

## How does HCV contribute to hepatocarcinogenesis?

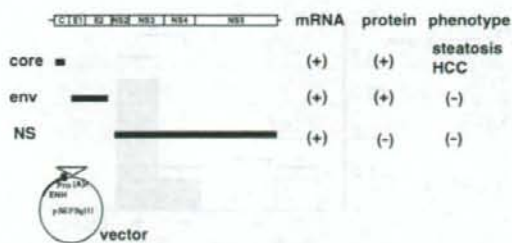
How HCV is involved in hepatocarcinogenesis is not yet clear, despite the fact that nearly 80% of patients with HCC in Japan are persistently infected with HCV.<sup>1,6,7</sup> HCV infection is also common in patients with HCC in other countries, albeit to a lesser extent. These lines of evidence force us to determine the role of HCV in hepatocarcinogenesis. Inflammation induced by HCV should be considered in a study on hepatocarcinogenesis in hepatitis viral infection; necrosis of hepatocytes due to chronic inflammation followed by regeneration enhances genetic aberrations in host cells, the accumulation of which culminates in HCC. This theory presupposes an indirect involvement of hepatitis viruses in HCC via hepatic inflammation. However, this context leaves us with a serious question: can inflammation alone result in the development of HCC in such a high incidence or is there a multicentric nature in HCV infection?

The other role of HCV would be weighed against an extremely rare occurrence of HCC in patients with autoimmune hepatitis in whom severe inflammation in the liver persists indefinitely, even after the development of cirrhosis. This background and reasoning led to a possible activity of viral proteins for inducing neoplasia. This possibility has been evaluated by introducing HCV genes into hepatocytes in culture with little success. One of the difficulties in using cultured cells is the carcinogenic capacity of HCV, if any, which would be weak and would take a long time to manifest. In fact, it takes 30–40 years for HCC to develop in individuals infected with HCV. On the basis of these viewpoints, we started to investigate carcinogenesis in chronic hepatitis C, *in vivo*, by transgenic mouse technology.

## Transgenic mouse studies revealed an *in vivo* oncogenic activity of HCV core protein

Transgenic mouse lines with parts of the HCV genome were engineered by introducing the genes from cDNA of the HCV genome of genotype 1b.<sup>8,9</sup> Three different transgenic mouse lines were established, which carry the core gene, envelope genes or non-structural genes (Fig. 1), respectively, under the same transcriptional control element. Among these mouse lines, only the transgenic mice carrying the core gene develop HCC in two independent lineages.<sup>9</sup> The envelope gene transgenic mice do not develop HCC, despite high expression levels of both E1 and E2 proteins.<sup>10,11</sup> The transgenic mice carrying the entire non-structural genes have not developed HCC.

The transgenic mice carrying the core gene express the core protein of an expected size, and the intrahepatic level of the core protein is similar to that in the liver of chronic hepatitis C patients. Early in life, these mice develop hepatic steatosis, which is one of the histological characteristics of chronic hepatitis C, along with lymphoid follicle formation and bile duct damage.<sup>12</sup> Thus, the core gene transgenic mouse model well reproduces the feature of chronic hepatitis C. Of note, any pictures of significant inflammation are not observed in the liver of this animal model. Late in life, these transgenic mice develop HCC. Notably, the development of steatosis and HCC has been reproduced by other HCV transgenic mouse lines, which harbor the entire HCV genome or structural



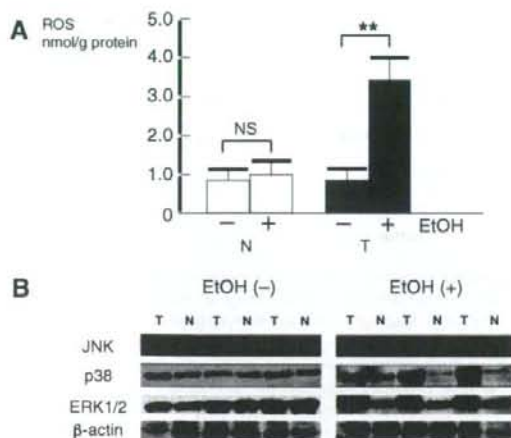
**Figure 1** Transgenic mouse lines carrying the hepatitis C virus (HCV) genome. Three different types of transgenic mouse lines, carrying the core gene, envelope genes or non-structural genes of HCV, respectively, were established under the control of the same regulatory elements. Among these mouse strains, only the transgenic mice carrying the HCV core gene developed hepatocellular carcinoma (HCC) after an early phase with hepatic steatosis in two independent lineages. The mice transgenic for the envelope genes or non-structural genes did not develop HCC. env, envelope genes; NS, non-structural genes.

genes including the core gene.<sup>13–15</sup> These outcomes indicate that the core protein per se of HCV has an oncogenic potential when expressed *in vivo*.

## Oxidative stress overproduction and MAPK activation as consequences to the core protein expression in the liver

It is difficult to determine the mechanism of carcinogenesis even for our simple model in which only the core protein is expressed in otherwise normal liver tissues. There is a notable feature in the localization of the core protein in hepatocytes: while the core protein predominantly exists in the cytoplasm associated with lipid droplets, it is also present in the mitochondria and nuclei.<sup>9,16</sup> On the basis of this finding, the pathways related to these two organelles, the mitochondria and nuclei, were meticulously analyzed.

One activity of the core protein is an increased production of oxidative stress in the liver. We would like to draw particular attention to the fact that the production of oxidative stress is increased in our transgenic mouse model in the absence of inflammation in the liver (hepatitis). This reflects a state of overproduction of reactive oxygen species (ROS) in the liver, or predisposition to it, which is staged by the HCV core protein without any intervening inflammation.<sup>17,18</sup> The overproduction of oxidative stress results in the generation of deletions in the mitochondrial DNA, an indicator of genetic damage. In addition, analysis of the anti-oxidant system revealed that some anti-oxidative molecules are not increased despite the overproduction of ROS in the liver of core gene transgenic mice; hemoxygenase-1 and glutathione peroxidase are not augmented whereas catalase and glutathione S-transferase levels are increased and enhanced by iron overloading (S Shinzawa *et al.*, unpubl. data, 2007). These results suggest that HCV core protein not only induces overproduction of ROS but also attenuates some of the anti-oxidant system, which may explain the mechanism underly-



**Figure 2** Alcohol administration enhances oxidative stress production and mitogen-activated protein kinase (MAPK) pathway activation in a synergistic fashion with hepatitis C virus (HCV) core protein. Administration of 5% alcohol for 3 weeks provoked an induction of reactive oxygen species (ROS) in HCV core gene transgenic mice, whereas it induced only a marginal increase in control mice, showing a synergy between the HCV core protein and ethanol in inducing ROS. Only the c-Jun N-terminal kinase (JNK) pathway is activated in the core gene transgenic mice before hepatocellular carcinoma (HCC) development, but feeding 5% alcohol for 3 weeks activated the other two pathways, p38 and ERK1/2, which was not observed in control mice. Thus, combining the effect of ethanol to that of the core protein resulted in the activation of all the MAPK pathways, among which only JNK was activated by the action of HCV core protein only in the absence of ethanol. ERK, extracellular signal-regulated kinase; EtOH, ethanol; N, non-transgenic control mouse; NS, statistically not significant; T, transgenic mouse. \*\* $P < 0.01$ .

ing the production of a strong oxidative stress in HCV infection compared to other forms of hepatitis.

