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Lymphotropic hepatitis C virus has an interferon-resistant phenotype

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SUMMARY. Hepatitis C virus (HCV) infects and associates with B cells, leading to abnormal B-cell activation and development of lymphoproliferative and autoimmune disorders. This immune perturbation may in turn be associated with the resistance of HCV against the host immune system. The objective of this study was to analyse the effects of HCV infection of B cells on the efficacy of interferon (IFN)-based therapy. The study enrolled 102 patients with chronic hepatitis C who were treated with pegylated IFN plus ribavirin. HCV RNA titres in B cells were compared in patients with rapid viral responder (RVR) *vs* non-RVR, sustained viral responder (SVR) *vs* non-SVR and null viral responder (NVR) *vs* VR. The levels of HCV RNA in B cells were significantly higher in non-RVR, non-SVR and NVR groups. Association between the therapy outcome and the positive B-cell HCV RNA was also investigated in relation to other known viral and host factors. Multivariable analyses showed that the positive B-cell HCV RNA and the minor single-nucleotide polymorphism near the IL28B gene (rs8099917) were independent factors associated with NVR in patients infected with HCV genotype 1. When these two factors were combined, the sensitivity, specificity, positive and negative predictive values for NVR were 92.3%, 98.2%, 92.3% and 98.2%, respectively. Genotype 1 and the presence of one or no mutations in the IFN-sensitivity determining region were associated with higher levels of B-cell HCV RNA. B-cell-tropic HCV appears to have an IFN-resistant phenotype. B-cell HCV RNA positivity is a predictive factor for resistance to IFN-based therapy.

Keywords: B-cell disorders, B-cell tropism, hepatitis C virus, interferon resistance, lymphoproliferative disorders.

INTRODUCTION

Hepatitis C virus (HCV) infects 200 million people worldwide, causing chronic hepatitis, liver cirrhosis and hepatocellular carcinoma [1,2]. In addition, HCV infection causes proliferative disorders of B cells, such as mixed cryoglobulinemia [3–5] and B-cell non-Hodgkin's lymphoma [6]. Even when no clinical extrahepatic manifestations are observed, 74% of patients infected with HCV present 'biological' extrahepatic manifestations, such as cryoglobulinemia, high levels of rheumatoid factor, hypocomplementemia and clonal expansion of B cells [7]. Molecular mechanisms of these B-cell abnormalities remain to be clarified. Replication of HCV is observed in the peripheral blood mononuclear cells (PBMCs), especially in B cells of patients with chronic hepatitis C (CH-C) [8–10]. In our previous report, HCV RNA was detected in B cells from 64.0% of CH-C patients and negative-strand HCV RNA in 5.3% of these patients [7]. The presence of lymphoproliferative disorders (LPDs) is associated with HCV RNA detection in B cells. Furthermore, the IFN responses of peripheral B cells from CH-C patients have been reported to be impaired [11]. Taken together, these results indicate that B-cell-tropic HCV exists and may play an important role in the immunological dysfunction of B cells.

As the discovery of HCV, rapid progress has been made in the development of antiviral therapies against HCV infection. The current standard treatment for CH-C, a combination of pegylated interferon (PEG-IFN) and ribavirin for 24 or 48 weeks, has improved clinical responses [12,13]. However, even with this regimen, half of CH-C patients do not achieve sustained clearance of HCV. The likelihood of the response varies greatly, depending on both host and viral factors. Viral factors associated with resistance to interferon (IFN)-based therapy include HCV genotype 1, high viral

Abbreviations: CH-C, chronic hepatitis C; HCV, hepatitis C virus; ISDR, interferon sensitivity determining region; LPD, lymphoproliferative disorders; NPV, negative predictive value; NVR, null viral responder; PBMCs, peripheral blood mononuclear cells; PEG-IFN, pegylated interferon; PPV, positive predictive value; RVR, rapid viral responder; SNP, single-nucleotide polymorphism; SVR, sustained viral responder; VR, viral responder.

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loads and reduced quasispecies diversity in the IFN-sensitivity determining region (ISDR) of NS5A [14], as well as mutations at the 70th and 91st amino acids of the HCV core region [15]. Host factors include age, race, gender, degree of hepatic fibrosis, insulin resistance [16] and interleukin (IL) 28B genotype [17]. Although many of these factors are used for prediction of the response to IFN therapy, it is currently impossible to determine who will attain viral eradication from hepatocytes. Furthermore, examination of all the markers increases medical expenses.

HCV replicates in lymphoid cells at low levels, but they may serve as an extrahepatic reservoir; this is implicated in the recurrence and persistence of HCV infection in immunosuppressed individuals [18]. In addition, low amounts of positive- and negative-strand HCV RNAs are still detected in lymphocytes of sustained viral response (SVR) patients with CH-C after the virus eradication from hepatocytes by IFN-based therapy [19,20]. HCV in lymphoid cells may represent a subpopulation resistant to innate immunity and/or a source of B-cell dysfunction.

The primary purpose of this study was to test the hypothesis that HCV associated with B cells confers resistance to IFN-based therapy and serves as a useful predictive parameter for therapy outcome. The study also evaluated other viral and host factors in conjunction with B-cell HCV RNA as predictive markers for the efficacy of antiviral therapy.

METHODS

Subjects

This study enrolled 102 patients with CH-C who were treated with PEG-IFN plus ribavirin therapy for 24, 48 or 72 weeks (Showa University Hospital, 2005–2010). Diagnosis of HCV infection was based on the detection of anti-HCV antibody and HCV RNA in the serum prior to the initiation of therapy. Clinical characteristics of the HCV-infected patients are shown in Table 1. Missense mutations in the ISDR and in codons 70 and 91 of the core region were analysed in patients infected with HCV genotype 1 by direct sequencing [14,15]. Liver biopsies were performed in 92 patients before the start of therapy; fibrosis was staged from F0 to F4 according to the scheme of Desmet *et al.* [21]. The study protocol was approved by the Ethics Committee of Showa University School of Medicine, Tokyo, Japan. Informed written consent was obtained from each participant and the study followed the ethical guidelines of the 1975 Declaration of Helsinki.

