fibrosis,[11–15] and higher COX-2 expression in the cirrhotic liver has been reported to be a significant independent risk factor for residual liver HCC recurrence after curative surgery for HCC.[15]

Various kinds of transcriptional regulatory factor binding sites and several single nucleotide polymorphisms (SNP) are present in the promoter region of the *COX-2* gene. Some of these SNP influence the risk of esophageal and prostate cancer.[16–19]

Single nucleotide polymorphisms (−616G>T, −443T>C, and −155−>G) are also present in the promoter region of the osteopontin (*OPN*) gene and a promoter/enhancer region of the forkhead box protein 3 (*FOXP3*) ([GT]n) gene. OPN is one of the extracellular matrix proteins that has been identified as an early T-lymphocyte activation antigen (Eta-1) and is produced by activated T cells.[20] It is a key cytokine for the initiation of T-helper cell (Th)1 type immune reaction and promotes tumor metastasis at the carcinoma site.[21] OPN has also been reported to affect various immune responses such as anti-infectious and antitumor immune responses and induce autoimmune disease.[22]

FOXP3 is a transcriptional factor that is mainly expressed in $CD4^+CD25^+$ regulatory T cells (Treg) and it suppresses immune responses. Treg deficiency is one of the mechanisms for initiation and promotion of autoimmune diseases. It has been reported that the frequency of Treg is much higher in people with chronic HCV than that in healthy controls and that Treg suppress HCV-specific immune response.[23–25]

In the present study, we examined the SNP in the promoter regions of the *COX-2* and *OPN* genes and the promoter/enhancer region of the *FOXP3* ([GT]n) gene in patients with HCV infection and studied the relationship between these SNP and susceptibility to HCV infection and the progression of liver disease. We showed that the SNP of the *COX-2* promoter region is involved in susceptibility to HCV infection and progression of liver disease in the Japanese and that the SNP of the *OPN* promoter region affects the inflammatory activities in HCV infection.

METHODS

Patients and blood samples

PERIPHERAL BLOOD SAMPLES were obtained from 104 Japanese patients with chronic HCV infection and 74 healthy Japanese donors. All HCV-infected patients were positive for HCV RNA but negative for hepatitis B surface antigen (HBsAg). Patient characteristics are shown in Table 1. Healthy controls were negative for HCV antibody and HBsAg, and had no autoimmune disease. All patients and controls gave written informed consent according to a protocol approved by the Ethical Committee of Showa University.

Table 1 Characteristics of patients with chronic hepatitis C virus (HCV) infection

Number	104
Age, years	55.8 (14.2)§
Male : female	57:47
Platelet (10^4/μL)	16.5 (6.6)
AST (IU/L)	57.6 (39.3)
ALT (IU/L)	76.6 (60.1)
γ-GT (IU/L)	72.7 (84.0)
ALP (IU/L)	296.3 (139.7)
Total bilirubin (mg/dL)	0.7 (0.3)
Albumin (g/dL)	4.0 (0.5)
HCV RNA levels† (high : low : ND)‡	92:5:7
HCV genotype (1 : 2 : other and ND)‡	57:28:19

†HCV RNA levels: high ≥100 KIU/mL, low <100 KIU/mL.
‡ND: not determined.
§Mean (SD).
ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GT, γ-glutamyl transpeptidase.

SNP analysis

Genomic DNA was extracted from peripheral blood using a DNA isolation and purification system (Magtration System 6GC; Precision System Science, Chiba, Japan). DNA polymorphisms in the promoter region of the *COX-2* gene were determined using a fluorescence-based polymerase chain reaction single-strand conformation polymorphism (PCR–SSCP) analysis.[26,27] Primer sequences for amplifying the DNA fragment containing the −1195G>A region were 5′-GAGCACTACCCATGA TAGATG-3′ (forward) and 5′-TGTTGTACTTTGATCC ATGGT-3′ (reverse) and those for the −765G>C region were 5′-ACAGGGTAACTGCTTAGGAC-3′ (forward) and 5′-ACAGCTATGTACACTGAAGG-3′ (reverse). The 5′-end of one of the primers was labeled with cyanine-5 or 6-carboxyfluorescein (6-FAM). The DNA fragments were amplified using ExTaq DNA polymerase (Takara, Shiga, Japan) containing 5% dimethylsulfoxide (DMSO) under the following cycling conditions: 94°C for 1 min followed by 30 cycles at 94°C for 1 min, 60°C (−1195G>A region), or 58°C (−765G>C region) for 30 s, and 72°C for 30 s. Nucleotide variations in the DNA fragments were analyzed by the SSCP method

using an ALFexpress automated DNA sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden) or an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The nucleotide variation of each sample was determined using the wave pattern. The nucleotide sequences of the DNA variations were confirmed by DNA sequence analysis using a BigDye Terminator ver. 3.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI PRISM 3100 Genetic Analyzer.

The polymorphisms in the promoter region of *OPN* gene were also determined using PCR–SSCP analysis. Primer sequences for amplifying the DNA fragment containing the −616G>T and −443T>C regions were 5′-ACGGTCTGGCTCCTGAAGCA-3′ (forward) and 5′-AGGCTATTGTTCAAGCCTGC-3′ (reverse). The 5′-end of the forward primer was labeled with 6-FAM. The DNA fragment was amplified with Phusion DNA polymerase (Finnzymes, Oy, Finland) containing 0.3% DMSO under the following cycling conditions: 98°C for 30 s, followed by 25 cycles at 98°C for 5 s, 60°C for 10 s, 72°C for 15 s and 72°C for 5 min. The primers used for amplifying the −155–>G region were 5′-ATGCTGAATGCCCATCCCGT-3′ (forward) and 5′-GTCATGAGGTTTTCTGCCAC-3′ (reverse). The 5′-end of the reverse primer was labeled with 6-FAM. The DNA fragment was amplified using ExTaq DNA polymerase containing 5% DMSO under the following cycling conditions: 94°C for 1 min followed by 30 cycles at 94°C for 1 min, 60°C for 30 s and 72°C for 30 s. The reaction mixture was applied to an ABI PRISM 3100 Genetic Analyzer.

For analysis of the promoter/enhancer region of the *FOXP3* gene, we amplified the intron zero containing the (GT)n microsatellite polymorphism with the primers 5′-GGTGCTGGACCTCTGCACGT-3′ (forward) and 5′-CCACCTGAGCCACGTGCACA-3′ (reverse). The 5′-end of the forward primer was labeled with 6-FAM. The DNA fragment was amplified with ExTaq DNA polymerase containing 5% DMSO under the following cycling conditions: 94°C for 1 min followed by 30 cycles at 94°C for 1 min, 65°C for 30 s and 72°C for 30 s. Genotyping was performed in a mixture of amplified products and internal size standard by an ABI PRISM 3100 Genetic Analyzer. Reagents and primers were obtained from Sigma Genosys (Hokkaido, Japan) and Exigen (Tokyo, Japan), respectively.

Luciferase assay

To compare the effects of nucleotide variations in the promoter region on COX-2 transcriptional activity, we analyzed promoter activity using a luciferase reporter assay. DNA fragments of the −1630 to the −1 region of the *COX-2* promoter containing the −1195G>A and −765G>C variations were synthesized with the primers 5′-GTAAAACTCGAGCCATGCAATAAATAGGAGTGCC-3′ and (forward) and 5′-GTAAAAAAGCTTGTCGCTAAC CGAGAGAACCT-3′ (reverse). DNA fragments were amplified using Phusion DNA polymerase (Finnzymes) containing 5% DMSO under the following cycling conditions: 98°C for 30 s followed by 30 cycles at 98°C for 10 s, 60°C for 30 s, 72°C for 1 min and 72°C for 10 min. The amplified DNA fragments were ligated with the luciferase reporter vector pGL4 (Promega, Madison, WI, USA). The nucleotide sequence of the fragment inserted into each plasmid was confirmed by DNA sequencing. The plasmids were transfected into the HCC cell lines, HepG2 and Huh7 cells, and the human epithelial cervical cancer cell line HeLa using FuGENE HD transfection reagent (Roche, Basel, Switzerland). The pRL-TK plasmid containing the Renilla luciferase gene (Promega) was co-transfected with the pGL4-derived plasmids as an internal standard. At 24 h after transfection, cell extracts were prepared and luciferase activity was measured by the Dual-Luciferase Reporter Assay System (Promega). Transcriptional activity was determined from the level of firefly luciferase after normalization against Renilla luciferase activity. The transfection process was repeated three times.

Statistical analysis

Odds ratios and 95% confidence intervals for the SNP in HCV infection were calculated by logistic regression and adjusted for sex and age. The relationships between the SNP and platelet counts or serum alanine aminotransferase (ALT) levels were analyzed using the Wilcoxon rank sum or Kruskal–Wallis tests. Activities of the luciferase assay were compared using Student's *t*-test. Statistical analyses were performed using JMP ver. 5 (SAS Institute, Tokyo, Japan). Statistical differences were identified at $P < 0.05$.