Thus, the core protein induces oxidative stress overproduction in the absence of inflammation, which may, at least in part, contribute to hepatocarcinogenesis in HCV infection. If inflammation were added to the liver with the HCV core protein, the production of oxidative stress would be escalated to an extent that cannot be scavenged any longer by a physiological antagonistic system. This indicates that the inflammation in chronic HCV infection would have a characteristic difference from those of other types of hepatitis, such as autoimmune hepatitis. The basis for the overproduction of oxidative stress may be ascribed to mitochondrial dysfunction.<sup>9,17</sup> The dysfunction of the electron transfer system of the mitochondrion is suggested in association with the presence of the HCV core protein.<sup>19</sup> Hepatic steatosis in hepatitis C, which is also attributed to the action of the core protein,<sup>8</sup> may work as fuel for oxidative stress overproduction.<sup>18,20,21</sup>

Other possible pathways would be the alteration of the expression of cellular genes, interacting with cellular proteins, and modulation of intracellular signaling pathways. For example,

tumor necrosis factor (TNF)- $\alpha$  and interleukin-1 $\beta$  have been found transcriptionally activated.<sup>22</sup> The core protein has also been found to interact with some cellular proteins, such as retinoid X receptor (RXR)- $\alpha$ , that play pivotal roles in cell proliferation and lipid metabolism.<sup>23</sup> The mitogen-activated protein kinase (MAPK) cascade is also activated in the liver of the core gene transgenic mouse model. The MAPK pathway, which consists of three routes, c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK), is involved in numerous cellular events including cell proliferation. In the liver of the core gene transgenic mouse model prior to HCC development, only the JNK route is activated. Downstream of the JNK activation, transcription factor activating protein (AP)-1 activation is markedly enhanced.<sup>22,24</sup> Far downstream, both the mRNA and protein levels of cyclin D1 and CDK4 are increased. Thus, the HCV core protein modulates the intracellular signaling pathways and confers an advantage for cell proliferation to hepatocytes. Interestingly, we found recently that a protein interacting with the core protein, proteasome activator 28 $\gamma$  (PA28 $\gamma$ ), is indispensable for the core protein to exert its function for the development of steatosis, insulin resistance and HCC.<sup>25,26</sup>

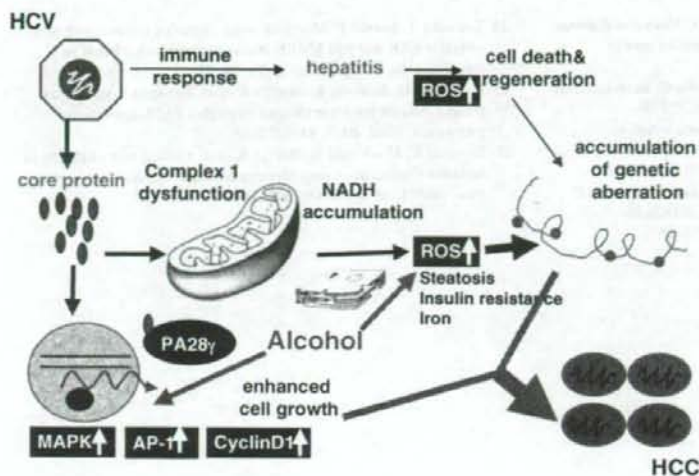
Such an effect of the core protein on the MAPK pathway, in combination with that on oxidative stress, may explain the extremely high incidence of HCC development in chronic hepatitis C.

### Molecular basis for the synergy between alcohol and HCV infection in hepatocarcinogenesis

As described above, the production of oxidative stress is increased in the liver of aged HCV core gene transgenic mice in the absence of inflammation. In young mice, the increase in oxidative stress is apparently marginal. However, feeding 5% ethanol to mice for 3 weeks induced ROS in the liver of core gene transgenic mice, whereas it induced only a minimal increase in control mice, demonstrating a synergy between the core protein and ethanol in inducing ROS (Fig. 2a).<sup>17</sup> In contrast, only the JNK pathway is activated in the core gene transgenic mice before HCC development, but feeding 5% ethanol for 3 weeks activated the other two MAPK pathways, p38 and ERK1/2 in the core gene transgenic mice, the activation of which is not present in control mice (Fig. 2b). Thus, combining the effect of ethanol to that of the core protein provoked the activation of all the MAPK pathways, affording advantage to cell proliferation.<sup>24</sup>

In a long-term observation experiment, feeding 2% ethanol to the core gene transgenic mice for 9 months resulted in the acceleration of HCC development (Moriya K *et al.*, unpubl. data, 2007). Screening by the high-throughput immunoblot analysis revealed differential expression of proteins in the liver with or without ethanol feeding; some proteins, the levels of which were either increased or decreased by the effect of the core protein, such as Rho GTPase activating protein (GAP) or caspase-8, are down- or upregulated by the effect of ethanol feeding.

In summary, we postulate that the induction of oxidative stress, together with the activation of MAPK cascade, followed by AP-1 activation and cyclin D1 overexpression, plays a pivotal role in the development of HCC (Fig. 3). Alterations in cellular gene expressions, such as TNF- $\alpha$  or suppressor of cytokine signaling-1, and the



**Figure 3** Molecular pathogenesis of hepatocellular carcinoma (HCC) development in hepatitis C virus (HCV) infection in association with alcohol. We postulate that induction of oxidative stress through the dysfunction in the mitochondrial electron transfer system, together with alterations in cellular gene expressions and the intracellular signaling pathways, including the mitogen-activated protein kinase (MAPK) cascade, play a pivotal role in the development of HCC. Alcohol activates both of these pathways and augments the development of HCC in HCV infection. AP-1, activating protein-1; NADH, nicotinamide adenine dinucleotide; PA28 $\gamma$ , proteasome activator 28 $\gamma$ ; ROS, reactive oxygen species; SOCS-1, suppressor of cytokine signaling-1; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

presence of steatosis and insulin resistance are co-accelerators to hepatocarcinogenesis in HCV infection. Finally, alcohol augments both of these pathways that are activated by the core protein, and further enhance the development of HCC in HCV infection (Fig. 3).

### Conflict of interest

No conflict of interest has been declared by the authors.

### References

- Saito I, Miyamura T, Ohbayashi A *et al.* Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc. Natl Acad. Sci. USA* 1990; **87**: 6547-9.
- Ikeda K, Saitoh S, Suzuki Y *et al.* Disease progression and hepatocellular carcinogenesis in patients with chronic viral hepatitis: a prospective observation of 2215 patients. *J. Hepatol.* 1998; **28**: 930-8.
- Donato F, Gelatti U, Limina RM, Fattovich G. Southern Europe as an example of interaction between various environmental factors: a systematic review of the epidemiologic evidence. *Oncogene* 2006; **25**: 3756-70.
- Hassan MM, Hwang LY, Hatten CJ *et al.* Risk factors for hepatocellular carcinoma: synergism of alcohol with viral hepatitis and diabetes mellitus. *Hepatology* 2002; **36**: 1046-9.
- Yuan JM, Govindarajan S, Arakawa K, Yu MC. Synergism of alcohol, diabetes, and viral hepatitis on the risk of hepatocellular carcinoma in blacks and whites in the U.S. *Cancer* 2004; **101**: 1009-17.
- Kiyosawa K, Sodeyama T, Tanaka E *et al.* Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. *Hepatology* 1990; **12**: 671-5.
- Yotsuyanagi H, Shintani Y, Moriya K *et al.* Virological analysis of non-B, non-C hepatocellular carcinoma in Japan: frequent involvement of hepatitis B virus. *J. Infect. Dis.* 2000; **181**: 1920-8.
- Moriya K, Yotsuyanagi H, Shintani Y *et al.* Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. *J. Gen. Virol.* 1997; **78**: 1527-31.
- Moriya K, Fujie H, Shintani Y *et al.* Hepatitis C virus core protein induces hepatocellular carcinoma in transgenic mice. *Nat. Med.* 1998; **4**: 1065-8.
- Koike K, Moriya K, Ishibashi K *et al.* Expression of hepatitis C virus envelope proteins in transgenic mice. *J. Gen. Virol.* 1995; **76**: 3031-8.
- Koike K, Moriya K, Yotsuyanagi H *et al.* Sialadenitis resembling Sjögren's syndrome in mice transgenic for hepatitis C virus envelope genes. *Proc. Natl Acad. Sci. USA* 1997; **94**: 233-6.
- Bach N, Thung SN, Schaffner F. The histological features of chronic hepatitis C and autoimmune chronic hepatitis: a comparative analysis. *Hepatology* 1992; **15**: 572-7.
- Lerat H, Honda M, Beard MR *et al.* Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. *Gastroenterology* 2002; **122**: 352-65.
- Naas T, Ghorbani M, Alvarez-Maya I *et al.* Characterization of liver histopathology in a transgenic mouse model expressing genotype 1a hepatitis C virus core and envelope proteins 1 and 2. *J. Gen. Virol.* 2005; **86**: 2185-96.
- Machida K, Cheng KT, Lai CK, Jeng KS, Sung VM, Lai MM. Hepatitis C virus triggers mitochondrial permeability transition with production of reactive oxygen species, leading to DNA damage and STAT3 activation. *J. Virol.* 2006; **80**: 7199-207.
- Moriya K, Fujie H, Yotsuyanagi H *et al.* Subcellular localization of hepatitis C virus structural proteins expressed in transgenic liver. *Jpn. J. Med. Sci. Biol.* 1997; **50**: 169-77.
- Moriya K, Nakagawa K, Santa T *et al.* Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocellular carcinogenesis. *Cancer Res.* 2001; **61**: 4365-70.
- Moriya K, Todoroki T, Tsutsumi T *et al.* Increase in the concentration of carbon 18 monounsaturated fatty acids in the liver with hepatitis C: analysis in transgenic mice and humans. *Biochem. Res. Commun.* 2001; **281**: 1207-12.
- Okuda M, Li K, Beard MR *et al.* Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. *Gastroenterology* 2002; **122**: 366-75.