Table 1 Clinical characteristics of HCV-infected patients (n = 102)

Age (years)	54.1 ± 11.4
Male/Female	53/49
ALT (IU/L)	73.8 ± 74.4
Platelets ($\times 10^4/mm^3$)	18.4 ± 6.5
Fibrosis (F0/F1/F2/F3/F4)	1/38/38/9/6
Outcome of IFN therapy (RVR/SVR/NVR)	46 (45%)/60 (59%)/15 (15%)
IL28B SNPs (T/T *vs* T/G, G/G)	76/99 (77%) *vs* 23/99 (23%)
Cryoglobulinemia	24/98 (24%)
IgG (mg/dl)	1712 ± 504
IgA (mg/dl)	253 ± 118
IgM (mg/dl)	119 ± 64
Rheumathoid factor (>10 IU/mL)	39/99 (39%)
C3 (<86 mg/dL)	16/99 (16%)
C4 (<10 mg/dL)	4/99 (4%)
CH50 (<20 U/mL)	59/97 (58%)
Presence of B cell clonality	9/71 (12.7%)
HCV genotype (1/2)	77 (75.5%)/25 (24.5%)
Log HCV RNA in serum (log/mL)	5.9 ± 0.82
Positive of HCV RNA in B cells (>10 log/100 ng)	58/102 (57%)

HCV, hepatitis C virus; NVR, null viral responder; RVR, rapid viral responder; SNP, single-nucleotide polymorphism; SVR, sustained viral responder
Continuous variables are presented as mean ± standard error.

Isolation of B cells

B cells were isolated using an auto-MACS™ Pro Separator ver.2.0.0 (Miltenyi Biotec K.K., Bergisch Gladbach, Germany). Briefly, PBMCs were obtained from whole blood (30 mL) by centrifugation. Non-B cells were labelled by a cocktail of specific antibodies that were conjugated to biotin, and the mixture was adsorbed with the MACS microbeads to capture the biotin-labelled cells. The cell–microbead mixture was then passed through the auto-MACS magnetic column, and B cells (the flow-through) were collected.

Quantitation of HCV RNA in B cells

Total RNA from each cellular compartment was extracted using the AllPrep® DNA/RNA/Protein Mini kit (Qiagen, Duesseldorf, Germany). HCV RNA levels were determined in 100 ng of each RNA sample by real-time RT-PCR using the primers described previously [22]; this assay has a detection range over 1.0–8.0 log copies. Samples were scored as positive for HCV RNA when titres exceeded 1.0 log copies/100 ng; this threshold excluded contamination of lymphoid cells with serum HCV RNA [7].

IL28B SNP genotyping assay

In 99 patients, the genotype of an IL28B-proximal single-nucleotide polymorphism (SNP) (rs8099917) was determined by real-time PCR. For each patient, genomic DNA (10–100 ng) was purified (Qiagen) and amplified using the

SNP Genotyping Assay specific for rs8099917 (Applied Biosystems, Foster City, CA, USA) according to the TaqMan® GTXpress™ Master Mix Protocol (Applied Biosystems).

Statistical analysis

The mean of continuous variables, with and without normal distribution, was compared by Student's *t* test or by the Wilcoxon test, respectively. Comparison of discontinuous variables was performed by the chi-squared test or Fisher's exact test. A *P* value of <0.05 was considered to be statistically significant. Values with normal distributions were expressed as the mean ± standard error (SE). For variables that were not distributed normally, data were transformed into log values as required. To examine the relation between patient parameters and the outcome of therapy, candidate independent variables were analysed by the Wald test of logistic regression modelling via multivariable analysis. All statistical analyses were performed using JMP ver. 9 software (SAS Institute, Cary, NC, USA).

RESULTS

The levels of HCV RNA in B cells and IFN treatment response

HCV RNA titres in B cells were compared in patients with rapid viral responder (RVR) *vs* non-RVR (Fig. 1a), SVR *vs* non-SVR (Fig. 1b) and null viral responder (NVR) *vs* VR (non-NVR) (Fig. 1c) using univariable analysis. The levels of HCV RNA in B cells were significantly different in all three comparisons (P = 0.0001, 0.0012 and 0.0020, respectively). The results suggest that patients whose B cells have less HCV RNA are more sensitive to IFN-based therapy. Mean HCV RNA titres in B cells of NVR patients showed the highest titre among the three groups (RVR: 1.0 ± 0.2, SVR: 1.2 ± 0.2 and NVR: 2.9 ± 0.4 log copies/100 ng RNA). The fraction of patients scoring positive for the presence of HCV RNA in B cells was also significantly different in each comparison: [RVR: 18/46 (39.1%) *vs* non-RVR: 40/56 (71.4%), P = 0.0010], [SVR: 26/60 (43.3%) *vs* non-SVR: 32/42 (76.2%), P = 0.0010] and [NVR: 14/15 (93.3%) *vs* VR: 44/87 (50.6%), P = 0.0016].

