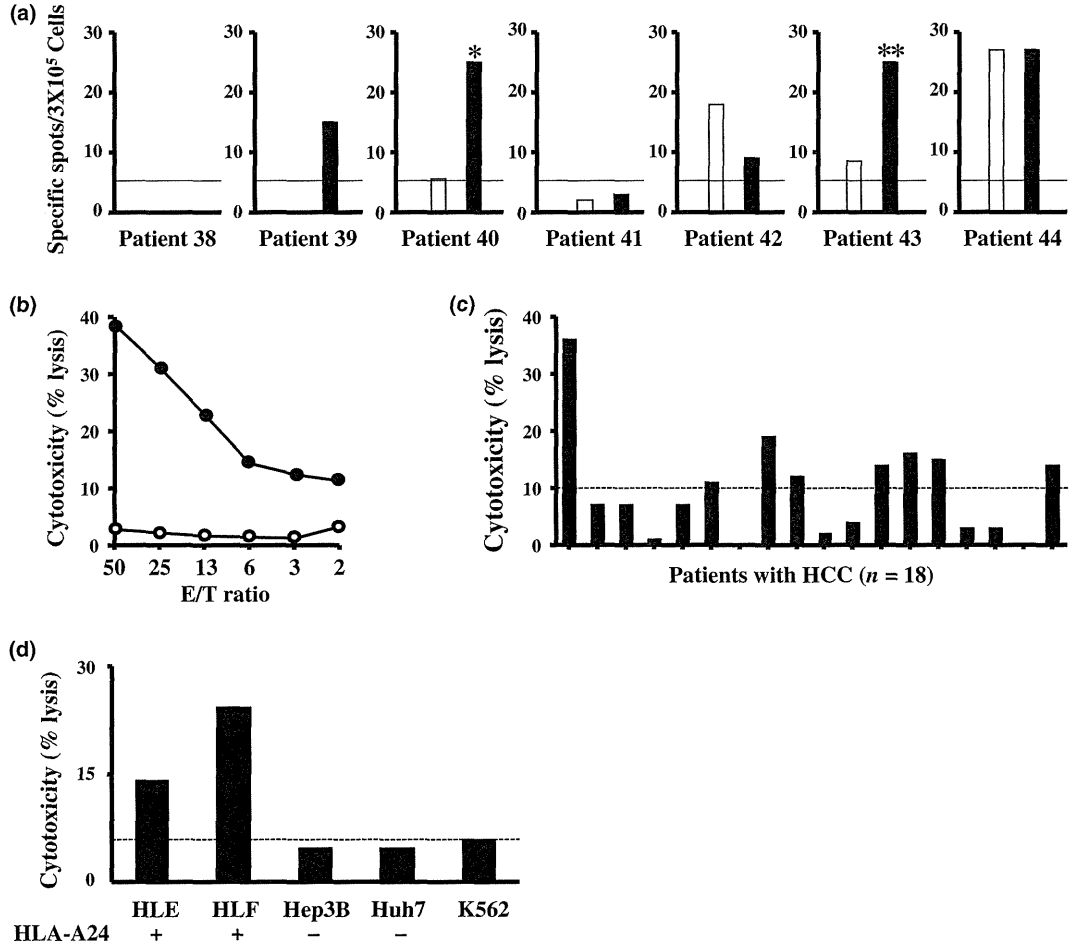

Fig. 3. Characteristics of chondroitin-glucuronate C5-epimerase-specific CTLs. (a) IFN-γ ELISPOT assay of PBMCs and TILs to one of the chondroitin-glucuronate C5-epimerase-derived peptide (peptide 3) in seven HCC patients. Open and solid bars show the frequency of chondroitin-glucuronate C5-epimerase-specific T cells in PBMCs and TILs respectively. *denotes 114 specific spots. **denotes 42 specific spots. (b) Representative results of the CTL assay. The closed and open circles show the cytotoxicity against C1R-A*2402 cells pulsed with and without a peptide respectively. (c) CTL assays (E/T ratio of 50:1) were performed in 18 HCC patients. Solid bars show the result for one patient. The results are shown as specific cytotoxic activity, which was calculated as follows: (cytotoxic activity in the presence of peptide) - (cytotoxic activity in the absence of peptide) and considered positive when higher than 10%. (d) Cytotoxicity of chondroitin-glucuronate C5-epimerase-specific T-cell lines derived with peptides was also measured against hepatoma cell lines. The cytotoxicity was considered positive when it was higher than that against K562 which shows non-specific lysis (E/T ratio of 50:1).