- 20 Shintani Y, Fujie H, Miyoshi H *et al.* Hepatitis C virus and diabetes: direct involvement of the virus in the development of insulin resistance. *Gastroenterology* 2004; **126**: 840–8.
- 21 Koike K, Moriya K. Metabolic aspects of hepatitis C: steatohepatitis distinct from NASH. *J. Gastroenterol.* 2005; **40**: 329–36.
- 22 Tsutsumi T, Suzuki T, Moriya K *et al.* Intrahepatic cytokine expression and AP-1 activation in mice transgenic for hepatitis C virus core protein. *Virology* 2002; **304**: 415–24.
- 23 Tsutsumi T, Suzuki T, Shimoike T *et al.* Interaction of hepatitis C virus core protein with retinoid X receptor- $\alpha$  modulates its transcriptional activity. *Hepatology* 2002; **35**: 937–46.
- 24 Tsutsumi T, Suzuki T, Moriya K *et al.* Hepatitis C virus core protein activates ERK and p38 MAPK in cooperation with ethanol in transgenic mice. *Hepatology* 2003; **38**: 820–8.
- 25 Miyamoto H, Moriishi K, Moriya K *et al.* Hepatitis C virus core protein induces insulin resistance through a PA28-dependent pathway. *J. Virol.* 2007; **81**: 1727–35.
- 26 Moriishi K, Mochizuki R, Moriya K *et al.* Critical role of PA28 $\gamma$  in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis. *Proc. Natl Acad. Sci. USA* 2007; **104**: 1661–6.

## Original Article

## Association between hepatitis B/C viral infection, chronic kidney disease and insulin resistance in individuals undergoing general health screening

Nobukazu Ishizaka,<sup>1</sup> Yuko Ishizaka,<sup>2</sup> George Seki,<sup>3</sup> Ryoza Nagai,<sup>1</sup> Minoru Yamakado<sup>2</sup> and Kazuhiko Koike<sup>4</sup>Departments of <sup>1</sup>Cardiovascular Medicine, <sup>4</sup>Infectious Diseases and <sup>3</sup>Nephrology, University of Tokyo Graduate School of Medicine, and <sup>2</sup>Center for Multiphasic Health Testing and Services, Mitsui Memorial Hospital, Tokyo, Japan

**Aim:** Previous studies have shown that hepatitis B virus (HBV) and hepatitis C virus (HCV) infection may be associated with glomerulonephritis.

**Methods:** In the current study, we investigated the possible association between HBV/HCV infection, estimated GFR (eGFR) and albuminuria by analyzing cross-sectional data from individuals undergoing general health screening.

**Results:** Of 12 535 individuals enrolled, 130 (1.0%) and 72 (0.6%) tested positive for HBV surface antigen and HCV core antigen, respectively. In comparison with hepatitis-negative individuals, the prevalence of low eGFR and albuminuria was significantly greater in individuals with HCV infection, but not in those with HBV infection. Logistic regression analysis adjusted for age, sex, systolic blood pressure and fasting plasma glucose showed that HCV infection was positively associated with low eGFR (odds ratio 1.63 [95% CI 0.95–2.80,

$P = 0.077$ ]) and with albuminuria (odds ratio 2.00 [95% CI 1.06–3.76,  $P = 0.003$ ]). By contrast, prevalence of neither low eGFR nor albuminuria was greater in individuals with HBV infection than in hepatitis-negative subjects. Further adjustment for either HOMA-IR or serum alanine aminotransferase levels abolished the statistical significance in the association between HCV infection and albuminuria.

**Conclusion:** Our data suggest that although both HCV and HBV infection are associated with increased insulin resistance, the different viruses may have different impacts on chronic kidney disease among Japanese individuals undergoing general health screening.

**Key words:** aminotransferase, chronic kidney disease, health screening, insulin resistance, viral hepatitis

## INTRODUCTION

IN JAPAN, MORE than 1 million people are estimated to be infected with hepatitis B virus (HBV) and over 2 million with hepatitis C virus (HCV);<sup>1</sup> HBV infection has been reported to be found in 0.8% and HCV infection in 0.5% of Japanese workers.<sup>2</sup> Although a major target organ of HBV and HCV infection is the liver, extrahepatic manifestations are also frequently observed in patients with acute and chronic viral hepatitis. In

HCV infected patients, even without clinical evidence of liver involvement, renal complications can occur, most commonly membranoproliferative glomerulonephritis (MPGN) and membranous glomerulonephritis (MGN), which are clinically characterized by hematuria, proteinuria and variable grade renal dysfunction. One study has reported that HCV antibody was found to be positive in a large proportion (60%) of Japanese patients with MPGN.<sup>3</sup> El-Serag *et al.* reported that HCV-infected subjects had a sevenfold increase in the odds of MPGN compared with control subjects without HCV infection.<sup>4</sup> HBV infection may also be associated with MGN and MPGN,<sup>5,6</sup> and about 3% of HBV-infected patients were reported to have glomerulonephritis.<sup>7</sup>

Until recently, few data have been available on the prevalence of chronic kidney disease (CKD) and its components in individuals with HBV or HCV infection

Correspondence: Dr Nobukazu Ishizaka, Department of Cardiovascular Medicine, University of Tokyo Graduate School of Medicine, Hongo 7-3-1 Bunkyo-ku, Tokyo 113-8655, Japan. Email: nobu@ishizaka-ky@umin.ac.jp

Received 8 August 2007; revision 15 December 2007; accepted 15 January 2008.

in a population-based study, Tsui *et al.* reported that HCV infection was associated with albuminuria, but not with decreased GFR, in a US population.<sup>8</sup> Huang *et al.* reported a significant association between proteinuria and HCV, but not HBV, infection in an HBV/HCV endemic area.

In the present study, we investigated whether HBV infection, diagnosed by HBV surface antigen (HBsAg) positivity, and HCV infection, diagnosed by HCV core antigen (HCcAg) positivity, were associated with CKD components in Japanese individuals who underwent general health screening.

## METHODS

### Study population

THE STUDY WAS approved by the Ethical Committee of the Mitsui Memorial Hospital. Between April 2004 and August 2006, 12 535 people (4481 women and 8054 men) underwent a general health screen at Mitsui Memorial Hospital, including an estimation of urinary excretion of albumin, and were enrolled in the present study. In Japan, regular health check ups for employees are a legal requirement; all or most of the costs of the screening are paid for either by the employee's company or by the subject himself.

### Laboratory analysis

Blood samples were taken from the subjects after an overnight fast. Serum levels of total cholesterol (TC), HDL-cholesterol (HDL-C) and triglycerides (TG), alanine aminotransferase (ALT) and creatinine were determined by the enzymatic method. Serum uric acid was measured by the uricase-peroxidase method and hemoglobin A1C was determined by latex agglutination immunoassay. The levels of HBsAg and HCcAg in the sera were determined using commercially available enzyme immunoassay kits, AxSYM HBsAg Dynapack (Abbott Japan, Osaka, Japan) and Lumispot "Eiken" HCV antigen (Eiken Chemical, Tokyo, Japan), respectively, according to the manufacturer's instructions. HCcAg of >8.0 pg/mL was considered to be positive. Plasma glucose was measured by the hexokinase method and serum insulin was measured by enzyme immunoassay. Homeostasis model assessment insulin resistance (HOMA-IR) was calculated in these individuals according to the following formula:  $HOMA-IR = (\text{fasting immunoreactive insulin } [\mu\text{U/mL}] \times \text{fasting plasma glucose [FPG; mg/dL]})/405$ . The median (range)

ALT values in each ALT quartile (IU/mL) were 12 (4–14), 17 (15–19), 23 (20–27) and 37 (28–677).