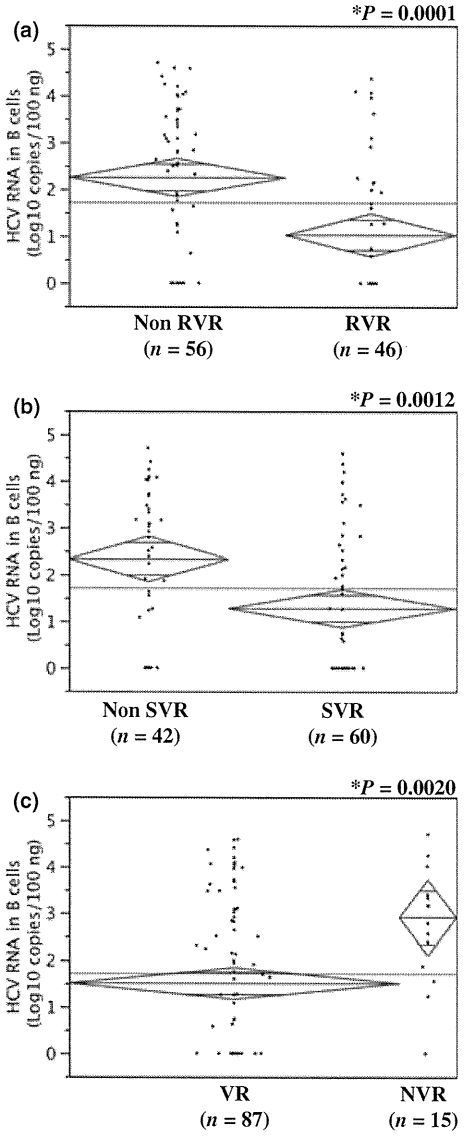

Fig. 1 Comparison of hepatitis C virus (HCV) RNA titres in B cells of patients classified by viral response status: (a) Rapid viral responder (RVR) *vs* non-RVR; (b) sustained viral responder (SVR) *vs* non-SVR; and (c) null viral responder (NVR) *vs* viral responder (VR). A horizontal grey line shows mean of all samples from both groups. Two grey diamonds indicate the averages and 95% confidence interval of each group. Statistical significance was determined by Student's *t* test.

HCV RNA in B cells as a predictive factor for viral response

We next analysed the factors associated with response (RVR, SVR or NVR) to IFN-based therapy. We used the presence of HCV RNA in B cells as a marker of LPD because it is associated with the presence of LPD [7]. Homozygosity for the major allele (T/T) of the IL28B SNP (rs8099917), HCV genotype 2 and low serum HCV RNA levels were significantly associated with RVR by univariable analysis (Table 2a), while the presence of HCV RNA in B cells was associated with non-RVR. When the independence of these factors was assessed by multivariable analysis using a multiple logistic regression model, homozygosity for the major allele (T/T) of the IL28B SNP and lower levels of HCV RNA in sera (<6 log copies/mL) were shown to be independent factors associated with RVR to IFN-based therapy.

In the comparisons of SVR with non-SVR, lower age, higher platelet number, fibrosis stage 1 or 2, the T/T allele of

Table 2 Univariable and multivariable analysis for RVR (a), SVR (b) and NVR (c)

(a) RVR

	Response for therapy		
Univariable analysis	RVR (n = 46)	Non-RVR (n = 56)	P value
Age (years)	52.6 ± 12.0	55.5 ± 10.7	NS
Men	27/46 (59%)	26/56 (46%)	NS
ALT (IU/L)	78.4 ± 68.9	70.0 ± 79.1	NS
Platelets ($\times 10^4/mm^3$)	19.2 ± 6.5	17.8 ± 6.4	NS
Fibrosis stages 1 or 2	36/39 (92%)	41/53 (77%)	NS
IL28B SNPs rs8099917 major allele (T/T)	**39/43 (91%)**	**37/56 (66%)**	**0.0040**
HCV genotype 2	**18/46 (39%)**	**7/56 (13%)**	**0.0019**
Log HCV RNA in serum (log/mL)	**5.45 ± 0.95**	**6.27 ± 0.43**	**<0.0001**
Presence of HCV RNA in B cells (>10 log/100 ng)	**18/46 (39%)**	**40/56 (71%)**	**0.0010**
Multivariable analysis	Odds ratio	95% CI	
IL28B SNP rs8099917 major allele (T/T)	**2.42**	**1.28–5.15**	**0.0116**
HCV genotype 2	1.53	0.84–2.85	NS
HCV RNA in serum <6.0 log/mL	**2.51**	**1.51–4.36**	**0.0006**
Absence of HCV RNA in B cells (<10 log copies/100 ng)	1.38	0.81–2.34	NS

(b) SVR

	Response for therapy		
Univariable analysis	SVR (n = 60)	Non-SVR (n = 42)	P value
Age (years)	**52.1 ± 11.6**	**57.1 ± 10.4**	**0.0247**
Men	32/60 (53%)	21/42 (50%)	NS
ALT (IU/L)	78.1 ± 70.8	67.2 ± 79.7	NS
Platelets ($\times 10^4/mm^3$)	**20.3 ± 7.0**	**15.7 ± 4.6**	**0.0003**
Fibrosis stages 1 or 2	**47/52 (90%)**	**30/40 (75%)**	**0.0477**
IL28B SNPs rs8099917 major allele (T/T)	**52/57 (91%)**	**24/42 (57%)**	**<0.0001**
HCV genotype 2	**21/60 (35%)**	**4/42 (10%)**	**0.0032**
Log HCV RNA in serum (log/mL)	**5.64 ± 0.94**	**6.27 ± 0.35**	**<0.0001**
Presence of HCV RNA in B cells (>10 log/100 ng)	**26/60 (43%)**	**32/42 (76%)**	**0.0010**
Multivariable analysis	Odds ratio	95% CI	
Age <50	1.39	0.77–2.57	NS
Platelets >17 × $10^4/mm^3$	**2.16**	**1.19–4.05**	**0.0139**
Fibrosis stage 1 or 2	1.22	0.56–2.77	NS
IL28B SNP rs8099917 major allele (T/T)	**3.47**	**1.76–7.84**	**0.0009**
HCV genotype 2	1.51	0.70–3.55	NS
HCV RNA in serum <6.0 log/mL	**2.58**	**1.33–5.63**	**0.0085**
Absence of HCV RNA in B cells (<10 log copies/100 ng)	1.66	0.88–3.24	NS

(c) NVR

	Response for therapy		
Univariable analysis	NVR (n = 15)	VR (n = 87)	P value
Age (years)	54.0 ± 2.9	54.2 ± 1.2	NS
Men	10/15 (67%)	43/87 (49%)	NS
ALT (IU/L)	90.5 ± 19.2	71.0 ± 8.0	NS
Platelets ($\times 10^4/mm^3$)	15.5 ± 1.7	19.0 ± 0.7	NS