RESULTS

−1195GG genotype in the promoter region of the *COX-2* gene was detected less frequently in patients with HCV infection

GENOTYPIC FREQUENCIES OF the SNP in the promoter region of the *COX-2* gene were analyzed in HCV-infected patients and healthy controls. The −1195GG genotype was detected less frequently in

Table 2 Genotype frequencies of the promoter regions of the *COX-2* and *OPN* genes in patients with chronic hepatitis C virus (HCV) infection and controls

Genotype	Controls $n = 74$ n (%)	HCV $n = 104$ n (%)	OR (95% CI)	*P*-value
COX-2				
−1195G>A				
AA	20 (27.0)	43 (41.3)	Reference	
GA	32 (43.2)	48 (46.2)	0.77 (0.36–1.62)	0.005
GG	22 (29.7)	13 (12.5)	0.23 (0.09–0.59)	
−765G>C				
GG	70 (94.6)	100 (96.2)	Reference	
GC	4 (5.4)	4 (3.8)	0.70 (0.16–3.08)	0.636
CC	0 (0.0)	0 (0.0)	Not calculated	
OPN				
−616G>T				
TT	7 (9.5)	9 (8.7)	Reference	
GT	24 (32.4)	39 (37.5)	1.14 (0.35–3.69)	0.445
GG	43 (58.1)	56 (53.8)	0.74 (0.24–2.28)	
−443T>C				
CC	15 (20.3)	13 (12.5)	Reference	
CT	30 (40.5)	55 (52.9)	2.07 (0.82–5.18)	0.271
TT	29 (39.2)	36 (34.6)	1.47 (0.57–3.80)	
−155–>G				
GG	8 (10.8)	9 (8.7)	Reference	
G–	23 (31.1)	41 (39.4)	1.40 (0.45–4.39)	0.326
– –	43 (58.1)	54 (51.9)	0.83 (0.27–2.49)	

Statistical analysis was performed using multiple logistic analysis and was adjusted for sex and age.
CI, confidence interval; OR, odds ratio.

HCV-infected patients than in healthy controls (Table 2). No significant difference was detected in the frequency of SNP of the −765G>C SNP between patients and controls. The frequencies of the −1195G>A and −765G>C SNP in the healthy controls were similar to those obtained from a large Japanese population study.[28] These results suggest that the −1195GG genotype is protective against HCV infection.

−1195GG genotype of the *COX-2* promoter region contributes to liver injury progression in patients with chronic HCV infection

We investigated the relationship between the SNP and platelet counts in patients with chronic HCV infection to evaluate whether the SNP of the *COX-2* promoter region was involved in the progression of liver disease because platelet counts have been reported to reflect the stages of chronic HCV infection and liver fibrosis.[29,30] Patients with the −1195GG genotype had significantly lower platelet counts than those with the −1195AA or AG genotype (median [range] $12.8 \times 10^4/\mu L$ [6.0–$25.9 \times 10^4/\mu L$] vs $16.9 \times 10^4/\mu L$ [5.2–$34.0 \times 10^4/\mu L$]) (Fig. 1). The results suggest that, once chronic HCV infection is established, the −1195GG genotype contributes to the progression of liver disease.

COX-2 promoter region containing the −1195G genotype showed significantly higher transcriptional activity than that containing the −1195A genotype in cell lines *in vitro*

To analyze the effect of SNP on transcriptional activity, we constructed a plasmid containing the −1195A and −765G SNP or the −1195G and −765G SNP. The plasmids were co-transfected with pRL-TK into the HCC cell lines HepG2 and Huh7 and the epithelial cervical cancer cell line HeLa. At 24 h after transfection, we

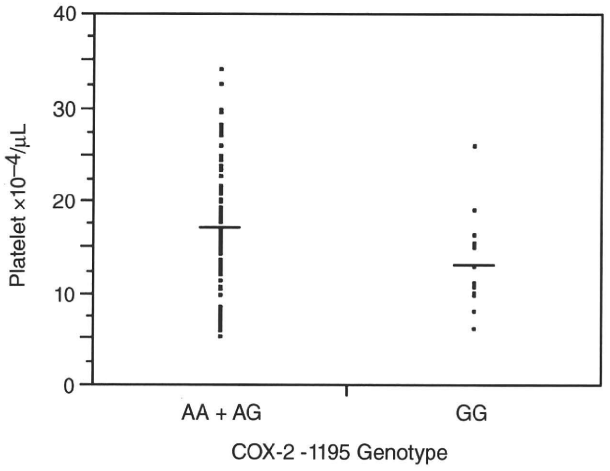

Figure 1 The −1195G>A genotype of the promoter region of the *COX-2* gene and platelet counts in patients with chronic hepatitis C virus infection. Platelet counts were significantly lower in patients with the −1195GG genotype of the promoter region of the *COX-2* gene than those with the −1195AA or AG ($P = 0.04$). The horizontal lines on the plots indicate the means.

determined the transcriptional activity by dual luciferase reporter analysis. When the activity of the promoter region containing −1195A was defined as 100%, the relative activity (mean ± standard deviation) of that containing −1195G was 169 ± 42% in HepG2 cells, 162 ± 38% in Huh7 cells and 154 ± 34% in HeLa cells (Fig. 2). The promoter region containing −1195G showed significantly higher transcriptional activity than that containing −1195A in all three cell lines. The results suggest that the COX-2 promoter region containing the −1195G allele increases transcriptional activity in liver cells and enhances COX-2 expression. Because the −1195GG genotype was more frequently observed in patients with low platelet counts, the high levels of COX-2 expression would be involved in the progression of liver injury.

Association observed between the −443T>C genotype of the OPN promoter region and ALT levels of patients with chronic HCV infection

We also examined the SNP of the promoter region of OPN (−616G>T, −443T>C and −155−>G) and found no significant differences between chronic HCV patients and controls (Table 2). However, patients with the −443TT genotype had significantly higher serum ALT levels than those with the −443CC or CT genotype (median [range] 76 IU/L [17–319 IU/L] vs 46 IU/L [9–266 IU/L]) (Fig. 3).

No relationship between microsatellite polymorphisms of the promoter/enhancer region of the *FOXP3* (GT)n gene and HCV infection.

We separately analyzed the polymorphisms in women and men because the *FOXP3* gene is located on chromosome Xp11.23. We determined the frequency of the $(GT)_{15}$ polymorphism because it has been reported that the major $(GT)_{15}$ dinucleotide repeat has stronger enhancer activity than that of the $(GT)_{16}$ repeat by a luciferase reporter assay using HeLa, COS-7 and Jurkat T cells.[31] However, we detected no differences in the polymorphisms between HCV positive patients and controls (Table 3).

DISCUSSION

IN THE PRESENT study, we found that the frequency of the −1195G>A genotype of *COX-2* was significantly different between patients with chronic HCV infection and healthy controls in the Japanese. We also found that the transcriptional activity of the *COX-2*

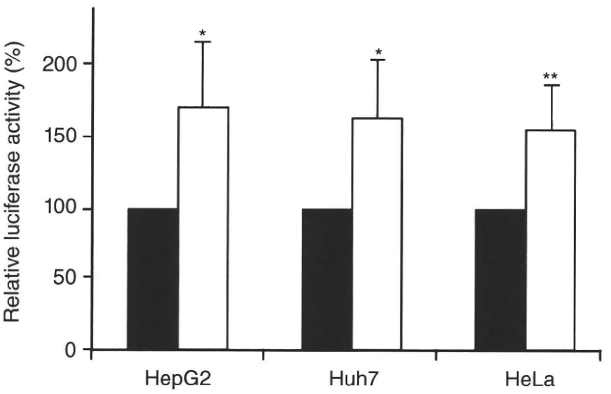

Figure 2 Comparison of the transcriptional activity of the *COX-2* promoter region containing the −1195A and −765G (closed bar) and that containing the −1195G and −765G (open bar) in HepG2, Huh7 and HeLa cells using a luciferase reporter assay as described in Methods. The average relative luciferase activity is shown from three independent transfection experiments, and each was performed in triplicate. The activity of the COX-2 promoter region containing the −1195A was defined as 100%. The vertical lines above bars indicate the standard deviations $*P < 0.05$, $**P = 0.05$.

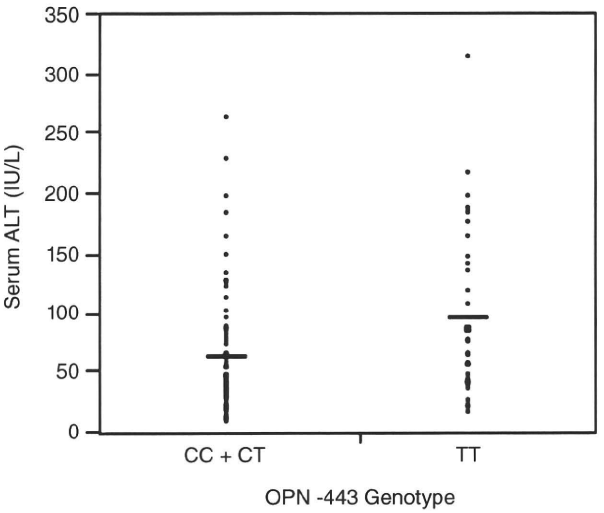

Figure 3 Relationship between the −443T>C genotype of the promoter region of the *OPN* gene and alanine aminotransferase (ALT) levels in patients with chronic hepatitis C virus infection. ALT levels were significantly higher in patients with the *OPN* promoter region containing −443TT genotype than those with the −443CC or CT genotype ($P = 0.01$). The horizontal lines on the plots indicate the means.

gene with the −1195G variant is significantly higher than that with the −1195A variant. We confirmed this by using three cell lines. However, one report states that the transcriptional activity of the *COX-2* gene with the −1195A variant is higher than that with the −1195G variant in HeLa cells,[16] which were also used in our experiment. These contrary results may be due to the different lengths of the DNA fragments used in the experiments. On the basis of our results, we assume that the −1195G variant has much more activity than the −1195A variant in hepatocytes. The −1195GG genotype was observed less frequently in patients with chronic HCV infection compared with HCV non-infected controls and was observed more frequently in patients with low platelet counts. These results suggest that the −1195G allele may resist HCV infection by inducing strong COX-2 expression. It has been reported that, indeed, prostaglandins affect both promotion and inhibition of virus replication.[32–35] However, once persistent HCV infection is established in hepatocytes, the −1195GG genotype promotes liver inflammation by inducing strong COX-2 expression and progressing liver injury. Genetic variation of this site may not only alter transcriptional gene activity and affect HCV infection susceptibility but also enhance HCV-induced liver disease progression.

