Measurement of Gene Expression in the Liver

Liver biopsy was performed immediately before starting the therapy. After extraction of total RNA from liver biopsy specimens, the messenger RNA (mRNA) expression of positive and negative cytoplasmic viral sensors (RIG-I, MDA5, and LGP2), the adaptor molecule (Cardif), related ubiquitin E3-ligase (RNF125), and the modulators of these molecules (ISG15 and USP18) was quantified by real-time quantitative polymerase chain reaction (PCR) using primers specific for target genes. In brief, total RNA was extracted by the acid-guanidinium-phenol-chloroform method using Isogen (Nippon Gene Co Ltd, Toyama, Japan) from the liver biopsy specimen, which was 0.2–0.4 cm in length and 13 gauge in diameter. Complementary DNA (cDNA) was transcribed from 2 μg total RNA template in a 140-μL reaction mixture using a SYBR RT-PCR Kit (Takara Bio Co Ltd, Otsu, Japan) with random hexamer. Real-time quantitative PCR was performed using Smart Cycler version II (Takara Bio Co Ltd) with the SYBR RT-PCR Kit (Takara Bio Co Ltd) according to the manufacturer's instructions, and intercalating SYBR Green I (Molecular Probes Inc, Eugene, Oregon) was detected. Assays were performed in duplicate, and the expression levels of target genes were normalized to expression of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene and hydroxymethylbilane synthase, which is stable in the liver, as quantified using real-time quantitative PCR as internal controls. For accurate normalization, a set of 2 housekeeping genes was used in the present study. Sequences of primer sets were as follows: RIG-I: 5′-AAAGCATGCATGGTGTTCCAGA-3′, 5′-TCATTCGTGCATGCTCACTGATAA-3′; MDA5: 5′-ACATAACAGCAACATGGGCAGTG-3′, 5′-TTTGGTAAGGCCTGAGCTGGAG-3′; LGP2: 5′-ACAGCCTTGCAAACAGTACAACCTC-3′, 5′-GTCCCAAATTTCCGGCTCAAC-3′; Cardif: 5′-GGTGCCATCCAAAGTGCCTACTA-3′, 5′-CAGCACGCCAGGCTTACTCA-3′; RNF125: 5′-AGGGCACATATTCGGACTTGTCA-3′, 5′-CGGGTATTAAACGGCAAAGTGG-3′; ISG15: 5′-AGCGAACTCATCTTTGCCAGTACA-3′, 5′-CAGCTCTGACACCGACATGGA-3′; USP18: 5′-TGGTTCTGCTTCAATGACTCCAATA-3′, 5′-TTTGGGCATTTCCATTAGCACTC-3′; GAPDH: 5′-GCACCGTCAAGGCTGAGAAC-3′, 5′-TGGTGGTGAAGACGCCAGT-3′. hydroxymethylbilane synthase: 5′-AAGCGGAGCCATGTCTGGTAAC-3′, 5′-GTACCCACGCGAATCACTCTCA-3′.

Sequential Measurement of Gene Expression in Peripheral Blood Mononuclear Cells Before and During Therapy

To understand transcriptional response of the genes to PEG-IFN-α-2b and ribavirin therapy, serial expression of RIG-I, RNF125, Cardif, ISG15, and USP18 were determined before and during treatment in peripheral blood mononuclear cells (PBMC) in 14 patients (7 were sustained viral responders [SVR] and 7 were NVR). PBMC was obtained from whole blood samples collected before and at 4, 8, 24, 48, and 168 hours after the initiation of PEG-IFN-α-2b and ribavirin combination therapy. After extraction of total RNA from the PBMC, the expression of mRNA was quantified at each specified time point using real-time quantitative PCR as described above. Gene expression levels at each time point during treatment were calculated relative to baseline expression levels measured prior to IFN treatment.

Western Blotting

Western blotting was carried out in 9 patients (5 were SVR and 4 were NVR) and 3 non-HCV control subjects as described previously.[21] Liver biopsy specimen of ~10 mg was homogenized in 100 μL Complete Lysis-M (Roche Applied Science, Penzberg, Germany). Twenty micrograms of the homogenates were separated by SDS-PAGE and blotted onto a polyvinylidene difluoride Western blotting membrane. The membrane was incubated with the primary antibodies followed by a peroxidase-labeled anti-IgG antibody and visualized by chemiluminescence using the ECL Western blotting Analysis System (Amersham Biosciences, Buckinghamshire, United Kingdom). The anti-VISA mouse monoclonal antibody (BioDesign, Saco, ME) and anti-β-actin antibody (Sigma Chemical Co, St. Louis, MO) were used.

HCV Dynamics in Serum

To analyze the viral dynamics, HCV RNA was quantified just before and at 4, 8, and 24 hours and 2, 7, 14, 28, 56, and 84 days after the initiation of PEG-IFN-α-2b and ribavirin combination therapy, using real-time detection PCR, as reported previously.[22] For each patient, the viral decline curve was plotted on a semilogarithmic scale, and the slopes of the exponential viral declines were calculated for each viral decline phase with a straight-line fit of the data.

Definitions of Response to Therapy

A patient negative for serum HCV RNA during the first 6 months after the completion of PEG-IFN-α-2b and ribavirin combination therapy was defined as an SVR, and a patient for whom HCV RNA became negative at the end of therapy and reappeared after completion of therapy was defined as a transient responder (TR). A patient who was positive for HCV RNA even during the course of therapy was defined as an NVR. HCV RNA was determined with the Amplicor qualitative assay (Roche Molecular Diagnostics Co, Tokyo, Japan). The detection sensitivity of this assay is approximately 50 IU/mL.

Statistical Analysis

Categorical data were compared by the χ^2 test and Fisher exact test. Distributions of continuous variables were analyzed by Mann–Whitney U test for 2 groups. Kruskal–Wallis test was used for multiple group comparisons. All tests of significance were 2-tailed, and P values $< .05$ were considered statistically significant.

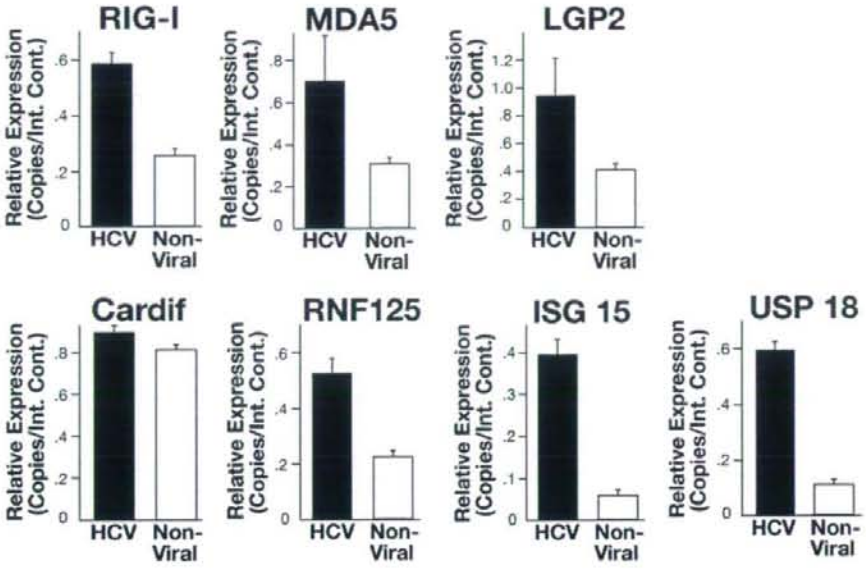

Figure 1. Comparison of hepatic gene expression levels between chronic hepatitis C patients (n = 74) and nonviral liver disease patients (n = 5). Expression levels of RIG-I, MDA5, LGP2, Cardif, RNF125, ISG15, and USP18 are shown. *Error bars* indicate the standard error. Upon HCV infection, expression of these genes except Cardif was stimulated. The *P* values determined by Mann–Whitney *U* test between 2 groups were as follows: RIG-I, *P* .02; MDA5, *P* .01; LGP2, *P* .005; Cardif, *P* .7; RNF125, *P* .06; ISG15, *P* .007; USP18, *P* .004.

Results

Patient Characteristics

According to the final virologic response, patients were classified into 3 groups: 30 were SVR, 24 were TR, and the remaining 20 were NVR, as shown in Table 1. Viral decline rates in NVR were significantly lower in both the first and second phases of HCV dynamics. It should be noted that most NVR patients exhibited no second-phase viral decline.

Data on factors that were available before starting the treatment were compared according to virologic response by univariate analysis. As shown in Table 1, only age and platelet count were associated with viral response, and no other clinical factors were predictive of NVR before initiation of the therapy.

Gene Expression Involving Innate Immunity in the Liver

First, we compared basal hepatic gene expression between the chronic hepatitis C patients (n = 74) and the nonviral liver disease patients (n = 5). As shown in Figure 1, levels of RIG-I, MDA5, LGP2, ISG15, and USP18 expression were significantly higher in the chronic hepatitis C patients than in the nonviral liver disease patients. However, there was no significant difference in levels of Cardif expression between the chronic hepatitis C and nonviral-related liver disease patients.