patients who showed immune responses to each peptide was 0–19% (10). In the present study, the frequency of chondroitin-glucuronate C5-epimerase-derived peptide-specific CTLs in HCC patients was 0–30 cells/ 3×10^5 PBMCs and the frequency of the patients who showed immune responses to the peptides was 11–27%. These results show that the frequencies of chondroitin-glucuronate C5-epimerase-specific CTLs in PBMCs and the patients with CTLs responsive to the TAA are very similar to those of previously identified immunogenic TAA-derived epitopes and suggest that the antigen and its CTL epitope are immunogenic. In addition, the CTLs were generated even in the early stages of HCC. These results suggest the advantages of using chondroitin-glucuronate C5-epimerase-derived peptides as a vaccine for immunotherapy of HCC.

For the next step to investigate the usefulness of chondroitin-glucuronate C5-epimerase as an immunotherapeutic target in HCC, we examined the safety and efficacy of chondroitin-glucuronate C5-epimerase-derived peptide as a cancer vaccine. In previous studies using chondroitin-glucuronate C5-epimerase-derived peptides for several cancers, they were reported to be safe. However, most patients with HCC have chronic liver disease. Therefore, safety of the peptide vaccine should be confirmed in the patients with chronic hepatitis or cirrhosis. The present vaccination study included nine patients with chronic liver diseases (four chronic hepatitis and five cirrhotic patients) confirmed by histological examination and there was no severe adverse event in all patients vaccinated. The induction of chondroitin-glucuronate C5-epimerase-specific CTLs

Table 3. Patient characteristics

Patient	Peptide Dose (mg)	Age	gender	Aetiology	Stage of HCC	ALT (IU/L)	AFP (ng/ml)	Child-Pugh (A/B/C)	Histology of liver	Treatment	Immune response	Toxicity (grade)
A1	0.03	73	F	HCV	I	26	12	A	F4A2	RFA	–	Pa(1)
A2	0.03	78	F	HCV	I	45	10	B	F4A2	RFA	–	P(1)
A3	0.03	59	M	NBNC	II	30	10	A	ND	RFA	–	None
B1	0.3	79	M	HCV	I	40	61	A	F3A1	RFA	–	R(1), S(1)
B2	0.3	72	M	NBNC	II	24	66	A	ND	RFA	–	R(1), S(1), P(1), H(1)
B3	0.3	78	M	HCV	II	45	10	A	F3A2	RFA	–	P(1)
C1	3.0	67	M	HCV	I	111	49	A	F3A1	RFA	+	P(1), S(1)
C2	3.0	73	M	NBNC	I	30	5	A	ND	RFA	–	None
C3	3.0	78	F	HCV	I	23	24	A	F4A2	RFA	+	P(1)
C4	3.0	75	M	HBV	I	21	15	A	F3A1	RFA	+	R(1), P(1)
C5	3.0	49	M	HBV	I	18	14	A	F4A1	RFA	+	None
C6	3.0	69	F	HBV	II	42	84	A	F4A2	RFA	–	Pa(1)

H, headache; Pa, pain; P, pruritus; R, rubor; S, skin induration.