Table 2 Continued

(c) NVR

Univariable analysis	Response for therapy: NVR (n = 15)	VR (n = 87)	P value
Fibrosis stages 1 or 2	4/14 (29%)	11/78 (14%)	NS
IL28B SNPs minor alleles (T/G or G/G)	**12/15 (80%)**	**11/84 (13%)**	**<0.0001**
HCV genotype 1	**15/15 (100%)**	**25/87 (29%)**	**0.0169**
Log HCV RNA in serum (log/mL)	**6.33 ± 0.21**	**5.83 ± 0.09**	**0.0279**
Presence of HCV RNA in B cells (>10 log/100 ng)	**14/15 (93%)**	**44/87 (51%)**	**0.0020**
Multivariable analysis	**Odds ratio**	**95% CI**	
IL28B SNP rs8099917 minor allele (T/G or G/G)	**8.51**	**3.52–27.45**	**<0.0001**
HCV RNA in serum >6.0 log/mL	1.54	0.51–4.85	NS
Presence of HCV RNA in B cells (>10 log copies/100 ng)	**4.80**	**1.57–24.85**	**0.0179**

HCV, hepatitis C virus; NVR, null viral responder; RVR, rapid viral responder; SNP, single-nucleotide polymorphism; SVR, sustained viral responder
Continuous variables are presented as mean ± standard error.
Bold text indicates statistically significant associations ($P < 0.05$). NS: not significant.

the IL28B SNP, HCV genotype 2 and lower serum levels of HCV RNA were significantly associated with SVR by univariable analysis (Table 2b). Higher platelet counts ($>17 \times 10^4/mm^3$), the T/T allele of the IL28B SNP and lower serum levels of HCV RNA (<6.0 log/mL) were independent factors for SVR by multivariate analysis.

In comparisons of NVR with VR, homozygosity (G/G) or heterozygosity (T/G) for the minor allele of the IL28B SNP, HCV genotype 1, higher serum levels of HCV RNA and the presence of HCV RNA in B cells were significantly associated with NVR by univariable analysis (Table 2c). As none of the NVR patients were infected with HCV genotype 2, multivariable analysis was performed using the 73 samples of HCV genotype 1-infected patients to determine the independent factors associated with NVR. Presence of minor alleles (G/G or T/G) of the IL28B SNP and the presence of HCV RNA in B cells were both found to be independent factors associated with NVR in patients infected with HCV genotype 1.

Stratified analysis of outcome for IFN-based therapy

To assess the utility of HCV RNA in B cells as a predictive factor for the outcome of IFN-based therapy, we repeated the analysis on stratified groups. When the patients were stratified by HCV genotype or by IL28B SNP genotype, the rates of RVR and SVR were higher among cases scoring negative for HCV RNA in B cells (Table 3). In HCV genotype 1-infected patients possessing a minor allele of the IL28B SNP, two of five patients (40%) lacking HCV RNA in B cells achieved RVR and SVR; none of these five patients had a NVR. In contrast, none of 14 patients with positive HCV

Table 3 Stratified analysis of outcome for the IFN-based therapy (n = 100)

	IL28B SNP rs8099917	HCV RNA in B cells	Total	RVR	SVR	NVR
HCV genotype 1 (n = 77)	T/T (n = 58)	Positive	38	15/37 (41%)	23/37 (62%)	2/37 (0.5%)
		Negative	20	11/20 (55%)	14/20 (70%)	1/20 (0.5%)
	T/G, G/G (n = 19)	Positive	14	0/14 (0%)	0/14 (0%)	12/14 (86%)
		Negative	5	2/5 (40%)	2/5 (40%)	0/5 (0%)
HCV genotype 2 (n = 23)	T/T (n = 19)	Positive	4	2/4 (50%)	2/4 (50%)	0/4 (0%)
		Negative	15	12/15 (80%)	15/15 (100%)	0/15 (0%)
	T/G, G/G (n = 4)	Positive	2	0/2 (0%)	1/2 (50%)	0/2 (0%)
		Negative	2	2/2 (100%)	2/2 (100%)	0/2 (0%)

HCV, hepatitis C virus; NVR, null viral responder; RVR, rapid viral responder; SNP, single-nucleotide polymorphism; SVR, sustained viral responder

RNA in B cells achieved either RVR or SVR; 12 of these 14 patients (86%) had a NVR. These results suggest that the absence of HCV RNA in B cells is a useful predictor of the response to IFN-based therapy. The ability to predict the response is defined based on sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) (Table 4). In genotype 1-infected patients, sensitivity, specificity, PPV and NPV for NVR according to genetic variation near IL28B were 100%, 87.7%, 65.0% and 100%, respectively. When both IL28B SNP variants and the presence of HCV RNA in B cells were combined, specificity and PPV were higher (specificity 98.2% and PPV 92.3%). In HCV genotype 2-infected patients possessing a major allele of the IL28B SNP, two of four patients (50%) with HCV RNA-positive B cells failed to achieve SVR. In contrast, all 15 patients with HCV RNA-negative B cells achieved SVR. Even in patients with minor alleles of the IL28B SNPs, both of two patients with HCV RNA-negative B cells achieved SVR. In genotype 2-infected patients, sensitivity, specificity, PPV and NPV for SVR according to the presence of HCV RNA in B cells were 90.0%, 100%, 100% and 60.0%, respectively.

These combined results indicate that the minor alleles of IL28B SNPs plus the presence of HCV RNA in B cells are useful predictors for NVR in genotype 1-infected patients, and the presence of HCV RNA in B cells is a predictor for non-SVR in genotype 2-infected patients.

Lymphotropic HCV has an IFN-resistant phenotype

We further characterized the viral phenotype of IFN resistance for B cell-tropic HCV and the effects of IL28B genotype on HCV RNA titre in B cells. Figure 2a shows that HCV RNA titres in B cells were significantly higher in genotype 1-infected s than in genotype 2-infected patients. The positive rates of HCV RNA in B cells were 68.8% (53/77) and 24.0% (6/25), respectively (P = 0.0001). These results suggest that genotype 1 HCV can infect and/or associate with B cells more efficiently than genotype 2. However, HCV RNA titres in B cells did not differ between patients bearing the major allele (T/T) of the IL28B SNP and those with the minor alleles (G/G or T/G) (data not shown), indicating that the IL28B genotype does not affect HCV infection of (or

Table 4 Predictive factors for outcomes of the IFN-based therapy in patients infected with HCV genotypes 1 and 2

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Genotype 1: Predictive factor for NVR				
IL28B T/G,G/G	100	87.7	65.0	100
IL28B T/G, G/G and HCV RNA in B cells (+)	92.3	98.2	92.3	98.2
Genotype 2: Predictive factor for SVR				
IL28B T/T	85.7	33.3	90.0	25.0
HCV RNA in B cells (−)	90.0	100	100	60.0

HCV, hepatitis C virus; NVR, null viral responder; PPV, positive predictive value; SVR, sustained viral responder.