Interferon (IFN) is used to eradicate HCV infection and reduce HCV-related liver damage. IFN treatment has been reported to reduce COX-2 expression in the liver in chronic HCV.[36] The promoter region of *COX-2* contains several transcription binding sites (C/EBP, AP2, SP1, NF-κB, CRE, Ets-1, PEA-3 and GATA-1).[37,38] The HCV NS3 protein enhances *COX-2* gene promoter activity, *COX-2* mRNA expression, COX-2 protein production and prostaglandin E2 release in HepG2 cells, all of which are regulated by NF-κB and multiple signaling components including JNK, ERK and PKD2.[9] The HCV core and NS5A proteins upregulate *COX-2* gene expression in hepatocyte-derived cells.[10] The genetic variant −1195G in the *COX-2* promoter region enhanced the promoter activity in our experiments. It is thought that the −1195G allele and HCV have an additive effect on enhancement of COX-2 expression, which affects transcription binding sites.

Because there is a close relationship between HCV infection and COX-2 expression, reduction of COX-2 expression may help control HCV-induced chronic liver injury. Unlike COX-1, COX-2 expression is undetectable in most normal tissues. In HCV-induced liver injury, inhibition of COX-2 expression may have two therapeutic potentials: improvement of hepatic inflammation and suppression of carcinogenesis. It has been reported that COX-2 inhibitors can promote apoptosis and sup-

Table 3 Genotype frequencies of the promoter/enhancer region of the *FOXP3* gene in patients with chronic hepatitis C virus (HCV) infection and controls

	Women			Men		
	n	Genotype		*n*	Genotype	
		$(GT)_{15}/(GT)_{15}$	others		$(GT)_{15}$	others
Control	32	7 (21.9)†	25 (78.1)	42	15 (35.7)	27 (64.3)
HCV	49	11 (22.4)	38 (77.6)	55	21 (38.2)	34 (61.8)

†Number (%).

press growth of a human hepatoma cell line.[39,40] Thus, COX-2 may be a target for preventing progression to cirrhosis, development of HCC, and recurrence of HCC after surgical or local therapy. Indeed, we showed that the −1195GG genotype was observed more frequently in patients with low platelet counts. This suggests that the −1195GG genotype would contribute to progression of liver injury. COX-2 inhibitors may reduce liver damage. This would be an attractive approach for patients who were not able to achieve sustained virological responses by IFN therapy. However, COX-2 inhibitors have several serious side-effects such as renal, gastrointestinal and cardiovascular problems. New COX-2 inhibitors without serious side-effects would be needed to treat patients with chronic HCV infection.

Although there was no difference in SNP of the promoter region of the *OPN* gene between healthy controls and patients with chronic HCV infection, the −443TT genotype was associated with increased levels of ALT. The frequency of the −443TT genotype has been reported to be higher in patients with high ALT levels.[41] Thus, an SNP of the *OPN* promoter region of −443T>C may affect hepatitis activity. No difference was observed in SNP of the promoter/enhancer region of the *FOXP3* gene between patients with chronic HCV infection and healthy controls.

In conclusion, our results suggest that the −1195GG genotype of the *COX-2* promoter region is protective against HCV infection in the Japanese. However, once chronic infection is established, the −443TT genotype of the *OPN* promoter region and the −1195GG genotype of the *COX-2* promoter are thought to promote inflammation and contribute to the progression of liver disease.

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Reactivation of Epstein–Barr Virus in B Cells of Patients With Chronic Hepatitis C

Yuu Shimozuma, Takayoshi Ito,* Momoko Inokuchi, Manabu Uchikoshi, Miyuki Miyashita, Hisako Nozawa, Tomoe Shimazaki, Kazumasa Hiroishi, and Michio Imawari

Division of Gastroenterology, Department of Medicine, Showa University School of Medicine, Tokyo, Japan

Hepatitis C virus (HCV) infection is associated with lymphoproliferative disorders. HCV infection of B cells is a predictive factor for lymphoproliferative disorders in patients with chronic hepatitis C, although its molecular mechanisms remain unknown. Epstein–Barr virus (EBV) is a B cell-tropic virus with the potential to cause lymphoproliferative disorders, and its reactivation is induced by several viruses and cytokines. The possibility that HCV infection triggers reactivation of EBV and induces lymphoproliferative disorders were investigated. Expression of EBV mRNAs was analyzed by RT-PCR in patients infected with HCV and control subjects, and correlations between reactivation of EBV and markers for lymphoproliferative disorders were investigated. BZLF1 mRNA, a starter molecule of reactivation, was detected in peripheral blood mononuclear cells from 12 of 52 (23%), patients infected with HCV and the frequency was higher than in healthy subjects [3 of 43 (9%), $P=0.032$]. But the presence of the BZLF1 mRNA was not associated with an abnormality of markers for lymphoproliferative disorders. This study on BZLF1 mRNA expression among lymphoid cell subsets showed that reactivation of EBV was observed specifically in B cells. The BZLF1 mRNA disappeared following anti-viral therapy and remained negative after eradication of HCV in patients with a sustained viral response, while the EBER1 RNA, a marker for persistence of EBV, was detected throughout the therapy. Infection with HCV induces reactivation of EBV in B cells, but this reactivation was not associated directly with lymphoproliferative disorders triggered by HCV. ***J. Med. Virol. 82:2064–2072, 2010.***

KEY WORDS: hepatitis C virus; Epstein–Barr virus; reactivation; BZLF1; lymphoproliferative disorders

INTRODUCTION

Hepatitis C virus (HCV) is a causative agent for chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) [Tong et al., 1995; Ikeda et al., 1998]. In addition, some patients infected with HCV develop proliferative disorders of lymphocytes, such as mixed cryoglobulinemia [Agnello et al., 1992; Frangeul et al., 1996; Donada et al., 1998] and B cell non-Hodgkin's lymphoma (NHL) [Ferri et al., 1994]. Although an epidemiological association has been noted between HCV infection and lymphoproliferative disorders, the underlying pathogenic mechanisms remain unclear. HCV is reported to infect B cells persistently, and somatic hypermutations in immunoglobulin genes have been observed in B cell lines infected with HCV as well as proto-oncogenes [Sung et al., 2003; Machida et al., 2004, 2005]. These observations indicate direct and/or indirect effects of HCV infection of B cells on the induction of lymphoproliferative disorders. In our previous study, infection of B cells and/or adsorption with HCV was observed in 64% of patients infected with HCV [Inokuchi et al., 2009]. Furthermore, the HCV RNA in B cells was an independent factor associated with the presence of markers for lymphoproliferation. HCV infection or phenomena induced by HCV may trigger the clonal proliferation of B cells, which leads to the development of lymphoproliferative disorders. The presence of monoclonal B cells circulating in patients infected with HCV was confirmed by analysis for rearrangement of the clonal immunoglobulin heavy-chain (IgH) gene, although its molecular mechanism remains unknown [Zuckerman et al., 2001].

Epstein–Barr virus (EBV) belongs to the human herpes viruses, is oncogenic and is disseminated widely. Antibodies against EBV have been confirmed in all

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*Correspondence to: Takayoshi Ito, Division of Gastroenterology, Department of Medicine, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8666, Japan. E-mail: tito@med.showa-u.ac.jp

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population groups worldwide; ~90–95% of adults are seropositive for EBV [Thorley-Lawson, 2001]. EBV transforms B cells and is associated with the development of lymphoproliferative disorders. EBV manifests two activity levels distinctly: latency, a state of limited expression of viral genes, and lytic infection, which ultimately leads to production of virions. Several viruses and cytokines have been reported to induce reactivation of EBV, even in healthy individuals, including human herpesvirus 6 (HHV-6), cytomegalovirus, and TGF β-1 [Flamand et al., 1993; Aalto et al., 1998; Fahmi et al., 2000]. The latency-to-lytic switch can be started by expression of activator genes for the lytic cycle of EBV, BZLF1, and BRLF1, which encode ZEBRA and Rta. These genes induce expression of viral and cellular genes related to replication of DNA and viral and cellular factors for reactivation [Amon and Farrell, 2005].

In order to examine the possibility that infection with HCV triggers reactivation of EBV to induce development of clonal expansion of B cells and lymphoproliferative disorders, profiles for expression of EBV mRNA associated with its reactivation were investigated in patients with chronic hepatitis C.

MATERIALS AND METHODS

Patients

From 2007 through 2008, 52 patients infected with HCV (chronic hepatitis, 41; and cirrhosis, 11) were enrolled in the study. Seven patients had complicating HCC. All patients were treated and/or followed up at Showa University Hospital. Diagnosis of infection with HCV was confirmed by detection of HCV RNA in the serum. Serving as controls, 43 healthy adults, 17 patients infected with hepatitis B virus (HBV), and 19 patients who had non-viral liver injury (alcoholism, 5; non-alcoholic steatohepatitis, 3; primary biliary cirrhosis, 9; and autoimmune hepatitis, 2) were enrolled in the study. Another 42 patents with chronic hepatitis C, who were admitted for treatment with pegylated interferon and ribavirin, were enrolled. Informed consent was obtained from all participants in the study, and the study was approved by the Ethics Committee of Showa University School of Medicine.

Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were obtained from 10 to 30 ml of whole blood in LeucoSep® tubes (Greiner Bio-One, Tokyo, Japan) according to the manufacturer's instructions. Cells were frozen and stored at −80°C until use.

Isolation of Lymphoid Cell Subsets

Beads with affinity for each type of lymphoid cell, MicroBeads® (Miltenyi Biote, Bergisch Gladbach, Germany) were added to PBMCs to isolate lymphoid cell subsets ($CD8^+$, $CD4^+$, $CD19^+$, and others) in 30 patients with chronic hepatitis C. Suspension of the cells was mixed well, incubated for 15 min at 4°C and centrifuged at 900*g* for 10 min in a tube. Then the tube was placed on a magnet, and the free floating cells were transferred to another tube. The pellet containing $CD8^+$ cells was collected and stored at −80°C until use. $CD4^+$ and $CD19^+$ cells were separated from the supernatant using similar procedures. The supernatant remaining was labeled as the fraction of "others." Free B cells were isolated from PBMCs, using a system for separation of touch-free cells (MagCellect B cell isolation kit II®; R&D Systems, Minneapolis, MN), of 12 patients with chronic hepatitis C.