Next, to assess the relationship between baseline hepatic gene expression and treatment efficacy, levels of gene expression were compared based on the final virologic response. As shown in Figure 2, the hepatic expression levels of RIG-I, MDA5, and LGP2 were significantly higher in NVR than in SVR and TR. In marked contrast, hepatic Cardif expression was significantly lower in the NVR group. The hepatic expression of RNF125, which is specific E3-ubiquitin ligase for RIG-I, MDA5, and Cardif, was also significantly lower in the NVR group. Because negative correlation was found between RIG-I and Cardif or RNF125 expression, we calculated the ratio of RIG-I to Cardif or RNF125 expression levels. As shown in Figure 2, the difference among the groups was conspicuous when comparison was made with the RIG-I/Cardif ratio or RIG-I/RNF125 ratio. Moreover, the RIG-I/Cardif expression ratio before treatment was negatively and significantly correlated with the exponential viral decline rate in both the first and the second phases of HCV dynamics (first phase, $r = -0.4$, $P < .0005$; second phase, $r = -0.5$, $P < .0001$). Similar correlation was found between RIG-I/RNF125 ratio and viral decline rate (first phase, $r = -0.4$, $P = .004$; second phase, $r = -0.2$, $P = .09$, data not shown).

Like RIG-I and MDA5, intrahepatic expression levels of ISG15 and USP18 were significantly higher in NVR than in SVR and TR (Figure 2). When we assessed the correlation of these 2 genes in individual patients, we found a strong and significant correlation between ISG15 and USP18 ($r^2 = 0.88$, $P < .0001$). Levels of ISG15 and USP18 expression before treatment were negatively correlated with the exponential viral decline rates calculated from

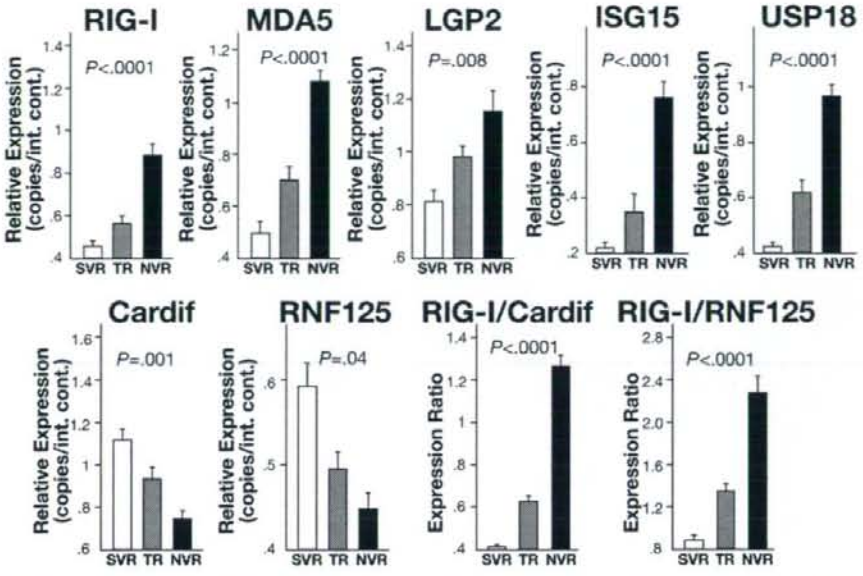

Figure 2. Comparison of hepatic gene expression levels according to final virologic outcome. Expression levels of RIG-I, MDA5, LGP2, ISG15, USP18, Cardif, RNF125, RIG-I/Cardif ratio, and RIG-I/RNF125 ratio are shown. *Open columns* indicate SVR (n = 30), *shaded columns* indicate TR (n = 24), and *solid columns* indicate NVR (n = 20). *Error bars* indicate the standard error. The *P* values were analyzed by the Kruskal–Wallis test.

the first and the second phases of HCV dynamics (ISG15, first phase, $r = -0.5$, $P < .0001$; ISG15, second phase, $r = -0.3$, $P = .02$; USP18, first phase, $r = -0.5$, $P < .0001$; USP18, second phase, $r = -0.3$, $P = .01$).

Receiver Operator Characteristic Analysis

To determine the usefulness of these gene quantifications as predictors, receiver operator characteristic (ROC) analysis was conducted (Figure 3). The area under the ROC curve for the RIG-I/Cardif ratio, ISG15, and USP18 was 0.91, 0.90, and 0.91, respectively, suggesting that quantification of these gene transcripts is of use for the prediction of NVR (Table 2). In addition, this analysis also suggested that RIG-I/Cardif ratio would be more specific for prediction of NVR, whereas ISG15 and USP18 would be more sensitive (Table 2).

Multivariate Analysis

Multivariate analysis for factors that were available before initiating therapy indicated that a higher ratio of RIG-I/Cardif and higher expression of ISG15 were independent factors that were associated with NVR (Table 3). In this analysis, USP18 was excluded because of its strong correlation with ISG15.

Protein Levels of Cardif in the Liver

Because hepatic expression of Cardif mRNA was significantly lower in NVR patients than in SVR patients,

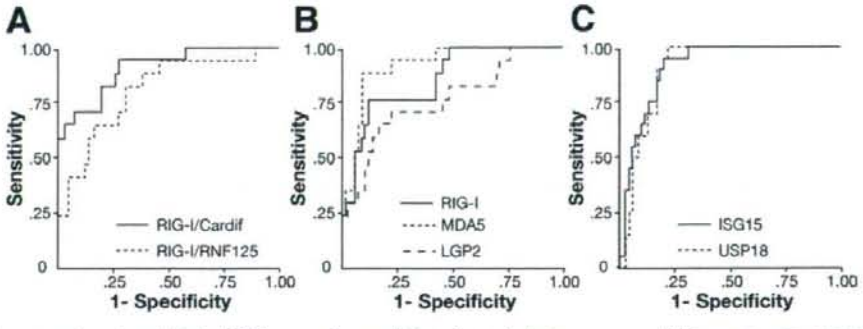

Figure 3. Receiver operator characteristic (ROC) curve for prediction of nonvirologic response. ROC curves were generated to compare (*A*) RIG-I/Cardif ratio (*solid line*) and RIG-I/RNF125 ratio (*shaded line*); (*B*) RIG-I (*solid line*), MDA5 (*shaded line*), and LGP2 (*dotted line*); and (*C*) ISG15 (*solid line*) and USP18 (*shaded line*).

Table 2. Area Under the ROC Curves, Sensitivity, Specificity, and Negative and Positive Predictive Values of Non-Virologic Responses

Variables	Az	95% CI	Cut-off	Sensitivity	Specificity	NPV[a]	PPV[b]
RIG-I	0.89	0.78–0.95	0.68	0.80	0.87	0.92	0.70
MDA5	0.92	0.86–0.98	0.84	0.82	0.89	0.93	0.74
LGP2	0.76	0.63–0.90	1.03	0.65	0.72	0.85	0.46
RIG-I/Cardif	0.91	0.84–0.99	0.88	0.75	0.91	0.91	0.75
RIG-I/RNF125	0.81	0.69–0.93	1.05	0.82	0.62	0.91	0.43
ISG15	0.91	0.85–0.97	0.36	0.90	0.81	0.96	0.64
USP18	0.90	0.84–0.96	0.67	0.90	0.83	0.96	0.67

[a]NPV, negative predictive value.
[b]PPV, positive predictive value.

we determined the basal protein expression levels of Cardif in the liver in NVR and SVR patients. Western blot analysis demonstrated a single Cardif product in all samples (Figure 4*A*). Similar to Cardif mRNA expression, mean Cardif expression in NVR patients was significantly lower than that in SVR (Figure 4*B*, $P = .01$). The cleavage product of Cardif, which has been reported by Loo et al,[23] was not detected in our analyses.

Transcriptional Responses to PEG-IFN-α-2b and Ribavirin Therapy in PBMC

Sequential analysis in response to PEG-IFN-α-2b and ribavirin demonstrated a rapid and strong induction of RIG-I, ISG15, and USP18 mRNA expression, which peaked 8 hours after PEG-IFN-α-2b administration (Figure 5). A greater fold change of these peak inductions was observed in SVR patients compared with NVR patients, although statistical significance was not achieved. In marked contrast, RNF125 expression profile in response to PEG-IFN-α-2b was triphasic, and consisted of (1) rapid and strong suppression peaked at 8 hours after administration, (2) increased 1.5- to 2-fold above baseline level during 24–48 hours after the administration, and (3) gradually decreased to baseline level (Figure 5). The rapid suppression and subsequent increase following PEG-IFN-α-2b administration tended to have a greater fold change in NVR patients compared with those in SVR patients. In contrast from RIG-I, ISG15, USP18, and RNF125, Cardif expression profile was relatively constitutive, and transcriptional response to PEG-IFN was weak (Figure 5).

Discussion

In the present study, we found that baseline expression levels of intrahepatic viral sensors and related regulatory molecules were significantly associated with the final virologic outcome in patients with chronic hepatitis C who were treated with PEG-IFN-α-2b and ribavirin combination therapy: up-regulation of RIG-I, MDA5, LGP2, ISG15, and USP18 and lower expression of Cardif and RNF125 could predict nonresponse to subsequent treatment with PEG-IFN-α-2b and ribavirin. The positive predictive value of a high ratio of expression of RIG-I to Cardif (>0.88) for NVR was the highest at a value of 0.75, and the negative predictive values of high expression of ISG15 (>0.36/internal control) and USP18 (>0.67/internal control) were the highest at values of both 0.96. These data may be of use in predicting clinical responses to the PEG-IFN-α and ribavirin combination before initiating therapy.