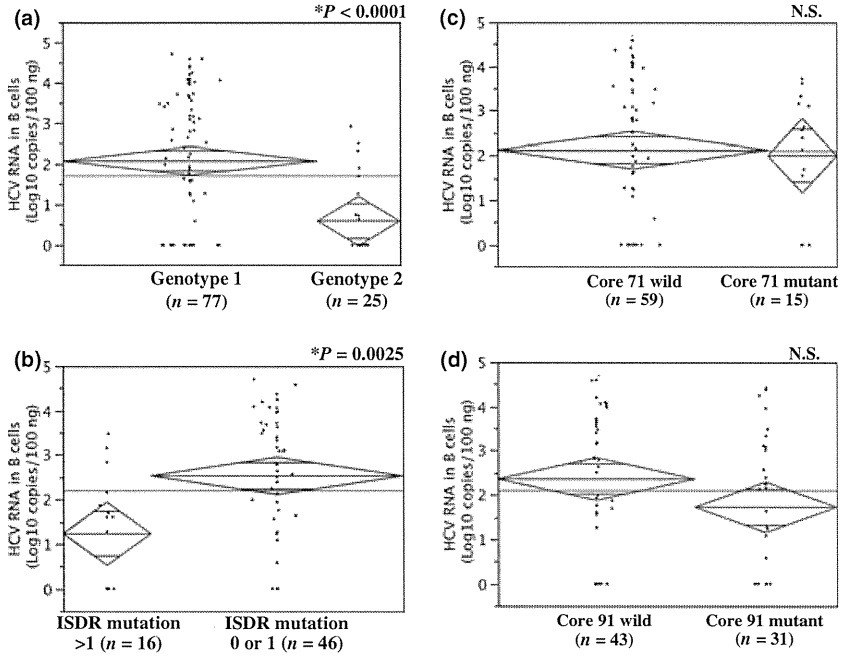

Fig. 2 Comparison of hepatitis C virus (HCV) RNA titres in B cells of (a) patients infected with HCV genotypes 1 and 2; (b) patients infected with HCV with 0-1 interferon sensitivity determining region amino acid mutations *vs* those with >1 mutations; (c) patients infected with HCV with codon 70 amino acid mutation of the core region *vs* those with wild type; and (d) patients infected with HCV with codon 91 amino acid mutation of the core region *vs* those with wild type. A horizontal grey line shows mean of all samples. Two grey diamonds indicate the averages and 95% confidence interval of each group. Statistical significance was determined by Student's t test. N.S.: not significant.

association with) B cells. We next analysed the effects of viral sequence and quasispecies diversity, which are known to affect the outcome of the IFN-based therapy, on HCV RNA titres in B cells. The number of nucleotide mutations in the ISDR of the NS5A region and missense mutations at codons 70 and 91 of the core region were determined in samples from patients infected with HCV genotype 1. Figure 2b shows that the HCV RNA levels in B cells were higher in patients infected with HCV with 0 or 1 mutation in the ISDR (ISDR 0-1) than in those harbouring HCV with more than one ISDR mutations (ISDR > 1). Additionally, the fraction of patients with HCV RNA in B cells was higher among those infected with ISDR 0-1 HCV [ISDR 0-1: 38/46 (82.6%) *vs* >1: 9/16 (56.3%), $P = 0.0463$]. However, substitutions at core positions 70 or 91 did not associate with HCV RNA titre in B cells (Fig. 2c,d). These results suggest that the number of mutations in ISDR, but not core mutations, affect the persistence of HCV in B cells.

DISCUSSION

While the human hepatocyte is the primary target for HCV infection, HCV also has lymphotropism, especially toward B cells [7,23]. Previous reports demonstrated the *in vitro* and *in vivo* association of HCV with B cells [7,24,25]. In our earlier report, we detected the replication of HCV in B cells in about 5% of HCV-infected patients and the presence of HCV RNA in about 64% of these patients [7]. Stamataki *et al.* [25] also demonstrated that HCV JFH1 bound (but did not replicate in) B cells in the cell culture system and that the virion binding B cells became more stable than free virions. These results suggest that the HCV isolates can be classified into three subgroups. The first subgroup consists of HCV isolates that neither infect nor adhere to B cells. A second subgroup (which encompasses most HCV isolates) can associate with B-cell surface receptors, but do not replicate efficiently in these cells. Such binding may induce activation and signalling, contributing to prolonged B-cell survival. The third subgroup (detected in only 5% of patients) consists of HCV isolates capable of infecting B cells and replicating efficiently in these cells.

The present study showed that HCV isolates that have infected and/or associated with B cells had an IFN-resistant phenotype. HCV RNA titres in B cells were significantly higher in patients with poor responses to IFN-based therapy (Fig. 1). Furthermore, the presence of HCV RNA in B cells was one of the factors determining the outcome of IFN-based therapy (Table 2). The precise mechanism of these clinical effects remains unknown; B-cell-associated viruses may be more stable than free virions [25]. Although current antiviral therapies can eliminate free HCV virions from sera, HCV is thought to survive in lymphocytes [19,20]. Peripheral blood memory B cells infected with HCV may be recruited to the liver of patients with CH-C [26]. Lymphotropic HCV may survive even after the IFN-based therapy and re-infect hepatocytes through the infiltration of HCV-infected B cells to the liver.