Detection of EBV mRNA by Nested RT-PCR

Total RNA was extracted from PBMCs or B cells using the RNeasy Mini Kit® (Qiagen, Tokyo, Japan). A portion was reverse transcribed by AMV® RT (Roche, Munich, Germany) using random primers under the conditions recommended by the manufacturer. The mRNAs of *Bam*HI Z leftward reading frame 1 (BZLF1), latent membrane protein 1 (LMP1), and Epstein–Barr virus-encoded small RNAs 1 (EBER1) were amplified by nested RT-PCR. GAPDH mRNA was amplified by RT-PCR as an internal control in each sample. First, PCR was performed using 2.5 μl of cDNA in 2.5 ml of mixture for RT reaction containing 0.25 μl of sense and anti-sense primers, 2.0 μl of dNTP mixture, and 0.5 U of Taq polymerase in reaction buffer. The PCR program was 94°C for 3 min for denaturation, 35 cycles of 94°C for 30 sec, 58°C for 1 min and 72°C for 1 min and 72°C for 7 min for extension. The outer sense primer for BZLF1 mRNA was 5′-ATT GCA CTT TGC CGC CAC CTT TG-3′, the outer anti-sense primer was 5′-CGG CAT TTT CTG GAA GCC ACC CGA-3′, the inner sense primer was 5′-GAC CAA GCT ACC AGA GTC TAT-3′, and the inner anti-sense primer was 5′-CAG AAT CGC ATT CCT CCA GCG A-3′. The outer sense primer for EBER1 RNA was 5′-AGG ACC TAC GCT GCC CTA GA-3′, the outer anti-sense primer was 5′-AAA ACA TGC GGA CCA GC-3′, the inner sense primer was 5′-GGT TTT GCT AGG GAG GAG AC-3′, and the inner anti-sense primer was 5′-GGT ACT TGA CCG AAG ACG GC-3′. The outer sense primer for LMP1 mRNA was 5′-TCC TCC TCT TGG CGC TAC TG-3′, the outer anti-sense primer was 5′-TCA TCA CTG TGT CGT TGT CC-3′, the inner sense primer was 5′-CTT GTC CTC TAT TCC TTT GC-3′, and the inner anti-sense primer was 5′-CAC AAT TCC AAG GAA CAA TGC C-3′. The sense primer for GAPDH mRNA was 5′-GCC TCC TGC ACC ACC AAC TG-3′, and the anti-sense primer was 5′-CGA CGC CTG CTT CAC CAC CTT CT-3′.

Serum Markers of Lymphoproliferative Disorders

Cryoglobulinemia was detected using a semi-quantitative method by centrifugation, as described previously [Inokuchi et al., 2009]. Rheumatoid factor was determined by the latex turbidimetric assay, and complement 4 and 50% hemolytic complement activity (CH50) were determined by nephelometry and Mayer's method,

respectively. Markers of LPD were determined in 52 patients with chronic hepatitis C.

PCR Amplification for Rearrangement of IgH Genes

Genomic DNA was extracted from PBMCs containing B cells by QIAamp DNA mini (Qiagen). To detect monoclonal rearrangement of IgH genes, the DNA was amplified by PCR using a semi-nested protocol, as described previously [Ngan et al., 1989]. The upstream primer was complementary to the third framework V region (FR3) conserved in the IgH gene, and the downstream primer annealed to an outer region conserved in the IgH joining (J) region in the first round of amplification and to an inner sequence conserved in the same J region in the second round. The upstream primer for FR3 (Fr3A; 5′-ACA CGG C[C/T][G/C] TGT ATT ACT GT-3′) and the downstream primer for JH (LJH; 5′-TGA GGA GAC GGT GAC C-3′) were used in the first round of amplification. In the second round, an inner downstream primer (VLJH; 5′-GTG ACC AGG GTN CCT TGG CCC CAG-3′) was substituted for LJH. PCR products were separated by 7.5% polyacrylamide gel electrophoresis. Clonal expansion was determined by the presence of one or more discrete, dominant bands within the range 72- to 118-bp, and polyclonal populations were indicated by the presence of smears with no specific dominant bands.

Anti-EBV Antibodies

Anti-Epstein–Barr virus nuclear antigen (EBNA) and anti-viral capsid antigen (VCA) IgG antibodies were determined in sera of healthy adults, patients with chronic hepatitis C, hepatitis B, and non-viral liver diseases using the SERION ELISA classic kit® (Virion-Serion, Würzburg, Germany) according to the manufacturer's instructions.

Statistical Analysis

The median of continuous variables, with and without normal distribution, was compared by Student's *t*-test and Wilcoxon's test, respectively. Comparison of discontinuous variables was performed by a chi-squared test and Fisher's exact test. A P-value <0.05 was considered to be statistically significant. Values with a normal distribution were expressed as means ± SD.

RESULTS

Expression Profiles of EBV Genes in PBMCs of Patients Infected With HCV

Figure 1 shows the profiles of expression for EBV mRNA in PBMCs from seven patients with chronic hepatitis C. EBER1 RNA was expressed in all patients, indicating that PBMCs were infected with EBV persistently. BZLF1 mRNA, which codes early proteins after reactivation of EBV immediately, was detected in

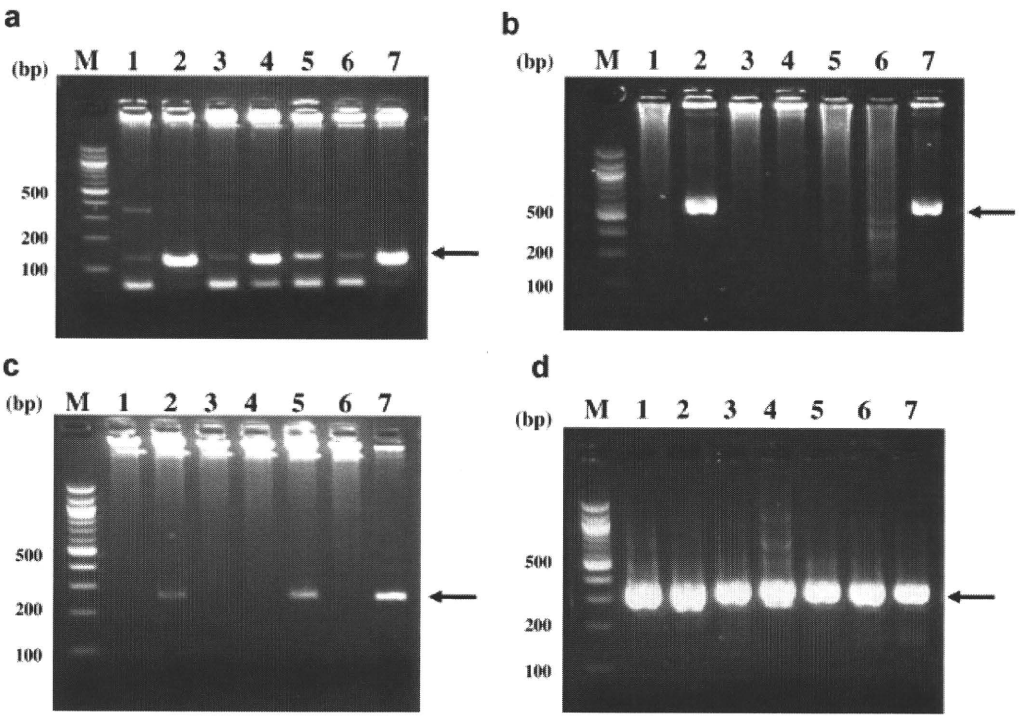

Fig. 1. Expression profiles of EBER1 RNA, and BZLF1 and LMP1 mRNAs from seven patients infected with HCV. **a**: All patients showed expression of EBER1 RNA in peripheral blood mononuclear cells (PBMCs) (lanes 1–7). Expression of BZLF1 (**b**) and LMP1 (**c**) mRNAs was observed in PBMCs from patients 2 and 7 (lanes 2 and 7), and patients 2, 5, and 7 (lanes 2, 5, and 7), respectively. **d**: GAPDH mRNA was expressed in PBMCs from all patients equally. Molecular markers are indicated in lane M. Arrows indicate appropriate sizes of the molecular markers for each RT-PCR product (EBER1: 126 bp; BZLF1: 556 bp; LMP1: 252 bp; and GAPDH: 310 bp).

TABLE I. Expression Profile of EBV mRNAs in PBMCs and Serum Antibodies Against EBV Among Patients Infected With HCV and Controls

	Healthy adults (N = 43)	HCV (N = 52)	HBV (N = 17)	Non-viral Liver diseases (N = 19)
Age	59.7 ± 19.1	66.2 ± 12.8, NS	55.9 ± 15.3, NS	59.7 ± 12.4, NS
Gender: male (%)	28/43 (65%)	22/52 (43%), $P = 0.027$*	11/17 (65%), NS	9/19 (47%), NS
EBER1 (%)	43/43 (100%)	49/52 (94%), NS	17/17 (100%), NS	19/19 (100%), NS
BZLF1 (%)	3/43 (9%)	12/52 (23%), $P = 0.032$*	1/17 (6%), NS	1/19 (5%), NS
LMP1 (%)	5/43 (12%)	9/52 (18%), NS	0/17 (0%), NS	4/19 (21%), NS
Anti-EBNA antibody (%)	29/35 (83%)	41/50 (82%), NS	17/17 (100%), NS	18/19 (95%), NS
Anti-VCA-IgG antibody (%)	33/34 (97%)	50/50(100%), NS	17/17 (100%), NS	19/19 (100%), NS

P, *P*-value; NS, not significant; PBMC, peripheral blood mononuclear cell; EBER1, Epstein–Barr virus-encoded small RNAs; BZLF1, *Bam*HI Z leftward reading frame 1; LMP1, latent membrane protein 1; EBNA, Epstein–Barr virus nuclear antigen; VCA, viral capsid antigen.
Data are no (%) or the mean ± SD.
*Significantly lower than healthy adults ($P < 0.05$).

patients 2 and 7. This suggests that reactivation of EBV occurred in these patients. LMP1, which is one of the later genes associated with transformation of host cells, was also identified in patients 2, 5, and 7. GAPDH mRNA was expressed in all studied samples equivalently.