Table 3. Multivariate Analysis for the Factors Associated With Non-Virologic Response

Variable	Odds ratio	95% CI	*P* value
RIG-I/Cardif Ratio (by 0.1)	1.5	1.1–2.1	.008
RIG-I/RNF125 Ratio (by 0.1)	1.2	1.0–2.5	.1
ISG15 (by 0.1/internal control)	1.5	1.1–2.0	.01
Age (by 1 y)	1.0	0.9–1.1	.6
Platelet count (by $1 \times 10^4/\mu L$)	1.2	0.9–1.5	.07

Previously, large randomized controlled trials identified several pretreatment factors associated with the final virologic outcome, such as genotype, HCV RNA level, degree of fibrosis, age, body weight, ethnicity, and steatosis.[24] However, these findings lead us to believe that predicting the final virologic response before initiating PEG-IFN-α and ribavirin is difficult. Indeed, only age and platelet count were associated with the outcome in our patients with genotype 1b and a high viral load. Currently, the final response can be gauged only after treatment has been initiated. Although an early viral response at 12 weeks suggests the eventual outcome with 60%–90% accuracy,[25] a 12-week regimen is associated with adverse effects and is expensive. Therefore, this study investigated the baseline expression of genes involving innate immunity that may have significant effects on clinical outcomes.

In the present study, we demonstrated that RIG-I and MDA5 were inducible upon HCV infection and that expression of these intrahepatic positive viral sensors was up-regulated in NVR. In vitro studies have suggested that RIG-I and MDA5 play a pivotal role in the regulation of IFN production and augment the production of IFN via an amplification circuit. These results suggest that expression of RIG-I and MDA5 and related amplification system may be up-regulated by endogenous IFN at a higher baseline level in NVR patients. However, HCV elimination by subsequent exogenous IFN is insufficient

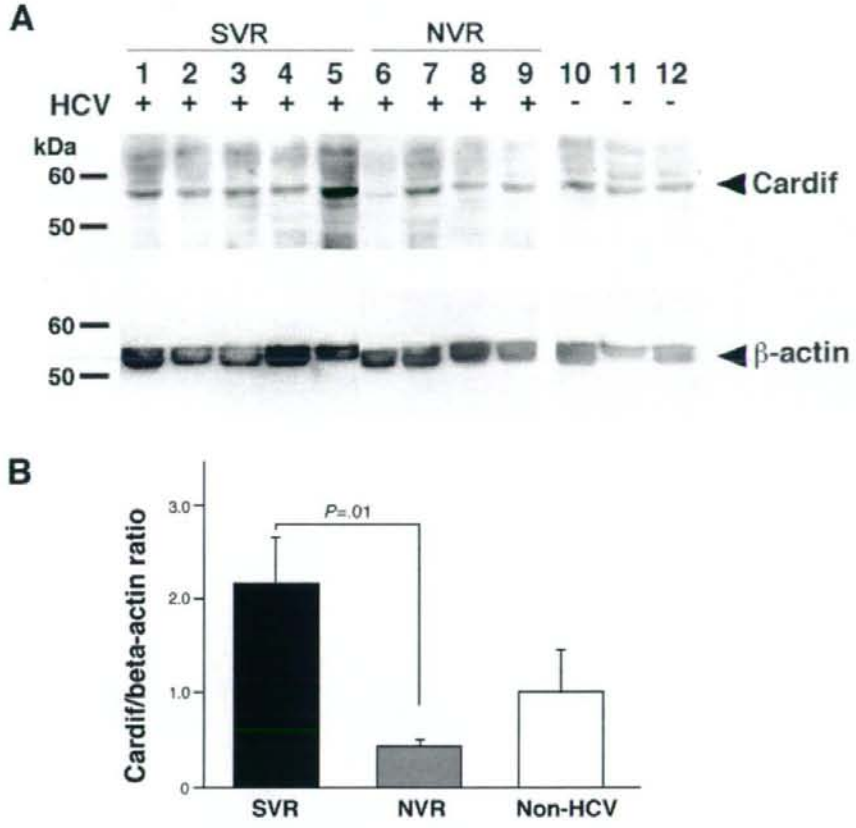

Figure 4. (*A*) Western blot analysis. Five lanes were SVR (*lanes 1–5*), 4 lanes were NVR (*lanes 6–9*), and 3 lanes were non-HCV control (*lanes 10–12*). Specific bands for Cardif and β-actin are indicated by *arrows*. (*B*) Expression level of Cardif protein normalized to β-actin in the liver biopsy specimens according to ultimate treatment response. *Error bars* indicate the standard error.

in these patients, suggesting that NVR patients may have adopted a different equilibrium in their immune response to the virus. In contrast to the expression of RIG-I and MDA5, Cardif mRNA, which was expressed in a relatively constitutive fashion, was significantly lower in NVR. Our ROC analysis highlights that lower expression of Cardif relative to that of RIG-I was one of the strongest predictors for NVR. Moreover, Western blot analysis further confirmed the down-regulation of Cardif in NVR patients, as demonstrated by its protein level. Because Cardif is one of the substantial target molecules of HCV evasion,[11,20] it is likely that Cardif expression is suppressed by HCV with resistant phenotype or is inadequate in NVR patients. Loo et al have demonstrated a Cardif cleavage product in 2 of 4 liver tissue samples of chronic HCV infection.[23] In our study, however, the Cardif cleavage product was not detected, presumably because the product could be unstable in vivo, resulting in rapid degradation. Although further studies are necessary to elucidate mechanisms of Cardif down-regulation, our findings of lower expression of Cardif in NVR suggested that the status of Cardif expression in the liver might have a significant effect on the ultimate outcome of antiviral treatment.

The antiviral effect brought by RIG-I/Cardif signaling is regulated by the coordination of negative and positive regulators. It has been shown that RNF125 functions as a negative regulator of RIG-I/Cardif signaling. RNF125 is an ubiquitin E3-ligase with activity against protein containing CARD domains, such as RIG-I, MDA5, and Cardif, and these ubiquitinated molecules undergo proteasomal degradation. In contrast, RNF125 do not have negative function against LGP2, a negative regulator of RIG-I signaling, because LGP2 lacks CARD domain. In contrast to RIG-I, RNF125 expression was rapidly suppressed by exogenous IFN; therefore, observed lower basal hepatic level of RNF125 in NVR could be explained by the suppressive effect of endogenous IFN, which may be up-regulated in NVR patients. Hence, RNF125 may constitute a negative regulatory circuit for IFN production and is responsible for responsiveness to PEG-IFN and ribavirin therapy.

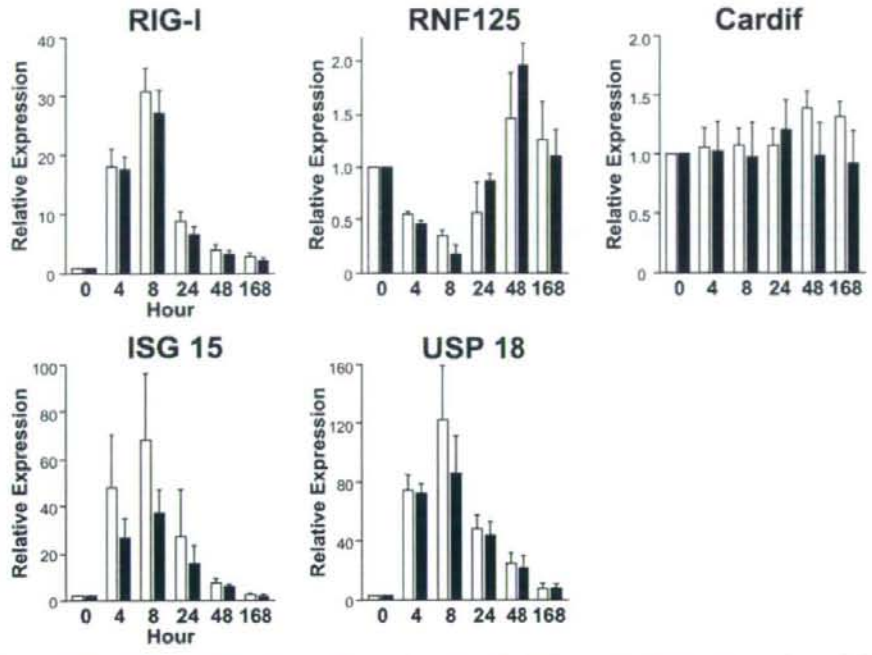

Figure 5. Transcriptional responses during PEG-IFN-α-2b and ribavirin therapy in PBMC (n = 14). *Open columns* indicate SVR (n = 7), and *solid columns* indicate NVR (n = 7). *Error bars* indicate the standard error. The *P* values determined by Mann–Whitney *U* test between 2 groups at 8 hours were as follows: RIG-I, *P* .3; RNF125, *P* .3; Cardif, *P* .7; ISG15, *P* .3; USP18, *P* .2.