Another important issue is that HCV infection of and/or association with B cells may also contribute to IFN resistance by inducing dysfunction in B cells. The patients with HCV-positive B cells had at least one abnormality of LPD markers, indicating that HCV infection of and/or association with B cells reflected the presence of B cell-disorders [7]. In fact, HCV has been reported to bind naïve B cells through CD81, leading to the abnormal activation of B cells in the absence of ligation with the B cell receptor [24].

Many predictive markers (both viral and host factors) are associated with IFN-based treatment outcomes. Prediction of NVR and/or non-SVR status might allow hepatologists to pay closer attentions to poorly responding patients while minimizing unnecessary therapy. Recently, the genotypes of IL28B SNPs have been reported to be a valuable predictor for the outcome of IFN-based therapy [17,27,28]. In the present study, multivariable analyses showed that the genotypes of IL28B SNPs and the presence of HCV RNA in B cells were independent predictive markers for NVR in genotype 1-infected patients (Table 2c). (No genotype 2-infected NVR patients were detected in this study.) The combination of IL28B minor alleles and the presence of HCV RNA in B cells are especially valuable for predicting the NVR *vs* VR (viral responder) distinction in patients infected with HCV genotype 1 and for predicting the SVR *vs* non-SVR distinction in patients infected with HCV genotype 2. In patients infected with HCV genotype 1, the PPV for NVR in individuals with the IL28B minor allele was significantly elevated from 65.3% to 92.3% by the addition of HCV RNA in B cells as a predictive marker (Table 4). Similarly, increases in sensitivity, specificity, PPV and NPV for SVR were all higher in HCV genotype 2-infected patients when the presence of HCV RNA in B cells was used as a marker. The detection of HCV RNA in B cells may serve as a useful parameter for predicting the outcome of IFN-based therapy in patients infected with HCV genotype 2 although further studies with an increased population size are warranted.

Multiple host and viral factors are proposed as predictors of outcomes for IFN-based therapy. Host genotype (IL28B SNP alleles) and mutations in the virus (including the ISDR and the HCV core region) have been reported as significant pretreatment predictors of response to PEG-IFN and ribavirin combination therapy [29,30]. The present study shows that patients with B-cell-associated HCV RNA typically are infected with genotype 1, and their HCV has fewer mutations in the ISDR. Lerat *et al.* [8] have shown that PBMCs from patients infected with genotype 1 exhibit a higher detection rate of positive- and negative-strand HCV RNA, and their results are consistent with our observations: HCV ISDR mutations, but not substitutions of the core region, affect HCV RNA titres in B cells. HCV ISDR mutations may affect HCV infection of B cells through the effects on IFN signalling [31]. Neither viral titres nor the detection of HCV

RNA in B cells was associated with the genotypes of IL28B SNPs, which are thought to be the strongest genetic factor predicting the outcome of IFN-based therapy. Detail mechanisms underlying the association of the genotype of IL28B SNPs with the outcome of the IFN-based therapy, remain unknown. Expression levels of interferon λ mRNA and/or *in situ* cytokine levels in liver may differ between patients with the major or minor alleles of IL28B SNPs. It is possible that the circumstance of the innate immunity against HCV in liver is different from that in B cells. It is speculated that the presence of HCV RNA in B cells is linked to the interferon-resistance phenotype of the virus itself and/or the host immune-disorders triggered by the abnormal activation of B cells in patients. In conclusion, HCV that infects or associates with B cells appears to present an IFN-resistant phenotype, and the presence of HCV RNA in B cells is a useful predictive marker for resistance to IFN-based therapy.

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Original Article

Cyclooxygenase-2 gene promoter polymorphisms affect susceptibility to hepatitis C virus infection and disease progression

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Aim: Because polymorphisms of cyclooxygenase-2 (*COX-2*) and osteopontin (*OPN*) promoter regions and a promoter/enhancer region of forkhead box protein 3 (*FOXP3*) gene are known to affect immune responses, we examined whether these polymorphisms can influence susceptibility to hepatitis C virus (HCV) infection and progression of liver disease.

Methods: Peripheral blood samples were obtained from 104 Japanese patients with chronic HCV infection and 74 healthy Japanese donors. Polymerase chain reaction single-stranded conformational polymorphism analysis of genomic DNA was performed to determine the polymorphisms.

Results: The risk of persistent HCV infection was decreased in subjects with –1195GG genotype of the *COX-2* promoter region. However, in patients with chronic HCV infection, the –1195GG genotype was associated with advanced-stage liver disease. A luciferase reporter assay performed to analyze the effect of single nucleotide polymorphisms (SNP) (–1195A or –1195G) in *COX-2* gene on transcriptional activity using the HepG2, Huh7 and HeLa cell lines indicated that the –1195G genotype showed higher transcriptional activity than the –1195A genotype. SNP of *OPN* and *FOXP3* did not differ between patients with chronic HCV infection and controls. However, the –443TT genotype of the *OPN* promoter region was associated with increased inflammatory activity of the liver.

Conclusion: These results suggest that the –1195GG genotype of the *COX-2* promoter region protects 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.

Key words: cyclooxygenase-2, forkhead box protein 3, hepatitis C virus, osteopontin, single nucleotide polymorphisms

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection causes chronic hepatitis (CH), liver cirrhosis (LC) and eventually leads to hepatocellular carcinoma (HCC). Immune responses are thought to play important roles in the pathogenesis of viral hepatitis and inflammation is thought to be an important factor in the progression of liver injury.