Table I shows a summary of expression of EBV mRNA in PBMCs among patients who had liver diseases associated with and without HCV. EBER1 RNA was expressed in almost all examined patients and control subjects, indicating that most adults were infected with EBV persistently. BZLF1 mRNA was detected in PBMCs from 12 of 52 (23%) patients with chronic hepatitis C, thus suggesting that reactivation of EBV was observed in these patients. The prevalence of antibodies against EBV protein in patients was not significantly different when compared to those of healthy adults. The frequency of positivity for BZLF1 mRNA was higher than in healthy subjects [3 of 43 (9%), $P = 0.032$]. The frequency in patients with other liver diseases was lower than that in patients with chronic hepatitis C (chronic hepatitis B: [1 of 17 (6%)] and non-viral liver diseases [1 of 19 (5%)]). The mRNA of EBV LMP1, which correlates with activity for the transformation of host cells, was also detected in 9 of 52 (18%) patients with chronic hepatitis C and 5 of 43 (12%) control subjects. Five of 52 (9.6%) patients with chronic hepatitis C were positive for mRNAs of both BZLF1 and LMP1. These results confirm that reactivation of EBV had occurred in these patients with chronic hepatitis C. Anti-VCA antibody (IgG class) was positive in almost all subjects examined in this study, except for one healthy adult, indicating that most of the subjects were infected with EBV previously. The frequency of positivity for anti-EBNA antibody in patients with chronic hepatitis C and non-viral liver diseases was lower than in healthy adults, although the differences were not significant statistically. This might be associated with reactivation of EBV in patients with chronic hepatitis C due to dysfunction of immunoglobulin secretion from B cells.

Clinical Characteristics of Patients Infected With HCV Who Were Positive for BZLF1 mRNA

The clinical characteristics of the 12 patients infected with HCV, who were positive for BZLF1 mRNA, are presented in Table II. A comparison of viral and host factors between patients who were BZLF1 mRNA-positive or -negative showed that positivity for BZLF1 mRNA was associated with higher age than negativity for BZLF1 mRNA (Table III). Abnormalities in markers for lymphoproliferative disorders were observed in

TABLE II. Clinical Characteristics of HCV-Infected Patients Whose PBMCs Were BZLF1 mRNA-Positive

	Age	Gender	Clinical stage	HCV group	HCV-RNA (log IU/ml)	ALT (IU/L)	Platelets ($\times 10^4/\mu l$)	Cg	C4 (mg/dl)	RF (IU/ml)	CH50 (mg/ml)	IgH clonality
1	72	F	CH	1	6.6	51	28.0	(−)	20.6	12.4	<12	(−)
2	61	M	CH	1	5.5	39	14.3	(−)	17.7	54.6	28.8	(−)
3	75	M	LC	1	6.3	26	6.3	(−)	13.1	<7	32.1	(−)
4	77	F	CH, HCC	1	3.7	15	10.6	(−)	20.5	<7	52.2	(+)
5	61	M	CH	1	5.9	17	15.0	(−)	22.6	<7	34.1	(−)
6	76	F	CH	1	6.6	56	14.8	(−)	21.4	19.6	28.8	(−)
7	87	M	LC, HCC	ND	6.7	31	13.7	(−)	15.6	23.2	37.1	(−)
8	80	F	CH	2	4.2	42	11.5	(−)	19.9	<7	21.0	(−)
9	58	M	CH	1	4.2	28	22.8	(−)	30.0	<7	47.0	(−)
10	79	F	LC, HCC	1	6.2	33	12.5	(+)	9.8	15.4	<12	(−)
11	57	F	LC	1	6.5	46	4.9	(−)	12.7	<7	<12	(−)
12	71	F	LC, HCC	1	5.0	43	6.0	(−)	17.9	17.5	44.5	(−)

PBMC, peripheral blood mononuclear cell; BZLF1, *Bam*HI Z leftward reading frame 1; ALT, alanine aminotranferase; Cg, cryoglobulinemia; C4, complement 4; RF, rheumatoid factor; CH50, 50% hemolytic complement activity; IgH, immunoglobulin heavy chain; CH, chronic hepatitis; LC, cirrhosis; HCC, hepatocelluar carcinoma; ND, not determined.

TABLE III. Comparison of Viral and Host Factors Between Patients Positive and Negative for BZLF1 mRNA

	BZLF1(+), N = 12	BZLF1(−), N = 40	*P*-value
Age	73.7 ± 9.9	63.9 ± 12.8	0.023
Gender (male)	5/12 (41%)	17/40 (43%)	NS
Clinical stage			
CH	7/12 (59%)	34/40 (85%)	0.047
LC	5/12 (41%)	6/40 (15%)	0.047
HCC	4/12 (33%)	3/40 (8%)	0.042
ALT (IU/L)	35.6 ± 12.9	44.7 ± 29.2	NS
Platelets ($\times 10^4/\mu l$)	13.4 ± 6.7	15.9 ± 6.8	NS
Cryoglobulinemia	1/12 (8%)	11/40 (28%)	NS
RF (>10 IU/ml)	6/12 (50%)	24/40 (60%)	NS
C4 (<10 mg/dl)	1/12 (8%)	6/40 (15%)	NS
CH50 (<20 mg/ml)	7/12 (58%)	22/40 (55%)	NS
Clonal IgH rearrangement	1/12 (8.3%)	5/39 (12.8%)	NS
Serum HCV-RNA (log/ml)	5.7 ± 1.1	5.5 ± 1.4	NS
Group			
1	10/11 (84%)	27/40 (68%)	NS
2	1/11 (8%)	8/40 (20%)	
LMP1 (+) (%)	5/12 (42%)	4/40 (10%)	0.011
EBNA antibody (+) (%)	8/12 (67%)	33/38 (87%)	NS

BZLF1, *Bam*HI Z leftward reading frame 1; NS, not significant; CH, chronic hepatitis; LC, cirrhosis; HCC, hepatocelluar carcinoma; ALT, alanine aminotranferase; RF, rheumatoid factor; C4, complement 4; CH50, 50% hemolytic complement activity; IgH, immunoglobulin heavy chain; LMP1, latent membrane protein 1; EBNA, Epstein–Barr virus nuclear antigen.
Data are no (%) or the mean ± SD.

patients infected frequently with HCV, but the frequency of abnormalities was not significantly different between patients who were BZLF1 mRNA-positive or -negative. The monoclonal IgH rearrangement was detected in PBMCs in only one patient, and results in Table III show that reactivation of EBV was not associated with the clonal expansion of B cells. The frequency of positivity for expression of LMP1 mRNA in patients positive for expression of BZLF1 mRNA was higher than that in patients negative for expression of BZLF1 mRNA, indicating that expression between the two genes is related. Three of 12 patients with reactivation of EBV were negative for anti-EBNA antibody, but the frequency was not higher significantly compared with the other group (data not shown). With regard to clinical stage, results showed that cirrhosis and HCC were associated with reactivation of EBV in only 11 and 7 patients examined, respectively.

Reactivation of EBV Induced in B Cells of Patients With Chronic Hepatitis C

EBV shows B cell tropism for replication, but several reports have shown that EBV is able to replicate in T, NK, and epithelial cells [Kawa, 2003]. To clarify which lymphoid subsets support reactivation of EBV, expression of BZLF1 mRNA was analyzed in four distinct fractions ($CD8^+$, $CD4^+$ T cells, $CD19^+$ B cells, and others) isolated from 30 patients using affinity beads. Furthermore, B and non-B cells isolated using the system for a touch-free cell isolation were examined for expression of BZLF1 mRNA in 10 patients. Expression of BZLF1 mRNA in B cells of 42 patients was screened first, and the results showed that reactivation of EBV occurred in 10 patients (23.8%). The frequency of positivity for expression of BZLF1 mRNA was almost the same as that in PBMCs from patients with chronic hepatitis C, as shown in Table I (23%). Subsequently, the same study was performed using fractions of other cell subsets from 10 patients. As shown in Figure 2a, BZLF1 mRNA was detected in only B cells from six patients, although it was detected in all fractions examined in one case. All samples from the seven cases showed the same levels as expression of GAPDH mRNA (data not shown). In the three patients studied, analysis of B cells isolated using the system of a touch-free cell isolation showed that reactivation of EBV occurred in only B cells (Fig. 2b). These results suggest that reactivation of EBV was induced in B cells of patients with chronic hepatitis C specifically.

Reactivation of EBV Was Terminated After Eradication of HCV by Interferon Therapy

In order to analyze the effects of eradication of HCV on reactivation of EBV, expression of BZLF1 mRNA in PBMCs was monitored in three patients that attained a sustained viral response (cases 1–3) through combination therapy with pegylated interferon and ribavirin. Figure 3 shows that expression of BZLF1 mRNA disappeared soon after of the start of therapy in these patients. BZLF1 mRNA remained negative even at 3 months after the end of treatment. EBER1 mRNA was expressed throughout the therapy, indicating that the cycle of EBV infection changed from lytic infection into persistent infection in some B cells in the patients. GAPDH mRNA was expressed in all cells investigated equally.