It has been shown that RIG-I function is modified by ISG15 via ISGylation.[17] Consistent with our data, Chen et al identified 18 genes, including ISG15 and USP18, whose expression differed between responders and non-responders.[26] Interestingly, a recent study has shown that USP18 negatively regulates IFN signaling independently of its isopeptidase activity toward ISG15 by binding to the IFNAR2 receptor subunit and blocking the interaction between Janus kinase and the IFN receptor.[27] Moreover, the siRNA knockdown of USP18 in human cells has consistently been shown to potentiate the ability of IFN to inhibit HCV RNA replication.[28] Therefore, USP18 is suggested as a novel in vivo inhibitor of signal transduction pathways that are specifically triggered by type I IFN. Consistent with a role for USP18 in down-regulating the antiviral IFN response, we confirmed that up-regulation of USP18 was one of the factors predicting a lack of response to treatment with IFN.

The mechanism underlying the association of gene expression involving innate immunity with resistance to therapy is not well understood. Our human study with HCV patients treated by PEG-IFN and ribavirin highlights RIG-I/Cardif, RIG-I/RNF125, and ISG15/USP18, which is partly responsible for the clinical responsiveness to antiviral therapy. RIG-I signaling by viral pathogens may affect a wide variety of responses in not only innate but also acquired immunity. Our study is the first to demonstrate the potential relevance between molecules involving innate immunity and the clinical response to antiviral therapy.

In addition, sequential analysis of expression profile during PEG-IFN-α-2b and ribavirin treatment was also performed in this study. Lanford et al demonstrated transcriptional response to IFN-α in chimpanzee by genome microarray analysis, which included RIG-I, ISG15, and USP18.[29] An association of transcriptional response with early phase of virologic response has been also reported in PBMC or liver biopsy specimen.[30-32] We recently reported that the transcriptional double-stranded RNA-activated protein kinase response during treatment with PEG-IFN-α-2b and ribavirin was associated with the ultimate clinical response.[30] Similarly, the present study demonstrated a strong and rapid increase of RIG-I, ISG15, and USP18 mRNA in response to clinical PEG-IFN treatment especially in SVR patients, although few patients were available to achieve statistical significance between SVR and NVR. In marked contrast, transcriptional response of RNF125 exhibited a triphasic pattern. Rapid suppression seen in the first phase was presumably because of a negative regulatory effect of IFN. However, increase of RNF125 mRNA in the second phase, which tended to be grater in NVR, may be responsible for inhibiting RIG-I expression seen 8–48 hours after PEG-IFN-α-2b administration. Although limitations includ-

ing the use of PBMC and small sample size still deserve mention, the sequential expression profile during treatment may provide further valuable information regarding the prediction of the clinical response to the therapy and the mechanism of action of antiviral treatment.

In the present study, we have included patients with genotype 1b because it is imperative to designate a virologically homogeneous patient group to associate individual treatment responses with different gene expression profiles that direct innate immune responses. We have preliminarily studied genotype 2 patients and found that Cardif and RNF125 gene expression levels in NVR patients were significantly lower than those with SVR patients ($P = .03$ and $P = .04$, respectively) and that RIG-I/Cardif and RIG-I/RNF125 ratios were significantly higher in NVR patients ($P = .02$ and $P = .009$, respectively, see Supplementary Figure 2 online at www.gastrojournal.org). These findings suggest that the differences in gene expression profiles between SVR and NVR were almost identical to those demonstrated in patients with genotype 1b. However, the correlation between treatment responses in all the genotypes and the different status of innate immune responses needs to be explored. Further studies may be necessary to clarify this issue.

In conclusion, the results of the present study offer potentially important clinical implications for patients with chronic hepatitis C who are treated with PEG-IFN-α and ribavirin. Quantifying hepatic gene expression of the RIG-I/Cardif system, including its regulators before treatment, is useful in identifying patients who are at a higher risk for NVR. The data from these assays can provide valuable information that may influence the decision about the treatment strategy in each individual patient. Finally, this clinical human study demonstrates the potential relevance of the molecules involving innate immunity to the clinical response to therapy. Our data will help understand the pathogenesis of HCV resistance and development of new antiviral therapy targeted toward the innate immune system.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.02.019.

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CORRESPONDENCE

External Validation of FIB-4: Diagnostic Accuracy Is Limited in Elderly Populations

To the Editor:

We read with interest the articles by Stering et al.[1] and Vallet-Pichard et al.[2] The former authors developed the FIB-4 index, a noninvasive method for assessing liver fibrosis in patients with HIV/HCV coinfection. The variables used are age, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and platelet (PLT) count, and the formula is as follows: (age [yr] × AST [U/L])/ ({PLT[10^9/L]) × (ALT[U/L])$^{1/2}$). They showed that over 70% of patients could be classified into either absence or presence of advanced fibrosis by cutoff of <1.45 or >3.25 respectively, with diagnostic accuracy of 87%. The latter authors expanded the applicability of the FIB-4 index to HCV-monoinfected patients and showed that 73% of patients were classified with diagnostic accuracy of 93%, an excellent performance in both classification and accuracy of diagnosis.

Because the mean age of patients was young in these studies (40 years[1] and 44 years[2]), we wondered whether this index could also fit to Japanese patients who are rather older than the Western patients. We validated the FIB-4 index in a retrospective cohort of 1,405 patients who underwent liver biopsy at our hospital. The mean age was 55 ± 12 years. The distribution of METAVIR fibrosis scores was as follows: 1.6% showed no fibrosis (F0), 44.8% showed mild fibrosis (F1), 29.5% showed moderate fibrosis (F2), 20.2% showed severe fibrosis (F3), and 3.9% showed cirrhosis (F4). The proportion of advanced fibrosis (F3 or F4) was slightly higher in our population compared to the former studies (24.1% vs. 20.7% [1] and 17.2% [2]). As shown in Table 1, only 53% of patients were classified to either <1.45 or >3.25, a much lower rate than previous reports. The diagnostic accuracy was excellent in patients with a FIB-4 index <1.45 (94%), however, it was relatively poor in patients with a FIB-4 index >3.25 (50%) making the overall accuracy as low as 67%.

We supposed this discordance with previous reports may be derived from the older age of our populations and thus we categorized patients into three groups according to age and analyzed separately. In patients with age ≤50 years, 64% of patients were classified, and the diagnostic accuracy was 94% for a FIB-4 index <1.45 and 68% for a FIB-4 index >3.25 making the overall accuracy of 90%, a result comparable to previous reports. In older patients, however, diagnostic accuracy was significantly low compared to those with age ≤50 years (56% for age 51-60 years, $P < 0.0001$ and 51% for age ≥60 years, $P < 0.0001$). Because patients with a FIB-4 index >3.25 increased according to age (6%, 34%, and 53% for ages ≤50, 51-60 and >60 years), and the diagnostic accuracy was low in these patients (48% to 50%), these results suggest that, in elderly patients, a variable "age" generates excessively high FIB-4 index leading to misclassification of no-moderate fibrosis (F0-F2) into a FIB-4 index >3.25.

In conclusion, the FIB-4 index could accurately differentiate advanced fibrosis in young Japanese patients with chronic hepatitis C but the diagnostic accuracy is limited in the elderly. Thus, in elderly patients, some sort of adjustment for the effect of age on FIB-4 index may be necessary for more precise classification.

Table 1. Comparison of FIB-4 Index and Liver Biopsy Results in Terms of Age

	METAVIR Fibrosis Score				Diagnostic Accuracy
	FIB-4	F0-2	F3-4	Total	
All patients					
	<1.45	283 (20%)	18 (1%)	301 (21%)	94%
	>3.25	228 (16%)	226 (16%)	454 (32%)	50%
	1.45-3.25	556 (40%)	94 (7%)	650 (47%)	
	Total	1067 (76%)	338 (24%)	1405 (100%)	67%
Age ≤50	<1.45	240 (54%)	16 (4%)	256 (58%)	94%
(Mean 40 yrs)	>3.25	9 (2%)	19 (4%)	28 (6%)	68%
	1.45-3.25	126 (28%)	38 (8%)	164 (36%)	
	Total	375 (84%)	73 (16%)	448 (100%)	90%
Age 51-60	<1.45	30 (7%)	2 (1%)	32 (8%)	94%
(Mean 56 yrs)	>3.25	76 (18%)	69 (16%)	145 (34%)	48%
	1.45-3.25	215 (50%)	36 (8%)	251 (58%)	
	Total	321 (75%)	107 (25%)	428 (100%)	56%
Age >60	<1.45	13 (2%)	0 (0%)	13 (2%)	100%
(Mean 66 yrs)	>3.25	143 (27%)	138 (26%)	281 (53%)	49%
	1.45-3.25	215 (41%)	20 (4%)	235 (45%)	
	Total	371 (70%)	158 (30%)	529 (100%)	51%

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Reply:

We thank Kurosaki and colleagues for interest in our article that developed a simple noninvasive index, FIB-4, to accurately assess fibrosis in patients coinfected with hepatitis C virus (HCV) and human immunodeficiency virus (HIV).[1] This model was recently validated in a large cohort of patients monoinfected with HCV, and had similar accuracy and was found to be as good as or better than other noninvasive models that use nonroutine tests.[2] In these 2 studies, the mean ages were 40 and 44 years, respectively. Therefore, the utility of this index in older patients is not known.