Cyclooxygenase-1 (COX-1) and COX-2 are enzymes that convert arachidonic acid into prostaglandins and thromboxanes. COX-1 is constitutively expressed in various tissues and plays important roles in homeostasis. In contrast, COX-2 is involved in inflammation, angiogenesis, anti-apoptosis and carcinogenesis.[1–4] COX-2 has been reported to be overexpressed in inflammatory tissues and cancers.[5–8]

The HCV core, NS3 and NS5A proteins are shown to stimulate COX-2 expression.[9,10] Overexpression of COX-2 has been reported in CH, LC and HCC tissues. Furthermore, the COX-2 expression level has been reported to correlate with HCV liver injury activity and

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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

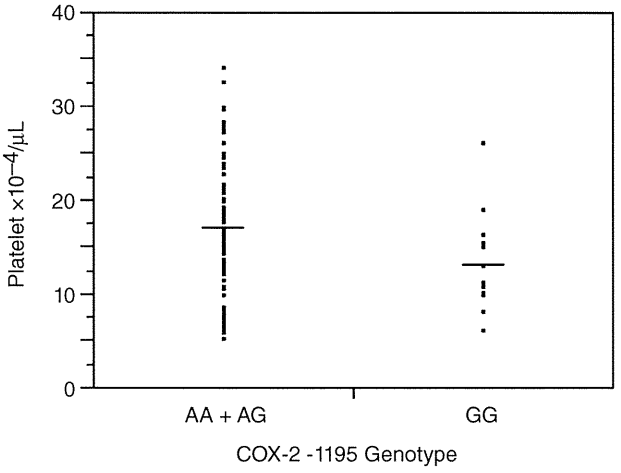

Figure 1 The −1195G>A genotype of the promoter region of the *COX-2* gene and platelet counts in patients with chronic hepatitis C virus infection. Platelet counts were significantly lower in patients with the −1195GG genotype of the promoter region of the *COX-2* gene than those with the −1195AA or 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*

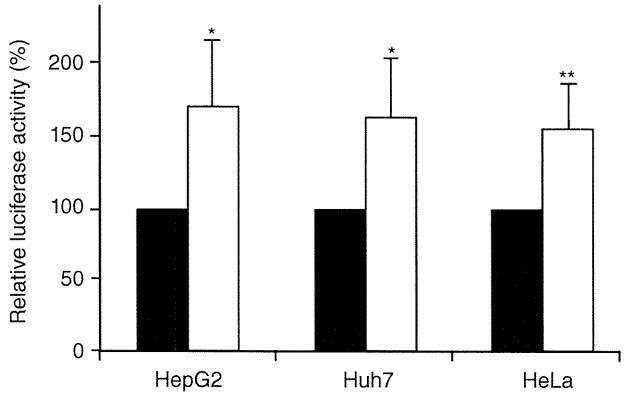

Figure 2 Comparison of the transcriptional activity of the *COX-2* promoter region containing the −1195A and −765G (closed bar) and that containing the −1195G and −765G (open bar) in HepG2, Huh7 and HeLa cells using a luciferase reporter assay as described in Methods. The average relative luciferase 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 $*P < 0.05$, $**P = 0.05$.

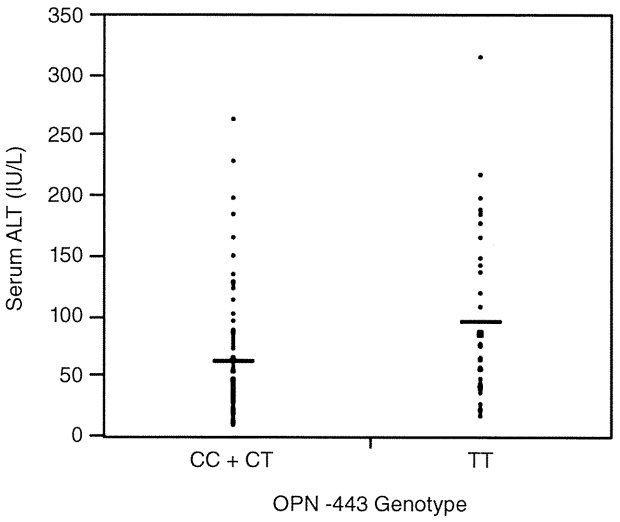

Figure 3 Relationship between the −443T>C genotype of the promoter region of the *OPN* gene and alanine aminotransferase (ALT) levels in patients with chronic hepatitis C virus infection. ALT levels were significantly higher in patients with the *OPN* promoter region containing −443TT genotype than those 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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Review Article

Immune Response of Cytotoxic T Lymphocytes and Possibility of Vaccine Development for Hepatitis C Virus Infection

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Immune responses of cytotoxic T lymphocytes (CTLs) are implicated in viral eradication and the pathogenesis of hepatitis C. Weak CTL response against hepatitis C virus (HCV) may lead to a persistent infection. HCV infection impairs the function of HCV-specific CTLs; HCV proteins are thought to actively suppress host immune responses, including CTLs. Induction of a strong HCV-specific CTL response in HCV-infected patients can facilitate complete HCV clearance. Thus, the development of a vaccine that can induce potent CTL response against HCV is strongly expected. We investigated HCV-specific CTL responses by enzyme-linked immuno-spot assay and/or synthetic peptides and identified over 40 novel CTL epitopes in the HCV protein. Our findings may contribute to the development of the HCV vaccine. In this paper, we describe the CTL responses in HCV infection and the attempts at vaccine development based on recent scientific articles.

1. Introduction

Hepatitis C virus (HCV) was first identified in 1989 [1]. The HCV is a member of the flavivirus family and is a type of positive-strand RNA virus. The discovery of HCV contributed to the diagnosis of hepatitis C; further, HCV has been implicated in many chronic non-A and non-B hepatitis infections. This virus spreads through needles used for vaccination or drug administration, and about 180 million people in the world are presumed to be infected with HCV. It has been clarified that HCV infection often persists, causing chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC).

Cytotoxic T lymphocyte (CTL) plays a part in viral eradication [2]. These cells have been also implicated in the immunopathogenesis of viral infection [3], because HCV, by itself, does not produce cytopathic effects in hepatocytes directly. It has been thought that hepatitis is caused by the destruction of HCV-infected hepatocytes by immune cells such as natural killer (NK) cells and CTLs. Thus, the investigation of the roles of CTL in immunopathogenesis of HCV would contribute to the development of a new treatment strategy for HCV-induced hepatitis.

Interferon (IFN) therapy alone or with ribavirin and polymerase/protease inhibitor combination therapy has shown positive outcomes in more than 80% of patients with acute HCV infection and 50% of patients with chronic HCV infection. However, IFN causes severe adverse effects including flu-like symptoms, pancytopenia, hyperglycemia, depression, lung fibrosis, and cerebral bleeding. Therefore, there is an urgent need to establish an alternative therapy, which can afford a high rate of sustained virological response and performed with few adverse effects. Immunotherapy with HCV vaccine is one of the candidates of such therapies.