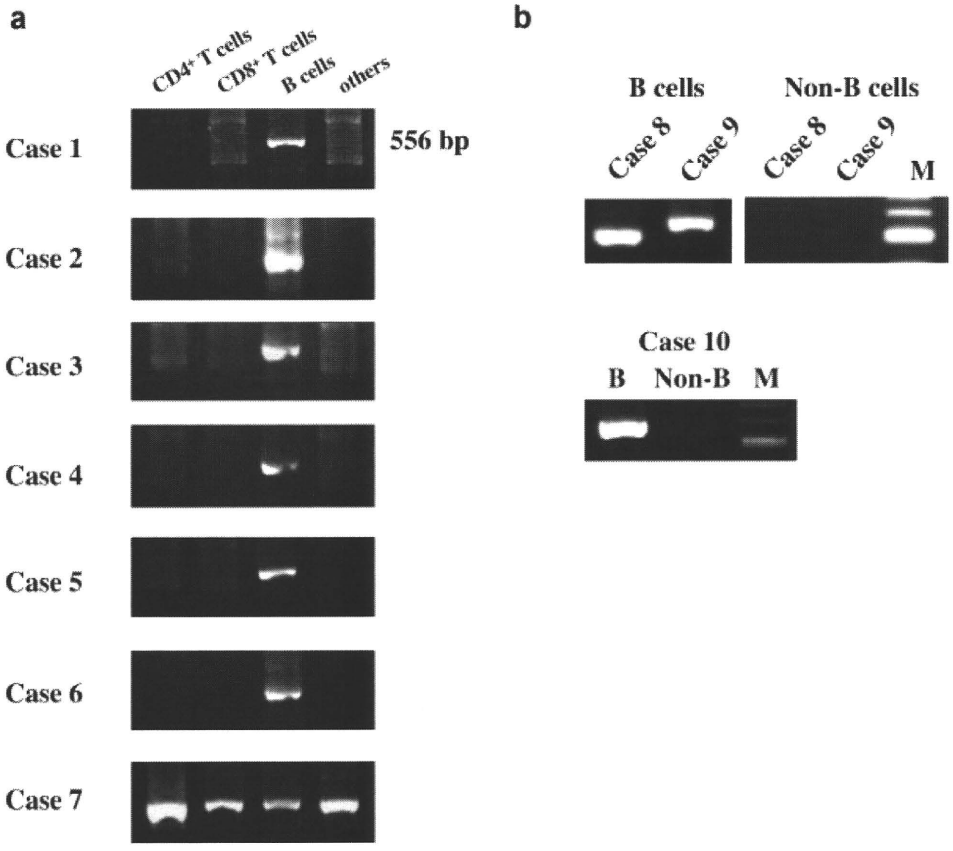

Fig. 2. **a**: Expression of BZLF1 mRNA in four fractions ($CD4^+$, $CD8^+$ T cells, $CD19^+$ B cells, and others) isolated from seven patients with chronic hepatitis C using affinity beads. **b**: Expression of BZLF1 mRNA (556 bp) in B and non-B cells isolated from three patients with chronic hepatitis C using the isolation system of a touch-free cell. Molecular markers are indicated in lane M.

DISCUSSION

HCV infection induces a number of extrahepatic manifestations [Cacoub et al., 1999; Zignego and Brechot, 1999], among which lymphoproliferative disorder is related to HCV infection most closely [Zignego et al., 2007], although the mechanisms remain unknown. Accordingly, there have been many reports speculating on the mechanisms responsible for lymphoproliferative disorders associated with HCV. It has been demonstrated that chronic infection with HCV can lead to the clonal expansion of B cells and that sustained proliferation of B cells promotes the occurrence of genetic mutations. Zignego et al. [2000] have observed translocation of t(14;18) and overexpression of bcl-2 in lymphoid cells from patients with lymphoproliferative disorders frequently in association with HCV infection, although the relationship between clonal expansion of B cells and t(14;18) has not been confirmed yet. Direct association between HCV and B cells is thought to accelerate the clonality of B cells; for example, binding of HCV-E2 to CD81 and infection of B cells with HCV [Machida et al., 2005]. In the previous study, the influence of HCV infection and/or association of B cells on the development of lymphoproliferative disorders were evaluated in patients with persistent infection of HCV [Inokuchi et al., 2009]. The results showed that HCV infection was more prevalent, and HCV RNA levels were higher in B cells than other lymphoid cell subsets. On multivariate analysis, HCV RNA in B cells was an independent factor associated with the presence of at least one marker for lymphoproliferation [Inokuchi et al., 2009]. Based on these results, correlations were

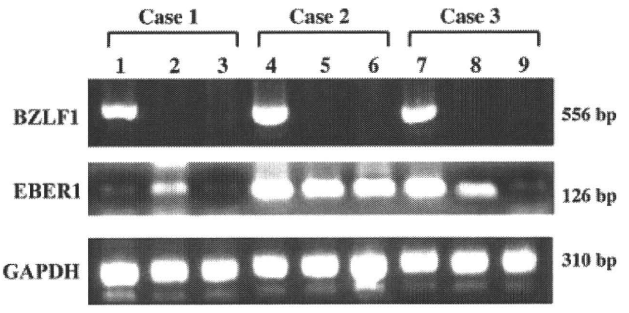

Fig. 3. Disappearance of BZLF1 mRNA in B cells after interferon therapy. BZLF1 mRNA in PBMCs was monitored in three patients (case 1: lanes 1–3; case 2: lanes 4–6; and case 3: lanes 7–9) whose B cells were positive before the start of therapy (lanes 1, 4, and 7). Putative sizes of molecular length for each PCR product are indicated on the right. All patients showed sustained virological responses after treatment with pegylated interferon and ribavirin. This therapy resulted in negativity for BZLF1 mRNA within 1 month (lane 2: 1 month; lane 5: day 1; and lane 8: day 3) and BZLF1 mRNA remained negative at 3 months after the end of therapy (lanes 3, 6, and 9).

demonstrated between infection and/or association of B cells with HCV and occurrence of lymphoproliferative disorders, although the detailed mechanisms remain unknown.

In the present study, BZLF1 mRNA was detected in B cells isolated from PBMCs of patients with chronic hepatitis C, thus suggesting the reactivation of EBV. EBV is a lymphotropic virus that induces lymphoproliferation and is associated with a wide range of tumors; for example, Burkitt's lymphoma, Hodgkin's lymphoma, and nasopharyngeal and gastric carcinoma [Niller et al., 2009]. Furthermore, this virus is also associated with autoimmune diseases; for example, rheumatoid arthritis and Sjögren's syndrome [Niller et al., 2008]. Thus, the pathobiology of EBV infection is similar to that of HCV infection. It is possible that HCV infection triggers reactivation of EBV, and that the two viruses induce host lymphoproliferation cooperatively, as well as prolongation of survival and transformation. In fact, EBV-encoded nuclear antigen 1 (EBNA1) enhances replication of HCV in vitro, and EBV DNA has been detected in the tissues of HCC in patients infected with HCV [Sugawara et al., 1999a,b, 2000], although the latter issue remains controversial [Chu et al., 2001; zur Hausen et al., 2003]. LMP1 mRNA, which is a latent viral protein related to the transformation of host cells, has also been detected in B cells of two patients with chronic hepatitis C. The expression of BZLF1 and LMP1 mRNAs confirms the switch from the persistent to the lytic cycle of EBV. This expression was detected by nested RT-PCR only, indicating that the number of reactivated B cells is extremely low.

Anti-VCA antibody was positive in all subjects except for one healthy subject. The subject without anti-VCA antibody had anti-EBNA antibody in the serum, indicating that all subjects studied were in a state of past infection for EBV. The frequency of positivity for anti-EBNA antibody, which is thought to be a neutralizing antibody, is almost the same (healthy adults, 82.8%; and patients infected with HCV, 82%). Furthermore, 8 of 12 (66.7%) patients infected with HCV, who were positive for BZLF1 mRNA, had anti-EBNA antibodies in their sera. This suggests that low levels of anti-EBNA antibody are not the main cause for reactivation of EBV in patients infected with HCV. The possibility that infection of B cells and/or association with HCV induce dysfunction of immunoglobulin itself could not be ruled out. In persistent infection of HCV, an increase in serum is commonly observed. This increase is polyclonal and is determined primarily by an increase of IgG levels. These immunoglobulins include both HCV-specific and non-specific antibodies. Nonetheless, memory B cells do not expand but rapidly differentiate to secrete immunoglobulin and undergo apoptosis [Racanelli et al., 2006]. HCV also induced hypermutation of immunoglobulin to reduce affinity and neutralizing activities against the envelope protein of HCV [Machida et al., 2008]. Based on these observations, HCV infection may impair the function of anti-EBNA antibodies to inhibit reactivation of EBV.

Analysis of EBV reactivation in lymphoid cell subsets showed that reactivation occurred in B cells specifically. EBV infects B cells mainly, but can establish latent infection in other cell types, including T lymphocytes and natural killer (NK) cells [Watry et al., 1991; Kanegane et al., 1996]. The results showed that EBV was reactivated in B cells mainly. In one patient with chronic hepatitis C, however, EBV reactivation was observed in all subsets of lymphoid cells. This patient did not have any lymphoproliferative disorders. HCV RNA in B cells was detected in only 5 of 10 patients studied (data not shown). These results suggest that reactivation of EBV may not be induced by HCV infection of B cells directly. Therefore, further studies are necessary to clarify the association between HCV infection and reactivation of EBV in B cells.