The current report in a large cohort of Japanese patients with HCV found that although FIB-4 had excellent accuracy (90%) in those < 50 years of age, it did not perform well in those > 50 (56% in those 51-60 years of age and 51% in those > 60 years old) despite an increasing proportion of advanced fibrosis in the older patients (25%-30% versus 16% in those < 50). The reason for the drop in performance of FIB-4 in older patients in the present study is not clear but may be due in part to patient demographics. Although the overall proportion of patients with advanced fibrosis were similar in the 3 cohorts, the proportion of patients with cirrhosis in the Japanese cohort was only 3.9% compared to 7.2% in the study by Vallet-Pichard[2] and 15% in our coinfected population.[1] Furthermore, we are not told the distribution of F4 cirrhosis among the 3 age groups in the current study, which could have affected their results.

Whenever an index includes both a numerator and denominator, changes in either can affect the results. Age was included in the numerator due to the observation that increasing age is associated with increasing fibrosis.[3] This is supported in the current analysis, which found increasing advanced fibrosis in those > 50 years of age. Another

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HEPATOLOGY

Insulin resistance and lichen planus in patients with HCV-infectious liver diseases

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Abstract

Background and Aim: Hepatitis C virus (HCV) causes liver diseases and extrahepatic manifestations, and also contributes to insulin resistance and type 2 diabetes mellitus (DM). The aims of the present study were to examine the incidence of extrahepatic manifestations including lichen planus in HCV-infected patients and to evaluate the relationship between lichen planus and insulin resistance.

Methods: Of 9396 patients with liver diseases presenting to the study hospital, 87 patients (mean age 60.0 11.5 years) with HCV-related liver diseases were identified and examined for the incidence of extrahepatic manifestations. Insulin resistance and the presence of *Helicobacter pylori* antibodies were also measured.

Results: The prevalence of DM was 21.8% (19/87), hypertension was 28.7% (25/87), thyroid dysfunction was 20.7% (18/87), and extrahepatic malignant tumor was 9.2% (8/87). The prevalence of lichen planus at oral, cutaneous, pharyngeal, and/or vulval locations was 19.5% (17/87). Characteristics of 17 patients with lichen planus (group A) were compared with 70 patients without lichen planus (group B). Prevalence of smoking history, presence of hypertension, extrahepatic malignant tumor, and insulin resistance (HOMA-IR) were significantly higher in group A than in group B. Significant differences were not observed for age, sex, body mass index, diagnosis of liver disease, alcohol consumption, presence of DM, thyroid dysfunction, liver function tests, or presence of *H. pylori* infection between the two groups.

Conclusions: Infection with HCV induces insulin resistance and may cause lichen planus. It is necessary for an HCV-infected patient to be assayed for insulin resistance, and to be checked for different extrahepatic manifestations of this infection, particularly lichen planus.

Introduction

The number of fatalities due to hepatocellular carcinoma (HCC) in Japan continues to increase, and it is estimated that this tendency will continue at least until 2015. Of the HCC cases in Japan, approximately 16% are caused by hepatitis B virus (HBV) infection and approximately 80% by hepatitis C virus (HCV) infection.[1] The average prevalence of HCV carriers in Japan is about 2%, with the absolute number estimated at 2 million.[2] The increase in HCC in Japan depends on the spread of HCV infection.[2]

Infection with HCV induces various extrahepatic manifestations as well as chronic liver diseases.[3,4] HCV infects cells or organs except hepatocytes and multiplies. Representative extrahepatic manifestations of HCV infection include lichen planus, diabetes mellitus (DM), malignant lymphoma, Sjögren's syndrome, cryoglobulinemia, and membranoproliferative glomerulonephritis. It has been reported that combined therapy using interferon and ribavirin is effective for different extrahepatic manifestations that are apt to be overlooked.[5,6]

At present, it has been shown that HCV multiplies in skin and oral mucosa leading to HCV-related lichen planus,[7,8] and that the risk of malignant transformation is higher in lichen planus with HCV infection than in lichen planus without HCV.[9] However, a mechanism for these extrahepatic manifestations has not been elucidated. Recently it was reported that there is a significant correlation between lichen planus and HCV and DM in southern Taiwan, particularly in HCV patients with elevated serum alanine aminotransferase (ALT) levels and atrophic-erosive oral lichen planus (OLP).[10] In our previous report, patients with lichen planus having DM were all found to be HCV-infected.[11]

In addition, it has been reported that DM is a risk factor for HCV-related hepatocarcinogenesis[12] and for decreased survival

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among liver cirrhosis patients.[13] In addition, the incidence of diabetes in patients having HCV-related liver cirrhosis is higher than that in patients with HBV-related liver diseases.[14]

We recently showed molecular mechanisms for HCV core-induced insulin resistance.[15] HCV core up-regulates the suppressor of cytokine signaling (SOCS) 3, and inhibits insulin signaling by down-regulation of insulin receptor substrate (IRS) -1 and IRS-2 in hepatocytes. Moreover, in an epidemiological survey, we demonstrated that a significant increase in the incidence of diabetes occurs in subjects with high titers of HCV core compared to subjects who are negative for anti-HCV antibody[16] and concluded that HCV infection induces insulin resistance, which causes an increase in the incidence of extrahepatic manifestations in HCV-infected individuals.[17]

In the current study, we surveyed the incidence of abnormal glucose tolerance in patients with or without lichen planus in a study population with HCV-related chronic liver disease, and investigated the relationship between lichen planus and insulin resistance.

Methods

Patients

A total of 105 984 consecutive patients had checkups for chronic liver disease for the first time in the Digestive Disease Center at Kurume University Hospital from April 1988 to August 2005. In the Digestive Disease Center, physicians, surgeons, radiologists, and an oral surgeon hold full-time positions. One of us (M.S.) is a hepatologist and examined 9396 of these 105 984 patients. There were 522 patients who were HCV antibody positive and who thereafter continued with regular hospital visits until April 2006.

Exclusion criteria were the following: (i) other causes of chronic liver disease or disease other than chronic HCV infection; (ii) liver disease related to HBV infection; and (iii) patients treated with interferon therapy at the time of study inclusion.

We examined the presence of extrahepatic manifestations of chronic HCV infection in 87 patients. Informed consent was obtained from all patients after the purpose and methods of the study were explained. The 87 patients were 44 men and 43 women with a mean age of 60.0 11.5 years.

The patients were monitored for the presence of extrahepatic manifestations of HCV infection such as lichen planus, DM, hypertension, thyroid dysfunction, and extrahepatic malignant tumor as well as liver disease. Biochemical tests were done and insulin values, blood glucose levels, and *Helicobacter pylori* antibody were measured in patient blood samples. Life histories were taken.

Clinical examinations

Patients received oral mucosa and cutaneous medical examinations by an oral surgeon and a dermatologist. The diagnosis of OLP was made on the basis of clinical and histopathological features. Diagnosis of type 2 DM was based on the American Diabetic Association (ADA) criteria of 1997.[18] Persons in whom diabetes was diagnosed before 30 years of age and who used insulin were categorized as type 1 DM and were excluded from our study.

The following definitions of cardiovascular disease were employed. Obesity was defined as a body mass index (BMI) >25 kg/m^2 or higher. Hypertension was defined as a systolic blood pressure (SBP) of 140 mmHg or higher, or a diastolic blood pressure (DBP) of 90 mmHg or higher according to the criteria of JNC-VI of the International Hypertension Society.[19] Thyroid hormones such as FT3, FT4 and thyroid stimulating hormone were measured for all patients, and thyroid echography examination was performed for some patients. Examination of the upper gastrointestinal tract or lower digestive tract was performed on patients for whom it was deemed clinically necessary.

We also took a history of smoking and alcohol consumption.

Serological assays

Serum samples from the 87 patients were collected and tested for platelets (PLT) and for the following liver function tests: serum ALT, aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (g-GTP), lactate dehydrogenase (LDH), total bilirubin (TBil), direct bilirubin (DBil), thymol turbidity test (TTT), zinc sulfate turbidity test (ZTT), total cholesterol (TC), total protein (TP), and albumin (Alb). Sera were also examined for the presence or absence of HCV or HBV infection. Anti-HCV was measured by a chemiluminescent enzyme immunoassay kit (Lumipulse II HCV, Fujirebio, Tokyo, Japan). HCV RNA in serum was detected using the Amplicore HCV test (Roche, Tokyo, Japan). Hepatitis B virus surface antigen (HBsAg) was assayed using a chemiluminescent immunoassay kit (Architect, HBsAg QT, Dainabot, Tokyo, Japan). Ultrasonographic examination for all patients was performed in order to investigate the shape of the liver and lesions occupying the liver. Computed tomography and liver biopsy were performed in some patients. Most patients underwent endoscopy for detection of esophagogastric varices. We used other possible predictors of liver cirrhosis progression, including serum albumin, TBil, prothrombin time, and PLT.

Plasma glucose levels were measured by a glucose oxidase method for all subjects and serum insulin levels were measured using a sandwich enzyme immunoassay kit (Eiken Chemical, Tokyo, Japan). Insulin resistance (IR) was calculated on the basis of fasting levels of plasma glucose and insulin, according to the homeostasis model assessment (HOMA-IR) method.[20] The formula for the HOMA-IR is: HOMA-IR = fasting glucose (mg/dL) ¥ fasting insulin (mU/mL)/405.

The presence of serum IgG antibodies against *H. pylori* antibody were measured by the SRL (Tokyo) using E Plate *H. pylori* antibody produced by Eiken Chemical.

Statistical analysis

The chi-squared test and the unpaired Student *t*-test were used for statistical analyses. Differences were judged significant for $P < 0.05$ (two-tailed). This study was approved by the Institutional Review Board/Ethics Committee of our Institution.