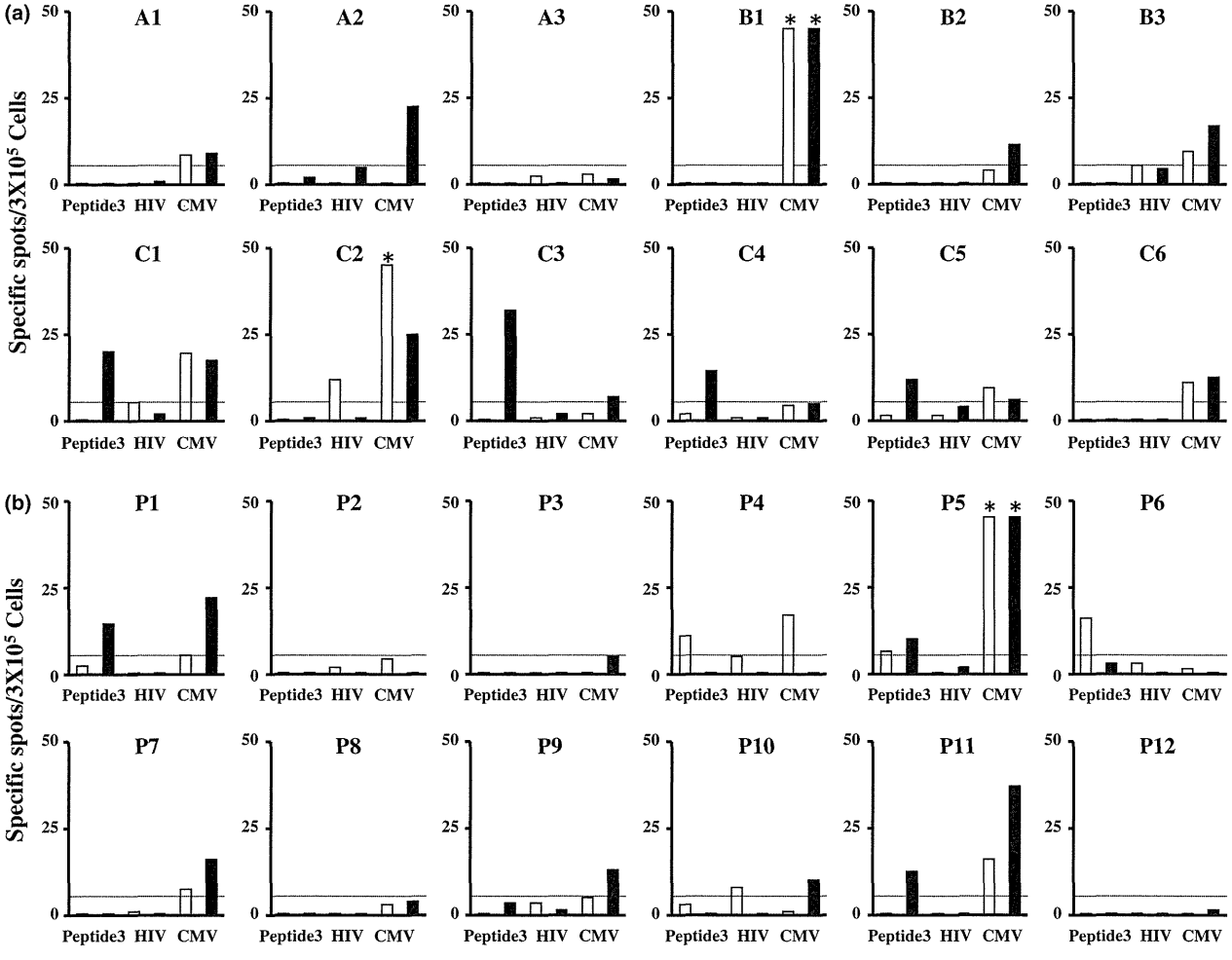

Fig. 4. IFN-γ ELISPOT assays of PBMCs to chondroitin-glucuronate C5-epimerase-derived peptide (peptide 3) or control peptides (peptides $HIVenv_{584}$ and $CMVpp65_{328}$) in HCC patients with RFA. (a) The assays were performed in the patients with peptide 3 vaccination. White and black bars show the T-cell responses before and after vaccination respectively. (b) The assays were also performed in the patients without vaccination. White and black bars show the T-cell responses before and after RFA respectively. *denotes more than 50 specific spots.

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was observed in four of six (66.7%) patients vaccinated with 3 mg of peptide, which is similar to the frequency of responded patients reported in other peptide vaccination studies (11, 20).

Apart from induction of CTLs, the efficacy of chondroitin-glucuronate C5-epimerase-derived peptides as a vaccine for advanced HCC is still unclear. In previous vaccine studies for advanced HCC, AFP, hTERT and glypican-3 have been targeted as tumour-associated antigens for the treatment (25, 30–32). In these studies, peptide-specific CTLs were reported to be induced in 10–80% of vaccinated patients. However, in spite of the induction of peptide-specific CTLs, it has been reported that the anti-tumour effect was very limited. Recent studies have shown that the frequency of myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) is increased in HCC patients and the cells inhibit the function of T cells (33, 34). Therefore, controlling their function might be important to develop more effective vaccination for advanced HCC.