No correlation was observed between reactivation of EBV and the occurrence of lymphoproliferative disorders. There were no differences in the markers for lymphoproliferative disorders between patients with PBMCs that were positive or negative for BZLF1 mRNA. The clonal expansion of B cells is reported to occur in 26% of Italian patients with chronic hepatitis C [Pozzato et al., 1999], while the clonality of B cells was detected in 11% of Japanese patients infected previously with HCV [Inokuchi et al., 2009]. The present study shows that induction of clonality was observed in one case only among patients infected with HCV with PBMCs positive for BZLF1. Several studies have focused on the important role of antigenic stimulation that is sustained, similar to lymphomagenesis due to infection with *H. pylori*, for its possible role in extra-nodal marginal-zone B cell lymphoma arising in lymphoid tissues on mucosae (MALT lymphoma) [Ivanovski et al., 1998; De Re et al., 2000; Sansonno et al., 2004].

Cirrhosis and hepatocelluar carcinoma may be associated with reactivation of EBV. Five of 12 (41%) patients with cirrhosis and 4 of 12 (33%) patients with hepatocelluar carcinoma had reactivation of EBV. These frequencies were statistically higher than those of patients without BZLF1 mRNA, indicating a possible association with reactivation of EBV and advance of clinical stages. But a large number of both patients with cirrhosis and with hepatocelluar carcinoma are needed to conclude this issue. EBV is also associated with the development of malignancies from both B cells and epithelial cells: Burkitt's lymphoma [Epstein et al., 1964], nasopharyngeal carcinoma [Andersson-Anvret et al., 1979], and Hodgkin's disease [Weiss et al., 1989]. Furthermore, about 10% of gastric carcinoma is associated with EBV [Fukayama et al., 1994]. As mentioned above, EBV DNA was detected in HCC tissues in patients infected with HCV [Sugawara et al., 1999a,b, 2000]. It is possible that reactivation of EBV is associated with neoplasm. Again, further large-scale studies are necessary to confirm these issues.

Reactivation of EBV is also associated with other medical problems such as lupus, malaria, and multiple sclerosis [Huggins et al., 2005; Christensen, 2006; Chene et al., 2007]. This may simply indicate that there

are several factors to induce reactivation of EBV in patients suffering from several medical problems. In order to confirm a correlation between HCV infection and reactivation of EBV, BZLF1 mRNA expression was followed in patients who succeeded in eradication of HCV after IFN therapy. After eradication of HCV, expression of BZLF1 mRNA disappeared in three patients with a sustained viral response. Expression of EBER1 RNA as a marker of EBV persistence was detected in PBMCs from all of these patients, although levels of expression were decreased in two of three patients. These results suggest that the interferon therapy eradicated HCV but not EBV in these patients. On the other hand, reactivation of EBV was not observed at 3 months after the end of treatment. Conceivably, the presence of HCV is required for reactivation of EBV in patients. It is possible that interferon therapy affects replication and/or reactivation of EBV directly. However, there have been few reports showing the effects of interferon on chronic infection of EBV directly. Further studies are necessary to clarify the molecular mechanisms responsible for the generation of lymphoproliferative disorders associated with HCV and its relationship with malignant lymphoma.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Strong CD8$^+$ T-cell responses against tumor-associated antigens prolong the recurrence-free interval after tumor treatment in patients with hepatocellular carcinoma

Kazumasa Hiroishi · Junichi Eguchi · Toshiyuki Baba · Tomoe Shimazaki · Shigeaki Ishii · Ayako Hiraide · Masashi Sakaki · Hiroyoshi Doi · Shojiro Uozumi · Risa Omori · Takuya Matsumura · Tatsuro Yanagawa · Takayoshi Ito · Michio Imawari

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Abstract
Aim We investigated whether tumor-specific CD8$^+$ T-cell responses affect tumor-free survival as well as the relationship between CD8$^+$ T-cell responses against tumor-associated antigens (TAAs) and the clinical course after tumor treatment in patients with hepatocellular carcinoma (HCC).
Methods Twenty patients with HCC that were treated by radiofrequency ablation or trans-catheter chemo-embolization (TACE) and in whom HCC was undetectable by ultrasonography, CT, and/or MRI 1 month after treatment were enrolled in the study. Before and after treatment for HCC, analyses of TAA (glypican-3, NY-ESO-1, and MAGE-1)-specific CD8$^+$ T-cell responses were evaluated with an interferon-γ enzyme-linked immunospot (ELISpot) assay using peripheral CD8$^+$ T-cells, monocytes, and 104 types of 20-mer synthetic peptide overlapping by 10 residues and spanning the entirety of the 3 TAAs.
Results Sixteen out of 20 patients (80%) showed a positive response ($\geq$10 TAA-specific cells/10^5 CD8$^+$ T-cells) before or after treatment. When we performed univariate analysis of prognostic factors for the tumor-free period in the 20 patients, platelet count, prothrombin time, and the number of TAA-specific CD8$^+$ T-cells after treatment were significant factors ($P = 0.027$, 0.030, and 0.004, respectively). In multivariate analysis, the magnitude of the TAA-specific CD8$^+$ T-cell response ($\geq$40 TAA-specific cells/10^5 CD8$^+$ T-cells) was the only significant prognostic factor for a prolonged tumor-free interval (hazard ratio 0.342, $P = 0.022$).
Conclusions Our results suggest that strong TAA-specific CD8$^+$ T-cell responses suppress the recurrence of HCC. Immunotherapy to induce TAA-specific cytotoxic T lymphocytes by means such as the use of peptide vaccines should be considered for clinical application in patients with HCC after local therapy.

Keywords Hepatocellular carcinoma · CD8$^+$ T-cell response · Cytotoxic T lymphocyte · ELISpot assay · Immunotherapy

K. Hiroishi · J. Eguchi · T. Baba · T. Shimazaki · S. Ishii · A. Hiraide · M. Sakaki · H. Doi · S. Uozumi · R. Omori · T. Matsumura · T. Yanagawa · T. Ito · M. Imawari (✉)
Division of Gastroenterology, Department of Medicine, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8666, Japan
e-mail: imawari@med.showa-u.ac.jp

Introduction

There are about 500,000 new patients with hepatocellular carcinoma (HCC) per year worldwide. Although vaccination against hepatitis B virus (HBV) and interferon (IFN)-based therapy against hepatitis C virus (HCV) will presumably reduce the number of HCC patients in the future, the incidence of HCC is still increasing in Asia and Africa because of the previous prevalence of infection with the virus. Progress in treatments for HCC has improved the prognosis of patients with HCC. However, HCC is usually associated with cirrhosis and often recurs even after complete treatment of the tumors in the remaining part of the cirrhotic liver. Thus, there is a strong need for the development of a new intervention therapy that suppresses the occurrence or recurrence of HCC effectively and that has fewer side effects. Immunotherapy may be such a treatment and may be applicable to the clinical treatment of HCC. In fact, some clinical trials have been performed [1–3].

Cytotoxic T lymphocytes (CTLs) are thought to be potent effector cells against cancers. CTLs recognize specific antigens, and the induction of CTLs specific for tumor-associated antigen (TAA) is an attractive procedure

for tumor therapy. The MAGE-1 gene was first identified as encoding a tumor-specific antigen on MZ-2-MEL cells, a melanoma cell line, in 1991 [4]. MAGE-1 gene and protein can be detected in many cancer tissues, and three articles reported the expression of MAGE-1 in HCC as 30, 68, or 78%, respectively, in a Japanese population [5–7]. In gastrointestinal tumors, immunotherapy using both dendritic cells and MAGE peptides has been performed for patients with primary malignant melanoma of the esophagus, and this therapy was able to induce peptide-specific immune responses [8].

NY-ESO-1 antigen, a member of the cancer-testis antigen family, was initially identified by a serological analysis of recombinant cDNA expression cloning in an esophageal cancer patient [9]. NY-ESO-1 mRNA was detected in 24–37% of HCCs by reverse transcription-polymerase chain reaction [10, 11].

Glypican-3 (GPC3) consists of 580 amino acids and is a heparan sulfate proteoglycan with a potential role in the control of cell division. GPC3 mRNA was detected in 74.8% of HCC tissues, but only in 3.2% of normal liver tissues [12], and GPC3 protein was detected in 72% of HCCs, but not in normal tissue using GPC-specific antibody [13]. The GPC3 protein can also be detected in sera of 40–53% of patients with HCC [14, 15].

These three antigens are thought to be attractive targets for cancer immunotherapy because they are expressed only in tumor tissues and testis, but not in normal tissues other than testis. On the basis of previous reports, it is assumed that most HCCs would express at least one of the three TAAs. Therefore, monitoring immune responses against these TAAs might help in the development of HCC immunotherapy, such as TAA-based vaccination. In this study, we investigated how the magnitude of $CD8^+$ T-cell responses against these TAAs determined by an IFN-γ enzyme-linked immunospot (ELISpot) assay is related to other clinical data and the tumor-free interval in patients with HCC, in order to explore the clinical application of such a TAA-based immunotherapy.

Methods

Patients

Twenty patients who were diagnosed with HCC at Showa University Hospital between 2006 and 2008 were enrolled in the study. They met the following study criteria: (1) pathologically confirmed as having HCC or a lesion with characteristic imaging features of HCC based on ultrasonography, CT, and/or MRI; (2) liver function classed as Child-Pugh A or B; (3) no extrahepatic metastasis or vascular invasion; (4) no previous or simultaneous cancers other than HCC; and (5) an indication for treatment such as radiofrequency ablation (RFA) or trans-catheter chemo-embolization (TACE). RFA was performed by well-trained hepatologists using usual methods according to previous reports [16]. A 16-gauge cooled-tip ablation electrode (Covidien, Boulder, CO) was used in the procedure. TACE was performed by well-experienced hepatologists and radiologists. A microcatheter was inserted from the femoral artery to the artery feeding the HCC superselectively after conventional hepatic angiography, and then a segmental or subsegmental TACE procedure was performed using gelatin, lipiodol, and either epirubicin hydrochloride or cisplatin. All patients were followed every 1–3 months by ultrasonography, CT, and/or MRI to examine the appearance of new lesions in the liver or other organs. The recurrence-free interval was defined as the period from the month of HCC treatment to the month when a recurrent and/or metastatic HCC was first detected after treatment. Clinical data (platelet count, prothrombin time, serum AST, ALT, albumin, total bilirubin level, and AFP level) were collected 1–7 days before HCC treatment. Chronic hepatitis C was diagnosed on the basis of detectable HCV RNA in serum using the Amplicor assay (Roche Diagnostics, Tokyo, Japan). Informed consent was obtained from each patient included in this study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Ethical Committee of Showa University.