Results

Among 87 patients with HCV-related liver diseases, the prevalence of lichen planus was 19.5% (17/87), DM was 21.8% (19/87),

Table 1 Clinical characteristics of 87 patients with HCV-related liver diseases according to presence of lichen planus (LP)

Clinical characteristic	All patients	Group A (with LP)	Group B (without LP)	*P* value (A *vs* B)
No. subjects	87	17	70	–
Age (years)	60.0 11.5	63.7 10.6	59.1 11.6	NS
Sex (M/F)	44/43	11/6	33/37	NS
BMI (kg/m^2)	22.8 2.9	23.9 2.8	22.5 2.9	NS
Smoking history	32 (36.8)	10 (58.8)	22 (31.4)	0.0356
Alcohol consumption percentage	50 (57.5)	10 (58.8)	40 (57.1)	NS
Diagnosis of liver disease				
Past history of HCV infection	1	0	1	NS
Chronic hepatitis C	69	11	58	
HCV-related liver cirrhosis	9	3	6	
HCV-related HCC	8	3	5	
Comorbidities				
Diabetes mellitus	19 (21.8)	4 (23.5)	15 (21.4)	NS
Hypertension	25 (28.7)	10 (58.8)	15 (21.4)	0.0022
Thyroid dysfunction	18 (20.7)	5 (29.4)	13 (18.6)	NS
Extrahepatic malignant tumor	8 (9.2%)	5 (29.4)[†]	3 (4.3)[‡]	0.0013

Values shown as *n* (%) or mean SD. BMI, body mass index; F, female; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; M, male; NS, not significant.

[†]Tumors were: gastric cancer (two), tongue cancer (one), larynx cancer (one), and renal and colon cancer (one). [‡]Tumors were: gastric cancer (one), colon cancer (one), and gallbladder cancer (one).

hypertension was 28.7% (25/87), thyroid dysfunction was 20.7% (18/87), and extrahepatic malignant tumor was 9.2% (8/87).

We compared characteristics of 17 patients who had lichen planus (group A) and 70 patients who did not have lichen planus (group B). The mean age in group A was 63.7 10.6 years; there were 11 men and six women. The mean age in group B was 59.1 11.6 years; there were 33 men and 37 women. Table 1 shows clinical features of groups A and B. The diagnoses of liver diseases in group A were chronic hepatitis C infection (11 patients), HCV-related liver cirrhosis (three patients), and HCV-related HCC (three patients). Those of group B were chronic hepatitis C infection (58 patients), HCV-related liver cirrhosis (six patients), HCV-related HCC (five patients) and past history of HCV infection (one patient) (Table 1).

The prevalence of smoking history ($P = 0.0356$), hypertension ($P = 0.0022$), and extrahepatic malignant tumor ($P = 0.0013$) were significantly higher in group A than in group B (Table 1). Diagnoses of extrahepatic malignant tumors in group A were: tongue cancer (one squamous cell carcinoma), larynx cancer (one squamous cell carcinoma), gastric cancer (one adenocarcinoma, one signet ring cell carcinoma), renal and colon cancer (one renal cell carcinoma). Diagnoses of extrahepatic tumor in group B were: gastric cancer (one adenocarcinoma), colon cancer (one adenocarcinoma), and gallbladder cancer (one adenocarcinoma). Significant differences were not observed for age, sex, BMI, liver disease, alcohol consumption, presence of DM, or thyroid dysfunction between these two groups.

We analyzed for differences between these two groups in liver assays, blood platelets, insulin, blood glucose, HOMA-IR, and presence of *H. pylori* infection. The laboratory data of both groups are shown in Table 2. Prevalence of insulin ($P = 0.0076$) and HOMA-IR ($P = 0.0113$) were significantly higher in group A than in group B (Table 2). Significant differences were not observed for serum AST, ALT, LDH, gGTP, TP, Alb, TBil, DBil, TTT, ZTT, TC, blood platelets, blood glucose, or presence of *H. pylori* infection between these two groups.

Seventeen patients had OLP at a total of 24 sites. The site of occurrence was: buccal mucosa in 13 (76.5%), lower lip in six (35.3%), upper lip in two (11.8%), gingiva in one (5.9%), tongue in one (5.9%), and floor of mouth in one (5.9%) (Table 3). The sites of lichen planus except oral mucosa were lower leg in four (23.5%), antebrachium in one (5.9%), skin extremities in two (11.8%), hypopharynx in one (5.9%), and vulva in one (5.9%). Biopsies of hypopharyngeal lichen planus were performed by an otolaryngologist, and of vulvar lichen planus by a gynecologist. The erosive and reticular variety, respectively, was found to be the prevalent form (Table 3).

Discussion

We performed an epidemiological survey for extrahepatic manifestations and HCC in an HCV hyperendemic area in Japan.[21,22] Anti-HCV positivity among residents of this area in 1990 was 23.6%.[23] We found that the prevalence of extrahepatic manifestations among individuals with HCV infection was higher than among those without HCV,[22] and found an association between HCV core, insulin resistance, and the development of type 2 DM.[16] Recently, we reported that insulin resistance in inhabitants who have an extrahepatic manifestation including OLP with HCV infection shows significantly greater increases than for inhabitants who have neither an extrahepatic manifestation nor HCV infection.[17] By the results of these epidemiological surveys we think that insulin resistance induced by HCV infection causes an increase in the incidence of extrahepatic manifestations in HCV-infected individuals.

In this study, we did long-term follow up for insulin resistance from the standpoint of lichen planus among patients who we identified as having HCV-related chronic liver disease at our hos-

Table 2 Laboratory data of 87 patients with HCV-related liver diseases according to presence of lichen planus (LP)

Laboratory assay	All patients	Group A (with LP)	Group B (without LP)	*P* value (A *vs* B)
AST (IU/L)	61.1 38.1	60.9 33.5	61.2 39.3	NS
ALT (IU/L)	68.2 46.7	62.4 39.6	69.6 48.5	NS
LDH (IU/L)	216.8 62.8	205.8 72.1	219.6 60.6	NS
g-GTP (IU/L)	64.1 68.4	63.5 50.0	64.2 72.5	NS
TP (g/dL)	7.7 0.5	7.7 0.5	7.7 0.5	NS
Alb (g/dL)	4.1 0.5	3.9 0.5	4.2 0.5	NS
PLT (/mm^3)	13.8 5.1	12.5 5.0	14.1 5.09	NS
TBil (mg/dL)	1.1 0.6	1.2 0.9	1.0 0.5	NS
DBil (mg/dL)	0.2 0.2	0.2 0.3	0.2 0.2	NS
TTT	16.2 6.7	18.4 4.7	15.8 7.0	NS
ZTT	20.6 6.9	21.8 5.8	20.3 7.2	NS
TC (mg/dL)	172.3 35.8	164.3 41.9	174.1 34.4	NS
Insulin (mU/L)	23.3 42.0	47.3 87.8	17.4 15.4	0.0076
Blood glucose (mg/dL)	97.4 30.1	103 33.2	96.1 29.5	NS
HOMA-IR	7.1 18.8	17.4 40.0	4.6 6.0	0.0113
Helicobacter pylori antibody (*n* (%))	58 (66.7)	10 (58.8)	48 (68.6)	NS

Values shown as mean SD. Alb, albumin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; DBil, direct bilirubin; g-GTP, gamma-glutamyl transpeptidase; HOMA-IR, homeostasis model assessment; LDH, lactate dehydrogenase; NS, not significant; PLT, platelets; TBil, total bilirubin; TP, total protein; TTT, thymol turbidity test; TC, total cholesterol; ZTT, zinc sulfate turbidity test.

Table 3 Location of lichen planus in 17 patients with hepatitis C virus-related liver diseases

No	Sex	Age (years)	Liver disease	Lichen planus location			Type
				Cutaneous	Oral	Other	
1	M	71	CH	Antebrachium	–	–	–
2	M	60	CH	Extremities	–	–	
3	F	70	LC	–	Gingiva	–	Erosive
4	M	72	LC	–	Lower lip	–	Reticular
5	F	64	LC	Leg	Buccal mucosa, upper lip, lower lip	–	Erosive
6	M	66	CH	Leg	Buccal mucosa, upper lip, lower lip	–	Erosive
7	M	59	CH	–	Buccal mucosa (reticular)	Pharynx (erosive)	Erosive + reticular
8	M	66	CH	Leg	Buccal mucosa, lower lip	–	Reticular
9	M	57	CH	–	Buccal mucosa	–	Reticular
10	M	50	CH	–	Buccal mucosa, tongue, lower lip	–	Erosive
11	F	77	CH	–	Buccal mucosa	–	Atrophic
12	F	75	CH	–	Buccal mucosa	–	Reticular
13	M	62	HCC	–	Buccal mucosa, lower lip	–	Erosive
14	F	83	HCC	Leg	Buccal mucosa (atrophic)	Vulva (erosive)	Atrophic + erosive
15	M	41	CH	–	Buccal mucosa	–	Reticular
16	M	58	HCC	Extremities	Buccal mucosa, floor of mouth	–	Erosive
17	F	53	CH	–	Buccal mucosa	–	Reticular

CH, chronic hepatitis C; F, female; LC, HCV-related liver cirrhosis; HCC, HCV-related hepatocellular carcinoma; M, male.

pital. Although there was no significant difference in fasting glucose levels and BMI between patients with and without lichen planus, fasting insulin levels and HOMA-IR values, an indicator of insulin resistance, were significantly higher in patients who had lichen planus than in those who did not.