In this review, we have summarized the findings of recent investigations on CTL responses against HCV and the trials for the development of HCV vaccine.

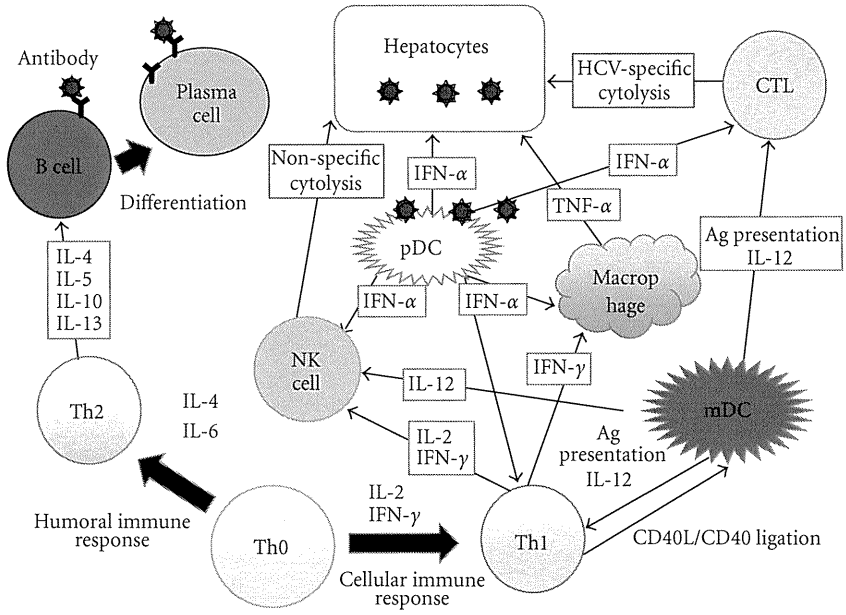

FIGURE 1: Cellular and humoral immune responses in HCV infection. Plasmacytoid dendritic cells (pDC) recognize HCV infection and produce IFN-α, which activates natural killer (NK) cells, helper T (Th) cells, macrophages, and cytotoxic T lymphocytes (CTLs). Activated NK cells destroy the HCV-infected hepatocytes in a nonspecific manner, whereas CTLs destroy the infected hepatocytes in an antigen-specific manner. Myeloid dendritic cells (mDC), which recognize dead hepatocytes, secrete IL-12, promoting the activation of NK cells, Th1 cells, and CTLs. Activated Th1 cells, in turn, promote DC maturation by interacting with the CD40/CD40 ligand. Macrophages stimulated by type 1 helper T (Th1) cells produce TNF-α, which accelerates local inflammation. In humoral immune responses, Th2 cells activate B cells. Plasma cells differentiated from B-cells secrete immunoglobulins to neutralize the circulating HCV. Abbreviated terms: CTL, cytotoxic T lymphocyte; pDC, plasmacytoid dendritic cells; mDC, myeloid dendritic cells; Th1 cell, type 1 helper T cell; Th2 cell, type 2 helper T cell; NK cell, natural killer cell; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

2. CTL Responses in HCV Infection

2.1. Innate Immune Responses in HCV Infection. HCV infection induces cellular and humoral immune responses (Figure 1). Similar to other viral infections, nonspecific immune responses are induced in the early stages of HCV infection for the eradication of HCV. Type I IFNs produced by HCV-infected hepatocytes and plasmacytoid dendritic cells (DCs) suppress viral replication by inducing enzymes such as 2′–5′ oligoadenylate synthetase (OAS) and RNA-dependent protein kinase (PKR) in hepatocytes [4]. The plasmacytoid DC recognizes HCV infection through toll-like receptor (TLR)-7, which interacts with single-stranded RNA [5]. The TLR-signaling upregulates PDC-TREM molecules on the cell surface, and PDC-TREM-dependent signal induces further production of IFN-α [6]. Activated OAS destroys viral RNAs, whereas PKR inhibits forming polysome of viral mRNA [4]. Moreover, type I IFNs activate innate immunity components such as natural killer (NK) cells [7]. The local inflammation further activates natural killer T-cells (NKT cells) and macrophages (Kupffer cells), thereby inducing the production of cytokines such as IFN-γ and tumor necrosis factor (TNF)-α. Hepatitis is thought to be initiated in this manner, and specific immune responses are generated if innate immune responses fail to eradicate HCV.

2.2. HCV-Specific Immune Responses and Immunopathogenesis of HCV-Specific CTLs. The process of HCV-specific CTL induction and the destruction of HCV-infected hepatocytes by CTLs are shown in Figure 2. The destruction of HCV-infected hepatocytes releases HCV fragments; these fragments are taken up by myeloid DCs, consequently activating the DCs. These DCs migrate to the draining lymph nodes and express HCV antigens on human leukocyte antigen (HLA) class II molecules. Then, they enhance expression of costimulatory molecules (CD80, CD86) that interact with and activate antigen-specific helper T (Th) cells [8]. In turn, the activated Th cells promote the maturation of DCs by the expression of CD40 ligand and TNF-α. Subsequently, mature DCs stimulate specific CTLs by antigen presentation on HLA class I molecule and enhance the expression of costimulatory molecules [8]. Cytokines such as IL-2 and IL-12 produced by Th1 cells and DCs further promote CTL activation. These CTLs infiltrate the liver and recognize HCV antigens presented on the surface of HCV-infected hepatocytes together with HLA class I molecules. Then, the effector CTLs release perforin, granzyme, and TNF-α, or express Fas ligand, and initiate a direct attack on HCV-infected hepatocytes [9, 10]. In the previous study, we demonstrated that Fas ligand and TNF-α can also destroy noninfected hepatocytes in the vicinity of the HCV-infected cells [11].

When appropriate CTL responses are induced in hosts, HCV eradication is achieved. However, HCV-specific CTL responses are usually not strong enough to eradicate the virus, hence contributing to persistent infection. On the