Synthetic peptides of TAA

Twenty-mer peptides overlapping by 10 residues and spanning the entire MAGE-1, NY-ESO-1, and GPC3 proteins were synthesized based on the amino acid sequences reported previously as PepSets™ and purchased from Mimotopes (Clayton South, Victoria, Australia). These peptides were >80% pure. A total of 30 MAGE-1, 17 NY-ESO-1, and 57 GPC3 peptides were synthesized, as shown in Table 1. A total of 10–11 TTA peptides were pooled in a mixture (total 10 mixtures) at a concentration of 10 μg/ml each.

Preparation of $CD8^+$ T cells and monocytes from patients with HCC

PBMCs were isolated from heparinized peripheral blood by gradient centrifugation using Ficoll-Paque (Pharmacia-LKB Biotechnology, Uppsala, Sweden). As reported previously, peripheral $CD8^+$ T-cells and monocytes were separated from PBMCs using CD8 microbeads (MACS system; Miltenyi Biotec, Bergisch Gladbach, Germany) and a Monocyte Isolation Kit II (Miltenyi Biotec), respectively [17]. These cells were isolated using an autoMACS™ Pro Separator (Miltenyi Biotec). The purity of the cells was >95% on flow cytometry (data not shown).

Table 1 Synthetic peptides and peptide mixtures used in this study

Tumor-associated antigen	Peptide	Amino acid sequence
Glypican-3	GL1	1–20
	GL2	11–30
	GL3	21–40
	⋮	⋮
	GL57	561–580
MAGE-1	MG-1	1–20
	⋮	⋮
	MG-30	290–309
NY-ESO-1	NY-1	1–20
	⋮	⋮
	NY-17	161–180

Mix 1	Mix 2	Mix 3	Mix 4	Mix 5	Mix 6	Mix 7	Mix 8	Mix 9	Mix 10
GL1	GL2	GL3	GL4	GL5	GL6	GL7	GL8	GL9	GL10
GL11	GL12	GL13	GL14	GL15	GL16	GL17	GL18	GL19	GL20
GL21	GL22	GL23	GL24	GL25	GL26	GL27	GL28	GL29	GL30
GL31	GL32	GL33	GL34	GL35	GL36	GL37	GL38	GL39	GL40
GL41	GL42	GL43	GL44	GL45	GL46	GL47	GL48	GL49	GL50
GL51	GL52	GL53	GL54	GL55	GL56	GL57	MG-1	MG-2	MG-3
MG-4	MG-5	MG-6	MG-7	MG-8	MG-9	MG-10	MG-11	MG-12	MG-13
MG-14	MG-15	MG-16	MG-17	MG-18	MG-19	MG-20	MG-21	MG-22	MG-23
MG-24	MG-25	MG-26	MG-27	MG-28	MG-29	MG-30	NY-1	NY-2	NY-3
NY-4	NY-5	NY-6	NY-7	NY-8	NY-9	NY-10	NY-11	NY-12	NY-13
NY-14	NY-15	NY-16	NY-17	–	–	–	–	–	–

IFN-γ ELISpot assay

The ELISpot assay was performed using an IFN-γ ELISpot assay kit (Mabtech AB, Stockholm, Sweden) as previously described [17]. Briefly, a 96-well microtiter plate with a nitrocellulose membrane bottom (Millititer; Millipore, Bedford, MA) was coated with 100 μl anti-IFN-γ monoclonal antibody at a concentration of 15 μg/ml in phosphate-buffered saline (PBS) overnight at 4°C. Unbound antibody was removed by washing 6 times in Hanks' balanced saline solution. After blocking with AIM-V medium (Invitrogen Japan, Tokyo, Japan) containing 10% fetal bovine serum, 1×10^5 $CD8^+$ T-cells, 1×10^4 autologous monocytes, and a TAA peptide mixture at 10 μg/ml of each peptide were placed and incubated in duplicate in 100 μl AIM-V medium at 37°C in a humid atmosphere with 5% CO_2. After incubation for 18 h, the cells were removed by washing the plate 8 times with PBS. Next, 100 μl of biotin-conjugated monoclonal antibody was added to each well, and the plates were incubated further for 2 h at room temperature. Wells were washed 5 times with PBS and incubated with 100 μl streptavidin–alkaline phosphatase for 2 h. Unbound antibodies were removed by washing 6 times with PBS. Then, 100 μl of alkaline phosphatase substrate (Bio-Rad Laboratories, Richmond, CA) was added to each well and incubated until dark spots emerged. Color development was stopped by washing 3 times with water, and the plates were allowed to dry. Using an ELISpot reader (KS ELISPOT compact; Carl Zeiss, Oberkochen, Germany), the number of spot-forming cells (SFCs) per well was counted. Numbers of TAA-specific SFCs for each peptide mixture were calculated by subtracting the mean number of SFCs of 2 control wells (without stimulus) from the mean number of SFCs of 2 wells stimulated by TAA antigens. An SFC number was calculated for each patient as the sum of SFCs in each peptide mixture. ELISpot assays were performed before and 3–7 days after treatment. When TAA-specific $CD8^+$ T-cell responses were analyzed in 10 normal subjects, we were unable to detect any responses against TAA peptides in the ELISpot assay (data not shown).

Statistical analyses

The relationship between the number of TAA-specific $CD8^+$ T-cells and the recurrence-free period was analyzed

using a parametric survival model. The log-rank test was used to compare recurrence-free data for 2 groups. The effects of multiple explanatory variables on recurrence-free interval were analyzed using a Cox proportional hazards model. Statistical analyses were performed using the statistical software JMP version 5 (SAS Institute Inc., Cary, NC). Differences were considered as significant when the P value was less than 0.05.

Results

TAA-specific CD8$^+$ T-cells were detected by ELISpot assay before and after HCC treatment in most HCC patients

The characteristics of the 20 patients enrolled in this study are shown in Table 2. The 20 patients had no HCC detected by ultrasonography, enhanced CT, and/or MRI 1 month after treatment for HCC. In those patients with HCCs who had up to 3 HCCs and in whom the diameter of each lesion was 3 cm or less, the treatment was usually RFA; the remaining patients were treated by TACE. However, in a few patients (patients 2 and 5) in whom the diameter of each lesion was less than 3 cm, the physician in charge of the patient selected TACE because they could not deny the existence of more lesions that were undetectable by conventional enhanced CT. The clinical courses of the patients were followed for 3–29 months after therapy for HCC. The ELISpot assay was performed to detect CD8$^+$ T-cell responses to TAAs before and 3–7 days after treatment. The data are shown in Table 3 as SFCs (total count of TAA-specific CD8$^+$ T-cells/1 $\times$ 10^5 CD8$^+$ T-cells). Sixteen out of 20 patients (80%) showed a positive response (10 or more SFCs) for TAA peptides either before and/or after treatment. The numbers of SFCs (mean $\pm$ SD) before and after therapy were 33.8 $\pm$ 51.4 (0–161, median 16.5) and 32.9 $\pm$ 34.7 (0–130, median 23.0), respectively. Of the 20 patients, 5 (25%) and 7 (35%) showed a high TAA-specific immune response (40 or more SFCs) before and after treatment, respectively.

When we analyzed the TAA peptides recognized by CD8$^+$ T-cells, we occasionally observed that different peptide mixtures were identified as positive before and after HCC treatment (data not shown).

Change in TAA-specific CD8$^+$ T-cell response induced by HCC treatment does not correlate with recurrence-free period

The number of SFCs increased in 11 of 20 (55%) patients after treatment. In these patients, TAA-specific CTLs might have been induced by the treatment. There were no significant differences between the increase in TAA-specific CD8$^+$ T-cell response induced by the treatment and either therapeutic procedure, laboratory data, or background of the patients (data not shown). The increase in TAA-specific CTLs after treatment did not predict a better prognosis of HCC.

Table 2 Characteristics of HCC patients before HCC treatment

	$n = 20$	Median
Age (years)[a]	68.8 $\pm$ 9.4	73.0
Gender		
M	11	
F	9	
AST (IU/l)[a]	70 $\pm$ 49	52
ALT (IU/l)[a]	63 $\pm$ 43	54
PLT ($\times 10^4/\mu$l)[a]	9.8 $\pm$ 5.3	8.5
PT (%)[a]	81 $\pm$ 11	78
Alb (g/dl)[a]	3.5 $\pm$ 0.4	3.4
T-Bil (mg/dl)[a]	0.9 $\pm$ 0.4	0.9
AFP (ng/ml)[a]	86 $\pm$ 157	16
Virus		
HCV	17	
NBNC	3	
Child-Pugh class		
A	12	
B	8	
HCC size (mm)[a]	23 $\pm$ 8	23
No. HCCs		
1	9	
2	4	
3	7	
>3	0	
Treatment		
RFA	13	
TACE	5	
RFA + TACE	2	

NBNC Negative for neither HBV nor HCV infection, *RFA* radiofrequency ablation, *TACE* trans-catheter chemo-embolization

[a] Results are shown as mean $\pm$ SD

Platelet count, prothrombin time, and the magnitude of TAA-specific immune response after treatment correlate with the recurrence-free period by univariate analysis

When we analyzed the relationship between TAA-specific SFCs detected by the ELISpot assay or other clinical variates and the HCC-free interval using a parametric survival model, we found that platelet count, prothrombin time, and the TAA-specific CD8$^+$ T-cell response after treatment significantly correlated with the HCC-free interval