In the present study, insulin levels (17.4 15.4 mU/L) and HOMA-IR values (4.6 6.0) in patients having HCV infection without lichen planus (group B) were higher than the normal range. Normal values for insulin are 3.06–16.9 mU/L, and for HOMA-IR are less than 2. Therefore, the significantly higher insulinemia in patients such as those in group A (among HCV infectious patients) might cause lichen planus.

In Japan, it is known that the prevalence of HCV infection in patients with lichen planus is high;[11] therefore, interferon therapy is often administered to patients with lichen planus and a persistent HCV infection. However, it has been reported that patients cannot

complete interferon therapy because of aggravation of lichen planus.[24,25] The measurement of insulin resistance as well as a search for lichen planus may be useful before performing interferon therapy. A large series of patients with OLP was evaluated for extraoral involvement by Eisen *et al.*[26] They concluded that any patient with OLP should undergo a through history and examination as part of an investigation of potential extraoral manifestations, because a high percentage of patients with OLP develop extraoral manifestations. In our 17 cases of lichen planus, cutaneous lichen planus was diagnosed in seven (41.2%), hypopharynx in one (5.9%), and vulva in one (5.9%). The simultaneous appearance of extraoral and oral lesions was noted among six (35.3%). Because the majority of OLP patients suffer from lichen planus of the genitalia,[27] clinicians should follow OLP patients with sufficient attention to the presence of extraoral manifestations.

Sikuker *et al.* evaluated an association between HCV infection and extrahepatic malignancies. Extrahepatic malignancies were found in 14.6% of anti-HCV positive patients.[28] The incidence of extrahepatic malignant tumor in our subjects was 9.2% (8/87). The insulin-like growth factor family of proteins plays a key role in cellular metabolism, differentiation, proliferation, transformation and apoptosis, during normal development and malignant growth.[29] The hyperinsulinemia that HCV infection causes may induce an extrahepatic malignant tumor as well as HCC.

Many studies have shown that *H. pylori* is involved in the pathogenesis of gastric cancer.[30] The seroprevalence of *H. pylori* is 71% in Japanese aged 50–59 years, and is 81% in those aged 60–69 years.[31] This is almost the same as the seroprevalence of our patients, which was 66.7% (58/87) overall and 82.6% (19/23) in those aged 60–69 years. Seroprevalence of *H. pylori* in our three subjects with gastric cancer was 66.7%. In our study, we did not find an association between *H. pylori* and lichen planus in patients with HCV-infectious liver diseases.

In conclusion, we investigated the association of insulin resistance and lichen planus among patients with HCV-infected chronic liver diseases. The significant factors for development of lichen planus were smoking history, presence of hypertension, extrahepatic malignant tumor, and insulin resistance (HOMA-IR). This supports our previous conclusion that insulin resistance in patients who have an extrahepatic manifestation of HCV infection increases more than insulin resistance of patients who have neither an extrahepatic manifestation nor HCV infection. HCV-infected patients with lichen planus should pay attention to the development of an extrahepatic malignancy. Cooperation with an oral surgeon and a hepatologist is vital for early diagnosis and treatment of any extrahepatic manifestations.

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HBV and HCV infection in Japanese dental care workers

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Abstract. Protective measures against occupational exposure to the hepatitis B virus (HBV) and hepatitis C virus (HCV) must be taken in order to prevent infection in dental care workers. To determine the best way to protect these workers, our study examined viral hepatitis infection in dental care workers in regions with a high prevalence of HCV infections in Japan. In total, 141 dental care workers (including dentists, dental hygienists and dental assistants) were enrolled. After a questionnaire to elicit demographic information was administered by an oral surgeon, hepatitis B surface antigen (HBsAg), antibody to HBs (anti-HBs), antibody to hepatitis B core antigen (anti-HBc) and antibody to HCV (anti-HCV) were measured. When necessary, HBeAg, anti-HBe, levels of HBV DNA, anti-HBc IgM and HCV RNA in serum were measured. Of the dental care workers included, 68 (48.2%) had been immunized with a HBV vaccine. Only 9 wore a new pair of gloves for each new patient being treated, 36 changed to a new pair only after the old gloves were torn and 24 did not wear any gloves at all. No one was positive for HBsAg or anti-HCV, though 73 (51.8%) and 17 (12.1%) workers were respectively positive for anti-HBs and anti-HBc. The positive rate of anti-HBc varied directly with worker age and experience. Of the 68 workers immunized with HBV vaccine, 51 (75%) were positive for anti-HBs. Of the 63 workers who were not so immunized, 17 (27%) were positive for anti-HBs and 15 of these were also positive for anti-HBc. Immunized workers were more protected against HBV infection than non-immunized workers, indicating that HBV vaccine was a useful measure for protection against the infection. The anti-HBc positive rate was significantly higher among dental care workers than general blood donors, suggesting that frequency of exposure to HBV was greater in dental care workers. HBV vaccination should be made compulsory for all dental care workers who handle sharp instruments.

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Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; anti-HBs, antibody to HBsAg; anti-HBc, antibody to hepatitis B core antigen; HCV, hepatitis C virus; anti-HCV, anti-bodies to HCV; HCC, hepatocellular carcinoma; CLEIA, chemiluminescent enzyme immunoassay

Key words: dentists, hepatitis B virus, hepatitis C virus, vaccine

Introduction

It is important to protect dental care workers (who perform invasive procedures daily) from nosocomial, blood-transmissible infections of the hepatitis B virus (HBV) and hepatitis C virus (HCV). There are ~1.5 million persistent HBV carriers and 2 million persistent HCV carriers in Japan. These carriers may develop hepatocellular carcinoma (HCC) decades later. The incidence of HCC continues to increase in Japan and ~80 and 10% of HCC are due to HCV and HBV, respectively (1). Treatment methods for hepatitis C and hepatitis B are now well established, continue to improve annually and their effects are dramatic.

The worldwide HBV infection rate is higher in dentists than in the general population: 6 times higher in the USA, 4 times higher in Germany and 2.5 times higher in Japan. The incidence of HBV infection among dentists is 10.8% in Brazil (2), 9% in the USA (3) and 7% in Germany (4). Among medical care workers, dentists have the highest incidence of HBV infection and this incidence increases with the length of clinical experience of the dentist (5,6). An investigation conducted in 1978 in Japan found approximately half of dentists with 5 or more years of clinical experience were infected with HBV or had a history of HBV infection (7). A study of 998 dentists conducted in 17 regions throughout Japan from 1978 to 1982 reported that 37 (3.7%) were hepatitis B surface antigen (HBsAg)-positive and 420 (42.1%) were antibody to HBs (anti-HBs)-positive (8). The results indicated that infection occurred at work without the dentists' knowledge. Thus dental care workers should be advised to receive a hepatitis B vaccine and it should be confirmed if they have acquired immunity to HBV.

What is the HCV infection rate in dentists in Japan? The anti-HCV-positive rate was 2.6% (10/382) according to the seroepidemiological survey of Shinozaki *et al* who used frozen-preserved serum obtained from dentists between 1986 and 1994 (9). However, the status of HCV infection was unclear, as the mean age of subject dentists and other information were not recorded. In New York city, the positive rate of anti-HCV was clearly higher among oral surgeons (9.3%) and other dentists (1.75%) than blood donors (0.14%) (10). The finding shows that morbidity in dentists differs by specialties.

In recent years, the infection rate in dentists in Japan remains unclear. The last estimates were made in the 1980s and

1990s, before more sensitive tests became available. Viral levels have never been measured (7-9). The status of hepatitis viral infection in dental care workers in the northern part of Kyushu, where the infection rates are the highest in Japan should be determined in order to assess the extent to which further health measures are needed to protect and maintain the health of dental care workers.

The present study screened for the presence of HBV and HCV infections in dental care workers in the Fukuoka prefecture (northern Kyushu). Since viral hepatitis is treatable, this investigation could contribute to the health maintenance of dental care workers.

Patients and methods

Patients. Participants included 141 dentists belonging to the X Dental Association in the Fukuoka prefecture and dental care workers (dental hygienists, assistants, mechanics and clerks) employed at dental clinics. Each member was notified by mail about the study before the examination. The examination was performed on 2 days (September 22 and 27, 2007).

Methods. Each participant gave informed consent and had a blood sample taken. An oral surgery specialist interviewed the subjects. Items of inquiry included gender, age, occupation, years employed as a dental care worker, disposable glove use, history of jaundice, history of blood transfusion, clinical history of liver diseases, family history of liver disease and hepatitis B vaccination status.

Viral markers of hepatitis were measured by chemiluminescent enzyme immunoassay (CLEIA) including HBsAg, anti-HBs and anti-HBc and by solid phase RIA including anti-HCV. When the serum was HBsAg-positive, HBeAg (CLEIA), anti-HBe (CLEIA), HBV DNA level (PCR method) and HBV genotype (PCR method) were assayed; when the serum was anti-HBc-positive, the anti-HBc IgM and HBV DNA level were assayed; and when the serum was anti-HCV-positive, RT-PCR was carried out to determine quantitative HCV RNA and HCV genotype.