### Estimated glomerular filtration rate, albuminuria and CKD

Serum creatinine was calibrated using the following formula: serum creatinine (Jaffe method) = 0.2 + serum creatinine (measured by enzymatic method). Serum creatinine was measured in mg/dL, and age in years; GFR was estimated using the equation from a simplified version of the Modification of Diet in Renal Disease (MDRD),<sup>9</sup> as follows: estimated GFR (eGFR; mL/min/1.73 m<sup>2</sup>) =  $186.3 \times (\text{serum creatinine})^{-1.154} \times (\text{age})^{-0.203} \times 0.881 \times 0.742$  (if female). In this MDRD formula, 0.881 is a coefficient for eGFR specific to the Japanese population.<sup>10</sup> For the diagnosis of albuminuria, spot urine samples were collected and expressed as urine albumin excretion ratio (UAE), which was expressed per g-creatinine. CKD was diagnosed when individuals had an eGFR of <60 mL/min/1.73 m<sup>2</sup>, designated as low eGFR, and/or UAE of  $\geq 30$  mg/g, designated as albuminuria.<sup>11</sup>

### Diagnosis of metabolic syndrome

Diagnosis of metabolic syndrome was made according to the criteria of the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP-III),<sup>12</sup> with body mass index (BMI) used as a surrogate for waist circumference.<sup>13</sup> Metabolic syndrome was said to be present when three or more of the following conditions were met: TG levels  $\geq 150$  mg/dL; HDL-C levels <40 mg/dL (men), <50 mg/dL (women); FPG levels  $\geq 110$  mg/dL or taking antidiabetic medication; systolic blood pressure (SBP)  $\geq 130$  mmHg or diastolic blood pressure (DBP)  $\geq 85$  mmHg or taking an antihypertensive medication; BMI  $\geq 25$  kg/m<sup>2</sup>.

### Statistical analysis

The data in this study were analyzed by one-way ANOVA with Bonferroni post hoc test,  $\chi^2$  test and by univariate and multivariate logistic regression analysis using the computer software StatView ver. 5.0 (SAS Institute, Cary, NC, USA). A value of  $P < 0.05$  was taken to be statistically significant. Results are expressed as the mean  $\pm$  standard deviation unless stated otherwise.

## RESULTS

### Baseline characteristics

THE BASELINE CHARACTERISTICS of the study subjects according to viral hepatitis infection are

Table 1 Clinical characteristics and laboratory data of study subjects

	Hepatitis negative (n = 12 333)	HBsAg positive (n = 130)	HCCAg positive (n = 72)	P-value
Male sex, n (%)	7916 (64)	93 (63)	45 (72)	0.21
Age, years	53.1 ± 10.6	55.3 ± 10.6	59.2 ± 10.5	<0.001
Body mass index, kg/m <sup>2</sup>	22.8 ± 3.1	23.9 ± 3.2	22.3 ± 2.8	<0.001
Systolic blood pressure, mmHg	122 ± 19	126 ± 20	123 ± 22	0.024
Diastolic blood pressure, mmHg	77 ± 12	79 ± 11	77 ± 13	0.077
WBC count, ×10 <sup>3</sup> cells/μL	5.3 ± 1.4	5.0 ± 1.2	5.0 ± 1.7	0.025
RBC count, ×10 <sup>4</sup> /μL	467 ± 43	473 ± 40	455 ± 48	0.020
Hemoglobin, g/dL	14.6 ± 1.5	14.8 ± 1.4	14.4 ± 1.5	0.17
Platelet count, ×10 <sup>4</sup> /μL	23.0 ± 5.1	20.1 ± 4.9	16.9 ± 5.8	<0.001
Serum data				
Total protein, g/dL	7.3 ± 0.4	7.3 ± 0.4	7.6 ± 0.5	<0.001
Albumin, g/dL	4.5 ± 0.2	4.5 ± 0.2	4.4 ± 0.3	<0.001
Total bilirubin, mg/dL	0.90 ± 0.36	0.92 ± 0.35	1.00 ± 0.47	0.040
ALT, IU/L	24 ± 19	27 ± 29	56 ± 46	<0.001
AST, IU/L	22 ± 12	25 ± 13	48 ± 27	<0.001
γ-GTP, IU/L	46 ± 67	38 ± 30	61 ± 57	0.061
Total cholesterol, mg/dL	211 ± 33	205 ± 31	175 ± 32	<0.001
HDL-cholesterol, mg/dL	59 ± 15	58 ± 14	53 ± 11	0.001
Triglycerides, mg/dL	117 ± 84	107 ± 83	89 ± 36	0.006
Fasting glucose, mg/dL	97 ± 19	98 ± 17	96 ± 15	0.82
Hemoglobin A1C, %	5.3 ± 0.7	5.3 ± 0.7	5.2 ± 0.7	0.30
HOMA-IR	1.5 ± 1.5	1.7 ± 1.1	2.4 ± 1.8	<0.001
Renal data				
Serum urea nitrogen, mg/dL	14.3 ± 3.6	14.6 ± 3.1	15.4 ± 6.4	0.031
Serum creatinine, mg/dL	0.78 ± 0.26	0.78 ± 0.14	0.81 ± 0.28	0.65
eGFR, mL/min/1.73m <sup>2</sup>	70 ± 10	70 ± 9	67 ± 13	0.087
Low eGFR, n (%)	1887 (15)	13 (10)	22 (31)	<0.001
UAER, mg/g	21 ± 129	12 ± 20	94 ± 428	<0.001
Albuminuria, n (%)	1157 (9)	8 (6)	14 (19)	0.006
Smoking status				
Never/former/current, %	52/25/23	43/29/28	60/24/17	0.18
Drinking status				
Never/former/current, %	20/5/75	19/5/75	32/17/51	<0.001

ALT, alanine aminotransferase; AST, aspartate aminotransferase; eGFR, estimated glomerular filtration rate; γ-GTP, gamma-glutamyltransferase; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment-insulin resistance; UAER, urine albumin excretion ratio; WBC, white blood cells; RBC, red blood cells.

described in Table 1. Of the 12 535 subjects enrolled, 130 (1.0%; 37 women, 93 men) and 72 (0.6%; 27 women, 45 men) were positive for HBsAg and HCCAg, respectively; no subjects were positive for both HBsAg and HCCAg. HCCAg-positive individuals were significantly older than hepatitis-negative individuals ( $P < 0.001$ ), whereas the age between HBsAg-positive and hepatitis-negative individuals did not differ significantly. All hepatitis-positive individuals enrolled in the current study, except one HBsAg-positive subject, underwent abdominal ultrasonography, and none was diag-

nosed as having advanced cirrhosis. The hematological data and aminotransferase levels of the individual who did not undergo abdominal ultrasonography were as follows: white blood cell count, 4000 (cells/microL); red blood cell count, 524 × 10<sup>4</sup> (cells/microL); Plt 25.4 × 10<sup>4</sup> (cells/microL); ALT 19 (IU/L); and AST 19 (IU/L). In the HCCAg-positive group, the mean serum TC level was lower than in the other two groups. Logistic regression analysis adjusted for sex, age, ALT, albumin and total bilirubin levels showed that an odds ratio of HBsAg-positivity and HCCAg-positivity for the lowest TC

**Table 2** Logistic regression analysis for HBV/HCV infection as independent variables, and low eGFR and albuminuria as dependent variables

	Dependent variables					
	CKD Odds ratio (95% CI)	P-value	Components of CKD			
			Low eGFR Odds ratio (95% CI)	P-value	Albuminuria Odds ratio (95% CI)	P-value
<b>Unadjusted</b>						
HBV/HCV negative	1.00	-	1.00	-	1.00	-
HBsAg positive	0.63 (0.39-1.01)	0.056	0.62 (0.35-1.09)	0.098	0.63 (0.31-1.30)	0.21
HCCAg positive	2.46 (1.54-3.94)	0.0002	2.44 (1.47-4.03)	0.0005	2.33 (1.30-4.19)	0.0047
<b>Adjusted for age and sex</b>						
HBV/HCV negative	1.00	-	1.00	-	1.00	-
HBsAg positive	0.53 (0.32-0.86)	0.011	0.51 (0.28-0.93)	0.027	0.57 (0.28-1.18)	0.13
HCCAg positive	1.77 (1.08-2.92)	0.025	1.64 (0.96-2.82)	0.071	1.86 (1.02-3.37)	0.042
<b>Adjusted for age, sex, SBP and FPG</b>						
HBV/HCV negative	1.00	-	1.00	-	1.00	-
HBsAg positive	0.49 (0.30-0.81)	0.0057	0.51 (0.28-0.92)	0.026	0.50 (0.23-1.05)	0.066
HCCAg positive	1.83 (1.10-3.05)	0.020	1.63 (0.95-2.80)	0.077	2.00 (1.06-3.76)	0.034

CKD, chronic kidney disease; FPG, fasting plasma glucose; HBV, hepatitis B virus; HCV, hepatitis C virus; SBP, systolic blood pressure.

quartile (TC < 187 mg/dL) was 1.42 (95% CI 0.95-2.12,  $P=0.89$ ) and 7.30 (95% CI 4.39-12.13,  $P<0.001$ ), respectively, compared with hepatitis-negative individuals. The finding that HCCAg-positive individuals had lower TC levels than non-hepatitis or HBsAg-positive individuals was in agreement with previous observations of ours and others.<sup>14,15</sup> Neither FPG nor HbA1c differed significantly between individuals positive for HBsAg or HCCAg and hepatitis-negative individuals; however, HOMA-IR was significantly greater in HCCAg-positive individuals than in hepatitis-negative ( $P<0.001$ ) or HBsAg-positive ( $P=0.003$ ) individuals. Serum albumin level was statistically significantly lower in HCCAg-positive subjects than in hepatitis-negative subjects, although the difference was very small (Table 1 and 4.4 g/dL vs. 4.5 g/dL). By Bonferroni post hoc analysis, serum bilirubin levels were not statistically significantly different between HCCAg-positive and hepatitis-negative individuals or between HBsAg-positive and hepatitis-negative individuals.