In contrast, other recent studies using chondroitin-glucuronate C5-epimerase-derived peptides for other advanced cancers have shown the induction of cellular immune responses and clinical responses for certain patients (9, 11). In the analysis of the prognosis of patients with RFA and chondroitin-glucuronate C5-epimerase-derived peptide vaccination in the present study, the recurrence rate in the patients with an increase in the peptide-specific CTLs after vaccination was lower than that in the patients without immune response. Although further studies are necessary to evaluate the efficacy of chondroitin-glucuronate C5-epimerase-derived peptides for HCC, the results of our study suggest that chondroitin-glucuronate C5-epimerase is a potential candidate for a target of HCC immunotherapy.

In conclusion, chondroitin-glucuronate C5-epimerase is a potential candidate for a tumour antigen with immunogenicity, and peptides derived from the protein would be useful for immunotherapy in cases of HCC.

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Conflict of interest: The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Cancer Research

Acyclic Retinoid Targets Platelet-Derived Growth Factor Signaling in the Prevention of Hepatic Fibrosis and Hepatocellular Carcinoma Development

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Abstract

Hepatocellular carcinoma (HCC) often develops in association with liver cirrhosis, and its high recurrence rate leads to poor patient prognosis. Although recent evidence suggests that peretinoin, a member of the acyclic retinoid family, may be an effective chemopreventive drug for HCC, published data about its effects on hepatic mesenchymal cells, such as stellate cells and endothelial cells, remain limited. Using a mouse model in which platelet-derived growth factor (PDGF)-C is overexpressed (*Pdgf-c Tg*), resulting in hepatic fibrosis, steatosis, and eventually, HCC development, we show that peretinoin significantly represses the development of hepatic fibrosis and tumors. Peretinoin inhibited the signaling pathways of fibrogenesis, angiogenesis, and Wnt/β-catenin in *Pdgf-c* transgenic mice. *In vitro*, peretinoin repressed the expression of PDGF receptors α/β in primary mouse hepatic stellate cells (HSC), hepatoma cells, fibroblasts, and endothelial cells. Peretinoin also inhibited PDGF-C–activated transformation of HSCs into myofibroblasts. Together, our findings show that PDGF signaling is a target of peretinoin in preventing the development of hepatic fibrosis and HCC. *Cancer Res; 72(17); 4459–71. ©2012 AACR.*

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide with a particularly poor patient outcome (1). It often develops as a result of chronic liver disease associated with hepatitis B or hepatitis C virus infection or with other etiologies such as long-term alcohol abuse, autoimmunity, and hemochromatosis (2–5). Despite the recent advances in antiviral therapy for hepatitis B or hepatitis C virus, these are insufficient to completely prevent the occurrence of HCC. Moreover, the recent increase in nonalcoholic fatty liver disease (NAFLD) associated with metabolic syndrome is a potential high-risk factor for the development of HCC (6).

HCC often develops during the advanced stages of liver fibrosis and is associated with deposits of extracellular matrix synthesized by activated stellate cells. During the course of chronic hepatitis, nonparenchymal cells, including Kupffer, endothelial, and activated stellate cells, release a variety of cytokines and growth factors. One of these growth factors is platelet-derived growth factor (PDGF), which is involved in fibrogenesis, angiogenesis, and tumorigenesis (7, 8). PDGF expression has been shown to be upregulated from the early stages of chronic hepatitis, suggesting its association with the development of fibrosis in chronic hepatitis C (CH-C; refs. 9 and 10). Overexpression of PDGF-C in mouse liver resulted in the progression of hepatic fibrosis, steatosis, and the development of HCC; this mouse model closely resembles the human HCC, which is frequently associated with hepatic fibrosis (7).

Peretinoin (generic name; code, NIK-333), developed by the Kowa Company, is an oral acyclic retinoid with a vitamin A–like structure, which targets the retinoid nuclear receptor. Oral administration of peretinoin was shown to significantly reduce the incidence of posttherapeutic HCC recurrence and improve the survival rates of patients in a clinical trial (11, 12). A large-scale clinical study including various countries is now planned to confirm its clinical efficacy.

Although peretinoin treatment can suppress HCC-derived cell line growth and inhibit experimental mouse or rat liver carcinogenesis (13, 14), the detailed mechanism of its effect has not been fully elucidated. Peretinoin has a high binding affinity to cellular retinoic acid-binding protein (15) and may interact with retinoic acid receptor-β and retinoid X receptor-α (16); however, the precise molecular targets for preventing HCC recurrence have not yet been elucidated.

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