Results were mailed to each participant. Ethical guidelines for the research were observed closely in order to protect participant confidentiality.

Results

There were 141 (43 males and 98 females) participants. Table I shows 43 were in their 20s, 35 in their 30s, 36 in their 40s, 17 in their 50s, 7 in their 60s, 2 in their 70s and 1 in his 80s. There were 42 dentists, 35 dental hygienists, 41 dental assistants, 8 dental mechanics and 15 clerks. Six subjects had a clinical history of liver disease that was unrelated to HBV or HCV infection.

As for hepatitis B vaccination, 68 (48.2%) were and 63 (44.7%) were not vaccinated. Dentists were the largest vaccinated group (39.7%, 27/68) and dental assistants were the largest unvaccinated group (34.9%, 22/63).

Regarding disposable glove use, only 9 people reported use of new gloves with every new patient. The highest number of people (36/141) said that they changed gloves only when the old pair of gloves were torn. Twenty-four workers did not wear gloves.

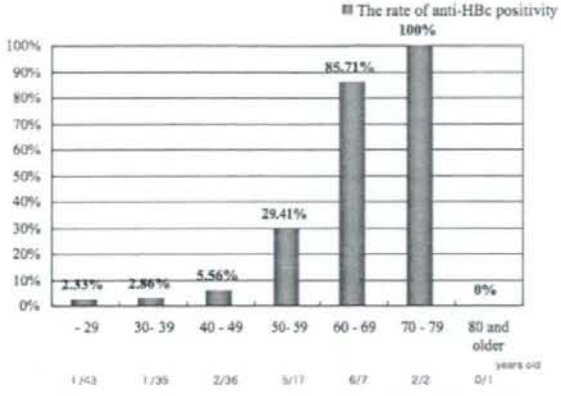

Figure 1. The rate of anti-HBc in 141 subjects classified according to age brackets. The rate of anti-HBc positivity increased with increased age.

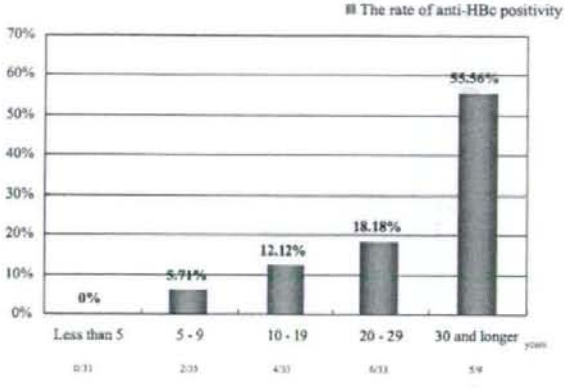

Figure 2. The rate of anti-HBc in 141 subjects classified according to years of experience in dental care. The rate of anti-HBc positivity increased with the number of years of dental care experience.

In hematological tests, no subjects were HBsAg-positive or anti-HCV-positive (Table II). However, 73 (51.8%) subjects were anti-HBs-positive and 17 (12.1%) were anti-HBc-positive. The rates of anti-HBc positivity increased with age: 85.7% of subjects in their 60s and 100% of subjects in their 70s (Fig. 1). The rate of anti-HBc positivity increased with the number of years of dental care experience (Fig. 2). As Table II shows, anti-HBs turned positive, indicating vaccine effectiveness, in 75% (51/68) of the vaccinated group and 27% (17/63) of the unvaccinated group. Fifteen of these 17 were anti-HBc-positive, indicating that these 15 were infected with HBV in the past.

Most (52.9%) of the 17 anti-HBc-positive subjects were dentists (Table III). The largest proportion of the anti-HBc-positive subjects were in their 60s (35.3%) and had 20 years of experience working in dentistry. Sixteen of the HBc-positive subjects (94.1%) were anti-HBs-positive. However, no HBV DNA was detected in the blood.

Table I. Background factors of 141 subjects classified by hepatitis B vaccination status.

	Total	Vaccination yes		Vaccination no		During vaccination (or Drop-out)		Unknown	
	(n)	(n)	(%)	(n)	(%)	(n)	(%)	(n)	(%)
		68	48.2	63	44.7	4	2.8	6	4.3
Sex									
Male	43	25	36.8	15	23.8	2	50.0	1	16.7
Female	98	43	63.2	48	76.2	2	50.0	5	83.3
Age									
29 years old	43	18	26.5	19	30.2	2	50.0	4	66.7
30-39	35	20	29.4	13	20.6	0	0.0	2	33.3
40-49	36	24	35.3	12	19.0	0	0.0	0	0.0
50-59	17	4	5.9	11	17.5	2	50.0	0	0.0
60-69	7	2	2.9	5	7.9	0	0.0	0	0.0
70-79	2	0	0.0	2	3.2	0	0.0	0	0.0
80 and older	1	0	0.0	1	1.6	0	0.0	0	0.0
Type of occupation									
Dentist	42	27	39.7	12	19.0	2	50.0	1	16.7
Dental hygienist	35	19	27.9	14	22.2	0	0.0	2	33.3
Dental assistant	41	15	22.1	22	34.9	2	50.0	2	33.3
Dental mechanic	8	4	5.9	4	6.3	0	0.0	0	0.0
Clerk	15	3	4.4	11	17.5	0	0.0	1	16.7
Years engaged in dental care									
<5 years	31	10	14.7	16	25.4	1	25.0	4	66.7
5-9	35	18	26.5	14	22.2	1	25.0	2	33.3
10-19	33	21	30.9	12	19.0	0	0.0	0	0.0
20-29	33	17	25.0	15	23.8	1	25.0	0	0.0
30 and longer	9	2	2.9	6	9.5	1	25.0	0	0.0
How to equip oneself with disposable gloves (Plural answers were given)									
Wear new pair with every new patient	9	6	8.8	3	4.8	0	0.0	0	0.0
Wear new pair with every 2 to 3 patients	31	19	27.9	9	14.3	1	25.0	2	33.3
Wear new pair when old one is torn	36	18	26.5	15	23.8	1	25.0	2	33.3
Wear new pair about twice a day	7	5	7.4	2	3.2	0	0.0	0	0.0

Table I. Continued.

	Total (n)	Vaccination yes (n)	(%)	Vaccination no (n)	(%)	During vaccination (or Drop-out) (n)	(%)	Unknown (n)	(%)
	68	48.2	63	44.7	4	2.8	6	4.3	
Wear when invasive treatment is performed	30	14	20.6	15	23.8	0	0.0	1	16.7
Wear when infected patients are treated	29	8	11.8	19	30.2	1	25.0	1	16.7
Wear when instrument is washed	2	0	0.0	2	3.2	0	0.0	0	0.0
Do not use	24	10	15	12	19	1	25.0	1	17
History of jaundice									
Yes	1	1	1.5	0	0.0	0	0.0	0	0.0
No	133	66	97.1	57	90.5	4	100.0	6	100.0
Unknown	7	1	1.5	6	9.5	0	0.0	0	0.0
History of blood transfusion									
Yes	5	1	1.5	4	6.3	0	0.0	0	0.0
No	133	66	97.1	57	90.5	4	100.0	6	100.0
Unknown	3	1	1.5	2	3.2	0	0.0	0	0.0
Clinical history of liver diseases									
Yes	6[a]	5	7.4	1	1.6	0	0.0	0	0.0
No	135	63	92.6	62	98.4	4	100.0	6	100.0
Unknown	0	0	0.0	0	0.0	0	0.0	0	0.0
Family history of liver diseases									
Yes	9	4	5.9	4	6.3	1	25.0	0	0.0
No	124	63	92.6	54	85.7	3	75.0	4	66.7
Unknown	8	1	1.5	5	7.9	0	0.0	2	33.3

[a]Fatty liver (n=3), acute hepatitis A (n=1), primary biliary cirrhosis (n=1) and hepatitis (unknown cause).

Table II. Hepatitis virus markers classified by hepatitis B vaccination status.

Hepatitis B virus markers	Total	Vaccination yes (n)	(%)	Vaccination no (n)	(%)	During vaccination (or Drop-out) (n)	(%)	Unknown (n)	(%)
		68	48.2	63	44.7	4	2.8	6	4.3
HBsAg									
Positive (+)	0	0	0.0	0	0.0	0	0.0	0	0.0
Negative (-)	141	68	0.0	63	0.0	4	0.0	6	0.0
Anti-HBs									
Positive (+)	73	51	75.0	17	27.0	2	50.0	3	50.0
Negative (-)	68	17	25.0	46	73.0	2	50.0	3	50.0
Anti-HBc									
Positive (+)	17	1	1.5	16	25.4	0	0.0	0	0.0
Negative (-)	124	67	98.5	47	74.6	4	100.0	6	100.0
Anti-HBs positive (+)									
Anti-HBc positive (+)	16	1	1.5	15	23.8	0	0.0	0	0.0
Anti-HBc negative (-)	57	50	73.5	2	3.2	2	50.0	3	50.0
Anti-HBs negative (-)									
Anti-HBc positive (+)	1	0	0.0	1	1.6	0	0.0	0	0.0
Anti-HBc negative (-)	67	17	25.0	45	71.4	2	50.0	3	50.0
Anti-HCV									
Positive (+)	0	0	0.0	0	0.0	0	0.0	0	0.0
Negative (-)	141	68	0.0	63	0.0	4	0.0	6	0.0