#### eGFR and urinary albumin excretion

Of the 12 535 subjects enrolled, 1179 (9.4%, 389 women, 790 men) had albuminuria, and 1922 (15.3%, 729 women, 1193 men) had low eGFR. Both of these conditions were present in 278 individuals (2.2%); therefore, 2823 (22.5%) subjects (1023 women, 1800 men) were diagnosed to have CKD. Among the 1179 (9.4%) individuals who had albuminuria, 1062 had an

UAER value between 30 and 299 mg/g (microalbuminuria), and the remaining 117 had an UAER value of  $\geq 300$  mg/g (macroalbuminuria). The median (interquartile range) of eGFR (mL/min/1.73 m<sup>2</sup>) was 69.6 (63.2-75.8) in HBV/HCV-negative individuals, 69.5 (64.3-77.2) in HBsAg-positive individuals, and 65.9 (58.4-76.9) in HCCAg-positive individuals. The median (interquartile range) of UAER (mg/g) was 6.4 (4.2-11.8) in HBV/HCV-negative individuals, 6.4 (4.2-11.6) in HBsAg-positive individuals and 8.0 (4.1-18.6) in HCCAg-positive individuals.

#### Association between HBsAg/HCCAg positivity and CKD

The prevalence of both low eGFR ( $P<0.001$ ) and albuminuria ( $P=0.007$ ) was significantly greater in HCCAg-positive than in HBV/HCV-negative individuals by  $\chi^2$  test (Table 1). In contrast, compared with HBV/HCV-negative individuals, the prevalence of either low eGFR ( $P=0.12$ ) or albuminuria ( $P=0.27$ ) was not different in HBsAg-positive individuals. After adjusting for age and sex, logistic regression analysis showed that HCCAg was statistically significantly positively associated with albuminuria (Table 2) and that it tended to be positively associated with low eGFR. In contrast, HBsAg positivity was inversely associated with low eGFR, whereas it was not significantly associated with albuminuria. Essentially the same results were obtained after further adjustment for SBP and FPG.



**Table 3** Logistic regression analysis for HBV/HCV infection as independent variables, and metabolic syndrome, increased insulin resistance and elevated ALT levels as dependent variables

	Dependent variables					
	Metabolic syndrome Odds ratio (95% CI)	P-value	Highest HOMA-IR quartile Odds ratio (95% CI)	P-value	Highest ALT quartile Odds ratio (95% CI)	P-value
Unadjusted						
HBV/HCV negative	1.00	-	1.00	-	1.00	-
HBsAg positive	1.21 (0.71-2.04)	0.49	1.60 (1.12-2.31)	0.011	1.42 (0.98-2.06)	0.068
HCCAg positive	0.25 (0.60-1.00)	0.050	3.39 (2.13-5.39)	<0.0001	10.3 (0.60-17.8)	<0.0001
Adjusted for age and sex						
HBV/HCV negative	1.00	-	1.00	-	1.00	-
HBsAg positive	1.09 (0.34-1.86)	0.75	1.57 (1.09-2.26)	0.016	1.36 (0.92-2.01)	0.12
HCCAg positive	0.23 (0.06-0.95)	0.042	3.18 (1.99-5.05)	<0.0001	16.53 (9.20-29.7)	<0.0001

ALT, alanine aminotransferase; HBV, hepatitis B virus; HCV, hepatitis C virus; HOMA-IR, homeostasis model assessment-insulin resistance.

### HBsAg/HCCAg positivity, metabolic syndrome and insulin resistance

Metabolic syndrome was diagnosed in 1304 individuals (10.4%, 160 women and 1144 men). The mean values of HOMA-IR in individuals with and without metabolic syndrome were  $3.1 \pm 3.1$  and  $1.4 \pm 1.0$ , respectively ( $P < 0.001$ ). Age and sex-adjusted logistic regression analysis showed that HCCAg positivity was inversely associated with metabolic syndrome, whereas HBsAg positivity was not (Table 3). On the other hand, after adjusting for the same variables, both HBsAg and HCCAg positivity was positively associated with the highest sex-specific HOMA-IR quartile, which was HOMA-IR of  $>1.39$  in women and  $>2.06$  in men.

### Relationship between metabolic syndrome, insulin resistance and CKD components

After adjusting for age and sex, logistic regression analysis showed that metabolic syndrome was positively associated with both low eGFR (odds ratio 1.43 [95% CI 1.23-1.67,  $P < 0.001$ ]) and albuminuria (odds ratio 3.84 [95% CI 3.31-4.47,  $P < 0.001$ ]). After adjusting for the same variables, the highest HOMA-IR quartile was also positively associated with both low eGFR (odds ratio 1.21 [95% CI 1.08-1.35,  $P = 0.0012$ ]) and albuminuria (odds ratio 2.86 [95% CI 2.52-3.23,  $P < 0.001$ ]).

The relationship between HBV/HCV infection and CKD components was analyzed after further adjustment for either metabolic syndrome or HOMA-IR (Table 4). The negative association between HBsAg positivity and low eGFR and the positive association between HCCAg

positivity and albuminuria remained statistically significant after further adjustment for metabolic syndrome. However, in the logistic regression analysis further adjusted for HOMA-IR, the association between HCCAg positivity and albuminuria did not remain statistically significant.

### Serum alanine aminotransferase activity and CKD components

Logistic regression analysis adjusted for age, sex, SBP and FPG showed that ALT was dose-dependently associated with albuminuria, but not with low eGFR (Table 5). When adjusted for age, sex, SBP, FPG and ALT, the positive association between HCCAg positivity and albuminuria did not remain statistically significant, whereas the negative association between HBsAg positivity and low eGFR remained statistically significant (Table 4).

## DISCUSSION

IN THE CURRENT study, by analyzing the data from individuals who underwent general health screening, it was found that HCCAg positivity was associated with a greater prevalence of low eGFR and albuminuria, both of which are components of CKD, than hepatitis-negative individuals. By contrast, the prevalence of neither low eGFR nor albuminuria was not different between HBsAg-positive and hepatitis-negative individuals. After adjusting for age, sex, SBP and FPG, the association of HCCAg with low eGFR (tendency) or with albuminuria (statistically significant) was still present.

**Table 4** Logistic regression analysis for HBV/HCV infection as independent variables, and low eGFR and albuminuria as dependent variables after further adjusting for HOMA-IR and ALT

	Dependent variables					
	CKD Odds ratio (95% CI)	P-value	Components of CKD			
			low eGFR Odds ratio (95% CI)	P-value	Albuminuria Odds ratio (95% CI)	P-value
Adjusted for age, sex and metabolic syndrome						
HBV/HCV negative	1.00	-	1.00	-	1.00	-
HBsAg positive	0.51 (0.31-0.84)	0.0082	0.50 (0.28-0.91)	0.024	0.54 (0.26-1.13)	0.10
HCCAg positive	1.92 (1.17-3.17)	0.010	1.70 (0.99-2.91)	0.055	2.19 (1.21-3.99)	0.010
Adjusted for age, sex, SBP, FPG and HOMA-IR						
HBV/HCV negative	1.00	-	1.00	-	1.00	-
HBsAg positive	0.49 (0.29-0.80)	0.0046	0.51 (0.28-0.92)	0.025	0.48 (0.23-1.02)	0.056
HCCAg positive	1.63 (0.97-2.74)	0.064	1.58 (0.92-2.72)	0.099	1.67 (0.88-3.19)	0.12
Adjusted for age, sex, SBP, FPG and ALT						
HBV/HCV negative	1.00	-	1.00	-	1.00	-
HBsAg positive	0.49 (0.30-0.81)	0.0050	0.51 (0.28-0.92)	0.025	0.49 (0.23-1.03)	0.060
HCCAg positive	1.55 (0.92-2.59)	0.098	1.49 (0.86-2.57)	0.16	1.59 (0.83-3.02)	0.16

ALT, alanine aminotransferase; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; HBV, hepatitis B virus; HCV, hepatitis C virus; HOMA-IR, homeostasis model assessment-insulin resistance; SBP, systolic blood pressure.

**Table 5** Logistic regression analysis for ALT quartiles as an independent variable and low eGFR, and albuminuria as dependent variables

	Dependent variables					
	CKD Odds ratio (95% CI)	P-value	Components of CKD			
			Low eGFR Odds ratio (95% CI)	P-value	Albuminuria Odds ratio (95% CI)	P-value
Unadjusted						
ALT-Q1	1.00	-	1.00	-	1.00	-
ALT-Q2	1.21 (1.07-1.36)	0.0020	1.21 (0.16-1.38)	0.0058	1.16 (0.96-1.40)	0.012
ALT-Q3	1.35 (1.20-1.53)	<0.0001	1.21 (1.06-1.39)	0.0057	1.55 (1.29-1.85)	<0.0001
ALT-Q4	1.33 (1.18-1.50)	<0.0001	0.95 (0.82-1.09)	0.45	2.05 (1.73-2.43)	<0.0001
Adjusted for age and sex						
ALT-Q1	1.00	-	1.00	-	1.00	-
ALT-Q2	1.03 (0.91-1.17)	0.63	1.02 (0.88-1.17)	0.80	1.04 (0.86-1.26)	0.66
ALT-Q3	1.27 (1.12-1.45)	0.0003	1.13 (0.97-1.31)	0.11	1.47 (1.22-1.77)	<0.0001
ALT-Q4	1.47 (1.28-1.67)	<0.0001	1.03 (0.88-1.20)	0.70	2.16 (1.80-2.59)	<0.0001
Adjusted for age, sex, SBP and FPG						
ALT-Q1	1.00	-	1.00	-	1.00	-
ALT-Q2	1.00 (0.88-1.14)	0.96	1.03 (0.89-1.19)	0.68	0.97 (0.80-1.18)	0.75
ALT-Q3	1.18 (1.03-1.34)	0.015	1.15 (0.99-1.34)	0.062	1.23 (1.02-1.49)	0.035
ALT-Q4	1.24 (1.08-1.41)	0.0023	1.08 (0.93-1.27)	0.32	1.45 (1.20-1.76)	0.0001

ALT-Q1, ALT-Q2, ALT-Q3 and ALT-Q4 indicate the first, second, third and fourth, respectively, serum alanine aminotransferase activity quartiles.

ALT, alanine aminotransferase; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; SBP, systolic blood pressure.

Both HCCAg positivity and HBsAg positivity were positively associated with increased insulin resistance. On the other hand, HCCAg positivity was inversely associated with metabolic syndrome.

Although renal involvement of hepatitis virus infection was first reported more than three decades ago,<sup>16</sup> knowledge of the association between HBV/HCV, proteinuria and low eGFR in the general population remains limited. Huang *et al.* analyzed data from individuals in southern Taiwan, an HBV/HCV-endemic area. They found that HBsAg and anti-HCV were positive in 13% and 7%, respectively, of the study population, and HCV infection, but not HBV infection, was associated with proteinuria.<sup>17</sup> Tsui *et al.* analyzed the data from a general population in the US and reported that HCV infection was associated with albuminuria, but not with low eGFR.<sup>8</sup> Our findings that albuminuria was positively associated with HCCAg positivity, but not with HBsAg, were therefore in agreement with these previous findings.

We showed that HCCAg positivity was associated with increased insulin resistance, defined as the highest HOMA-IR quartile. Several previous studies have shown that HCV infection was associated with diabetes as well as insulin resistance.<sup>18,19</sup> We have shown previously that HCV infection induces insulin resistance by the virus itself, which may influence the progression of chronic liver disease.<sup>20,21</sup> Compared to HCV infection, the relationship between HBsAg and insulin resistance has been less extensively studied. Custro *et al.* reported that both HBV and HCV infections increased the incidence of impaired glucose metabolism, and that the impact on glycemic homeostasis evoked by these two infections seemed to be similar.<sup>22</sup> In contrast, by analyzing subjects in Taiwan, where the prevalence of HBV infection is very high, Wang *et al.* showed that HBV carriers were not associated with insulin resistance.<sup>23</sup> We showed here that HBsAg positivity was also associated with increased insulin resistance, although to a lesser extent than HCCAg positivity (Table 3). Serum ALT levels, a marker for the extent of liver injury, is known to affect the degree of insulin resistance.<sup>24</sup> In the current study, the mean ALT levels were greater in HCCAg-positive than in HBsAg-positive individuals. The relative impacts of virus infection per se and liver injury for the development of hepatitis-related insulin resistance in our study population should be investigated further in future studies.

It was of note that the positive association between HCCAg positivity and albuminuria lost its statistical significance after adjusting for HOMA-IR, which suggested that the observed association between HCCAg positivity

and albuminuria was confounded by insulin resistance. Insulin resistance is one of the background features of albuminuria,<sup>24</sup> and albuminuria is one of the diagnostic components of metabolic syndrome in WHO criteria.<sup>12</sup> In contrast to the positive association between HCV infection and increased insulin resistance, however, we found an apparent *negative* association between HCCAg positivity and metabolic syndrome (Table 3). Several previous studies also reported that the prevalence of metabolic syndrome was lower in HBV or HCV-infected individuals.<sup>25,26</sup> Together with these reports, our data suggest that increased insulin resistance, which may play a role in the development of albuminuria in HCV infection, may not be recognized as a phenotype of metabolic syndrome in HCCAg-positive individuals. In addition, our data suggest the possibility that increased insulin resistance, but not metabolic syndrome phenotype, enhances the risk for albuminuria and CKD in these individuals.

In the current study, the association between HBsAg and low eGFR or albuminuria was not statistically significant by univariate analysis (Table 1). However, after multivariate adjustment, there was an inverse mode association between HBsAg positivity and low eGFR (statistically significant) or albuminuria (tendency). Whether or not there is truly an inverse relationship between HBsAg positivity and CKD components should be investigated further after increasing the number of HBsAg-positive individuals. Nevertheless, we may be able to conclude from the current study that there is a difference in the mode of association with CKD components between HCCAg positivity and HBsAg positivity in individuals who underwent general health screening, and had, if present, only minor liver damage.

The current study had several limitations. First, GFR was not determined by a direct measurement, but instead by the MDRD formula with the Japanese coefficient of 0.881. A recent study has suggested that estimation of GFR by this method may result in an underestimation of GFR when insulin clearance is over 60 mL/min/1.73 m<sup>2</sup> in Japanese.<sup>10</sup> Second, we could not assess data of anti-HBe positivity, which might affect the prevalence of extrahepatic manifestations in HBV infection.<sup>7</sup> Third, due to the cross-sectional nature of the study, we could not derive the causal and resultant relationship between HBV/HCV infection and CKD components. Fourth, as the liver is the primary organ of insulin clearance, C-peptide concentration may be a better marker of secreted insulin levels and insulin resistance than parameters derived from insulin,<sup>27</sup> such as HOMA-IR; however, serum C-peptide data were not available in

the current study. Finally, interferon therapy may affect albuminuria and renal function, which may be either reversible or irreversible.<sup>28-30</sup> Although information on the history of interferon therapy was not available in the current study, this point should be taken into account in future studies.

## CONCLUSION

**I**N CONCLUSION, BY analyzing the cross-sectional data of 12 535 individuals who underwent general health screening, we have investigated a possible association between viral hepatitis infection and CKD components. There was a positive association between HCCAg positivity, but not HBsAg positivity, and CKD components (low eGFR and albuminuria). The observed associations were confounded by the degree of insulin resistance and serum ALT levels. Although HCCAg positivity was associated with increased insulin resistance, HCCAg positivity was negatively associated with metabolic syndrome. These data collectively indicate that some differences may exist between HCV infection and HBV infection in terms of association with CKD components in Japanese individuals who undergo general health screening.

## ACKNOWLEDGMENTS

**T**HIS WORK WAS supported in part by grants from the Smoking Research Foundation, Chiyoda Mutual Life Foundation, St Luke's Grant for the Epidemiological Research and Daiwa Securities Health Foundation.

## REFERENCES

- Higuchi M, Tanaka E, Kiyosawa K. Epidemiology and clinical aspects on hepatitis C. *Jpn J Infect Dis* 2002; 55: 69-77.
- Narai R, Oyama T, Ogawa M et al. HBV- and HCV- infected workers in the Japanese workplace. *J Occup Health* 2007; 49: 9-16.
- Yamabe H, Johnson RJ, Gretch DR et al. Hepatitis C virus infection and membranoproliferative glomerulonephritis in Japan. *J Am Soc Nephrol* 1995; 6: 220-3.
- El-Serag HB, Hampel H, Yeh C, Rabeneck L. Extrahepatic manifestations of hepatitis C among United States male veterans. *Hepatology* 2002; 36: 1439-45.
- Johnson RJ, Couser WG. Hepatitis B infection and renal disease: clinical, immunopathogenetic and therapeutic considerations. *Kidney Int* 1990; 37: 663-76.
- Tang S, Lai FM, Lui YH et al. Lamivudine in hepatitis B-associated membranous nephropathy. *Kidney Int* 2005; 68: 1750-8.
- Cacoub P, Saadoun D, Bourliere M et al. Hepatitis B virus genotypes and extrahepatic manifestations. *J Hepatol* 2005; 43: 764-70.
- Tsui JJ, Vittinghoff E, Shlipak MG, O'Hare AM. Relationship between hepatitis C and chronic kidney disease: results from the Third National Health and Nutrition Examination Survey. *J Am Soc Nephrol* 2006; 17: 1168-74.
- Manjunath G, Sarnak MJ, Levey AS. Prediction equations to estimate glomerular filtration rate: an update. *Curr Opin Nephrol Hypertens* 2001; 10: 785-92.
- Imai E, Horio M, Nitta K et al. Estimation of glomerular filtration rate by the MDRD study equation modified for Japanese patients with chronic kidney disease. *Clin Exp Nephrol* 2007; 11: 41-50.
- National Kidney Foundation. K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. *Am J Kidney Dis* 2002; 39: S1-266.
- Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med* 1998; 15: 539-53.
- Ishizaka N, Ishizaka Y, Toda E, Nagai R, Yamakado M. Association between serum uric acid, metabolic syndrome, and carotid atherosclerosis in Japanese individuals. *Arterioscler Thromb Vasc Biol* 2005; 25: 1038-44.
- Moriya K, Shintani Y, Fujie H et al. Serum lipid profile of patients with genotype 1b hepatitis C viral infection in Japan. *Hepatol Res* 2003; 25: 371-6.
- Serfaty L, Andreani T, Giral P, Carbonell N, Chazouilleres O, Poupon R. Hepatitis C virus induced hypobetalipoproteinemia: a possible mechanism for steatosis in chronic hepatitis C. *J Hepatol* 2001; 34: 428-34.
- Combes B, Shorey J, Barrera A et al. Glomerulonephritis with deposition of Australia antigen-antibody complexes in glomerular basement membrane. *Lancet* 1971; 2: 234-7.
- Huang JF, Chuang WL, Dai CY et al. Viral hepatitis and proteinuria in an area endemic for hepatitis B and C infections: another chain of link? *J Intern Med* 2006; 260: 255-62.
- Tai TY, Lu JY, Chen CL et al. Interferon-alpha reduces insulin resistance and beta-cell secretion in responders among patients with chronic hepatitis B and C. *J Endocrinol* 2003; 178: 457-65.
- Shaheen M, Echeverry D, Oblad MG, Montoya MI, Teklehaimanot S, Akhtar AJ. Hepatitis C, metabolic syndrome, and inflammatory markers: results from the Third National Health and Nutrition Examination Survey [NHANES III]. *Diabetes Res Clin Pract* 2007; 75: 320-6.
- Shintani Y, Fujie H, Miyoshi H et al. Hepatitis C virus infection and diabetes: direct involvement of the virus in the development of insulin resistance. *Gastroenterology* 2004; 126: 840-8.

- 21 Koike K. Hepatitis C virus infection can present with metabolic disease by inducing insulin resistance. *Intervirology* 2006; 49: 51-7.
- 22 Custro N, Carroccio A, Ganci A *et al*. Glycemic homeostasis in chronic viral hepatitis and liver cirrhosis. *Diabetes Metab* 2001; 27: 476-81.
- 23 Wang CC, Hsu CS, Liu CJ, Kao JH, Chen DS. Association of chronic hepatitis B virus infection with insulin resistance and hepatic steatosis. *J Gastroenterol Hepatol* 2008; (in press).
- 24 Niskanen L, Laakso M. Insulin resistance is related to albuminuria in patients with type II (non-insulin-dependent) diabetes mellitus. *Metabolism* 1993; 42: 1541-5.
- 25 Jan CF, Chen CJ, Chiu YH *et al*. A population-based study investigating the association between metabolic syndrome and hepatitis B/C infection (Keelung Community-based Integrated Screening study, 10). *Int J Obes (Lond)* 2006; 30: 794-9.
- 26 Luo B, Wang Y, Wang K. Association of metabolic syndrome and hepatitis B infection in a Chinese population. *Clin Chim Acta* 2007; 380: 238-40.
- 27 Bonora E, Coscelli C, Orioli S *et al*. Hyperinsulinemia of chronic active hepatitis: impaired insulin removal rather than pancreatic hypersecretion. *Horm Metab Res* 1984; 16: 111-14.
- 28 Jones GJ, Itri LM. Safety and tolerance of recombinant interferon alfa-2a (Roferon-A) in cancer patients. *Cancer* 1986; 57: 1709-15.
- 29 Quesada JR, Talpaz M, Rios A, Kurzrock R, Gutterman JU. Clinical toxicity of interferons in cancer patients: a review. *J Clin Oncol* 1986; 4: 234-43.
- 30 Lederer E, Truong L. Unusual glomerular lesion in a patient receiving long-term interferon alpha. *Am J Kidney Dis* 1992; 20: 516-18.

## Original Article

Effect of treatment with interferon  $\alpha$ -2b and ribavirin in patients infected with genotype 2 hepatitis C virusYoshihiko Nagase,<sup>1</sup> Hiroshi Yotsuyanagi,<sup>1,2</sup> Chiaki Okuse,<sup>1</sup> Kiyomi Yasuda,<sup>3</sup> Tomohiro Kato,<sup>4</sup> Kazuhiko Koike,<sup>2</sup> Michihiro Suzuki,<sup>1</sup> Kusuki Nishioka,<sup>4</sup> Shiro Iino<sup>3</sup> and Fumio Itoh<sup>1</sup>

<sup>1</sup>Department of Internal Medicine, Division of Gastroenterology and Hepatology and <sup>2</sup>Department of Bioregulation and Proteomics, St. Marianna University, Kawasaki, <sup>3</sup>Department of Infectious Diseases, Internal Medicine, Graduate School of Medicine, University of Tokyo and <sup>4</sup>Center for Liver Diseases, Kiyokawa Hospital, Tokyo, Japan

**Aim:** Nearly 20% of chronic hepatitis C (CHC) patients with genotype 2 hepatitis C virus (HCV) infection are not curable, even by interferon (IFN)-ribavirin combination therapy. The aim of this study is to investigate the factors that determine the efficacy of combination therapy in patients with genotype 2 HCV infection.

**Methods:** Fifty patients with CHC who underwent a treatment of 6 MU IFN  $\alpha$ -2b with ribavirin for 24 weeks were retrospectively analyzed.

**Results:** All the patients showed no serum HCV-RNA within 12 weeks after starting the therapy. Forty-one of the 50 patients (82%) achieved a sustained virological response (SVR). The age, sex, genotype (2a vs. 2b) and grade/stage of the liver by histopathology and pretreatment viral load were

not different between the sustained responders and relapsers. Univariate analysis showed that an earlier viral clearance from blood and a larger number of amino acid substitutions in the interferon sensitivity determining region (ISDR) were predictors of SVR. Multivariate analysis showed that a large number of amino acid substitutions in the ISDR was a predictor of SVR.

**Conclusion:** The characterization of the amino acid sequences of ISDR may be helpful for predicting a relapse after combination therapy in patients with genotype 2 HCV infection.

**Key words:** genotype, hepatitis C virus, interferon, ISDR, ribavirin

## INTRODUCTION

CHRONIC HEPATITIS C (CHC) is an infection that affects more than 150 million people worldwide. Up to 50% of these people develop chronic liver disease leading to liver cirrhosis.<sup>1–3</sup> Once liver cirrhosis has developed, up to 7% of these patients per year develop hepatocellular carcinoma.<sup>4–6</sup> Antiviral treatment is crucial for the control of this disease.

Before the use of ribavirin, interferon (IFN) monotherapy was the only effective treatment for CHC.

Although many clinical trials and several meta-analyses have documented the efficacy of IFN monotherapy, the rate of sustained virological response (SVR) is low, particularly in patients with genotype 1 or 4 hepatitis C virus (HCV) infection.<sup>7–9</sup>

The combination therapy of IFN and ribavirin has been shown to be more effective than IFN monotherapy for CHC.<sup>10–14</sup> The baseline level of serum HCV-RNA before treatment and HCV genotype are predictors of a SVR to IFN therapy.<sup>15</sup> With regard to HCV genotype, patients who are infected with genotype 2 or 3 HCV can achieve a higher SVR rate than those with genotype 1 HCV. However, even with genotype 2 HCV infection, combination therapy for 24 weeks failed to eradicate the virus in about 20% of patients,<sup>12–14</sup> although the reason for this is still unclear.

Besides HCV genotype and viral load, mutations in the interferon sensitivity determining region (ISDR, aa 2209–2248) of the non-structural region 5A (NS5A) of

Correspondence: Dr Hiroshi Yotsuyanagi, Department of Infectious Diseases, Internal Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Email: hyotsu-iky@umin.ac.jp

Grant sponsor: Japanese Ministry of Health, Labor and Welfare. Received 7 September 2006; revision 28 June 2007; accepted 16 July 2007.

HCV have also been reported to influence the efficacy of IFN. In genotype 1 HCV infection, the number of amino acid substitutions in ISDR is reported to be related to the efficacy of IFN therapy in Japan and Europe,<sup>16–20</sup> although this correlation is still controversial.<sup>21</sup> In genotype 2 infection, the amino acid sequence of ISDR has been reported to also correlate with SVR to IFN monotherapy.<sup>22–24</sup> Therefore, the efficacy of IFN-ribavirin combination therapy in genotype 2 HCV-infected patients may be determined by the amino acid sequence of ISDR, which has not yet been studied.

The aim of this study is to elucidate factors that determine the response to IFN-ribavirin combination therapy in patients with genotype 2 HCV infection.

## METHODS

### Patient selection

FROM 2001 TO 2003, 140 patients (84 men and 56 women; mean age, 53.8 ± 11.3 years) were treated with recombinant IFN  $\alpha$ -2b (Intron A; Schering-Plough, Kenilworth, NJ) and ribavirin (Rebetol; Schering-Plough, Kenilworth, NJ) combination therapy. Eighty-five patients had genotype 1 HCV infection (54 men and 31 women; mean age, 56.3 ± 10.5 years) and 55 patients had genotype 2 HCV infection (30 men and 25 women; mean age, 50.0 ± 11.6 years). All the patients with genotype 2 HCV infection were treated daily with IFN  $\alpha$ -2b at 6 MU for two weeks, followed by treatment three times a week with IFN  $\alpha$ -2b 6 MU for 22 weeks in combination with ribavirin. Ribavirin was given orally twice a day at a total daily dose of 600 mg for 24 weeks for patients who weighed 60 kg or less and 800 mg for patients who weighed more than 60 kg. Fifty of the 55 patients with genotype 2 HCV infection with available clinical data were retrospectively analyzed.

### HCV markers

HCV genotype was determined by a direct sequencing of the amplified products generated during the Amplicor Monitor test (Roche Diagnostics, Branchburg, NJ)<sup>25</sup> with an ABI 3700 DNA sequencer (Perkin Elmer, Applied Biosystems, Foster City, CA).<sup>26</sup> HCV-RNA level was determined using Amplicor-M version 2 (Chugai-Roche Diagnostics, Tokyo, Japan).

### Polymerase chain reaction (PCR) and determination of sequences of ISDR

Complementary DNA (cDNA) was prepared by reverse transcription using an RNA-PCR kit (Takara Bio, Shiga,

Japan). In brief, 1  $\mu$ L of RNA solution, extracted from 100  $\mu$ L of serum and dissolved in 25  $\mu$ L of RNase-free distilled water, was mixed with 4  $\mu$ L of 1.5 mM MgCl<sub>2</sub> solution, 2  $\mu$ L of 10 $\times$  RNA-PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl), 8.5  $\mu$ L of RNase-free distilled H<sub>2</sub>O, 2  $\mu$ L of a dNTP mixture (10 mM dATP, dCTP, dGTP, dTTP), 1  $\mu$ L of random 9-mers (5'-NNNNNNNNN-3'), 0.5  $\mu$ L of RNase inhibitor (Takara Bio, Shiga, Japan) and 1  $\mu$ L of reverse transcriptase (Takara Bio, Shiga, Japan), was reverse transcribed at 42°C for 30 min.

The first round PCR was performed using the external primers (sense primer; nt 6824–6846; 5'-TCTCAG CTCCCTGCGATCCTGA-3' and antisense primer; nt 7155–7139; 5'-GATGGTATCGAAGGCTC-3') and 2.5 U of Ex Taq polymerase (Takara Bio, Shiga, Japan) with proofreading activity. The amplification conditions consisted of 94°C for 16 min followed by 40 cycles of 94°C for 1 min, 50°C for one minute and 72°C for one minute. One microliter of the first PCR product was used for the second PCR with internal primers (sense primer; nt 6950–6968; 5'-AGCTCTCA GCGAGC CAGCT-3', and antisense primer; nt 7104–7085; 5'-GATGGTATCGAAGGCTC-3') and 0.5  $\mu$ L of amplitaq gold (Roche Diagnostics, Branchburg, NJ). The amplification conditions of the second PCR were the same as those of the first PCR. The second PCR products were analyzed by 2% agarose gel electrophoresis, stained with ethidium bromide and visualized by UV transillumination.

Amplification products were purified on Wizard PCR Preps DNA purification resin (Promega, Madison, WI) and sequenced bidirectionally with the Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer, Applied Biosystems, Foster City, CA) using the above PCR primers. Sequencing was performed using an automated DNA sequencer ABI 377 (Perkin Elmer, Applied Biosystems, Foster City, CA).

### Histopathology

A liver biopsy was performed on each patient within six months before the start of therapy. The histopathological findings were assessed by grading inflammatory activity and the staging of fibrosis using the classification of Desmet *et al.*<sup>27</sup> by an experienced pathologist who had no knowledge of the clinical data of the patients.

### Statistical analysis

The collected data were analyzed using the SPSS program, version 11.0J (SPSS, Chicago, IL). The distributions of continuous variables were analyzed using the

Table 1 Clinical background of patients

	Genotype of HCV			Difference P (2a vs. 2b)
	2 (n = 50)	2a (n = 32)	2b (n = 18)	
Age (years)	49.2 ± 11.8	50.6 ± 10.1	46.6 ± 12.2	0.25
Male	30 (60%)	20 (63%)	10 (56%)	0.63
Viral load (KIU/mL)	491.6 ± 286.2	420.3 ± 264.8	618.2 ± 279.0	0.02
Histopathology				
Grade (0/1/2/3)	0/29/17/2	0/16/13/1	0/13/4/1	0.34
Stage (0/1/2/3/4)	1/23/14/9/1	1/10/10/8/1	0/13/4/1/0	0.02
SVR	41 (82%)	27 (84%)	14 (78%)	0.15

SVR, sustained virological response.

Mann-Whitney *U*-test. Differences in proportions were tested using Fisher's exact test. Independent factors that may influence the response to combination therapy were identified using stepwise multiple logistic regression analysis. Variables with  $P < 0.1$  at univariate analysis were retained for the multivariate logistic regression analysis. The significance of correlation was evaluated by Spearman's rank analysis. A two-tailed  $P$ -value of  $< 0.05$  was considered to indicate statistical significance.

## RESULTS

### Baseline characteristics of treated patients

TABLE 1 SHOWS the clinical background of the treated patients with genotype 2 HCV infection. The patients comprised 30 men and 20 women with a mean age of  $49.2 \pm 11.8$  years. The patients with genotype 2a have lower viral loads and more severe fibrosis than those with genotype 2b HCV infection. The rate of SVR was 84% (27 of 32) in the patients with genotype 2a and 78% (14 of 18) in those with genotype 2b.

### Amino acid sequence of ISDR

The amino acid sequence of ISDR was determined in 29 of the 32 patients with genotype 2a and 17 of the 18

patients with genotype 2b. The number of amino acid substitutions in ISDR was positively correlated with viral load (Spearman's rank correlation coefficient  $r = -0.53$ ,  $P < 0.001$ ). Figure 1 shows the amino acid sequences of ISDR. The prototype sequences of genotype 2a (D10749)<sup>28</sup> and 2b (D10988)<sup>29</sup> were determined to be the reference sequence for genotype 2a and 2b, respectively. The rate of SVR in the patients with no amino acid substitutions (wild type) in their ISDR sequence was 57% (8/14). In the patients with one to three amino acid substitutions (intermediate) and four or more substitutions (mutant) in their ISDR sequences, the rates of SVR were 85% (22/26) and 100% (8/8), respectively. In the patients with genotype 2a HCV infection, the rates of SVR in the wild, intermediate and mutant type ISDR were 63% (5/8), 80% (12/15) and 100% (8/8), respectively. In genotype 2b HCV infection, the rate of SVR in wild and intermediate type ISDR was 50% (3/6) and 91% (10/11), respectively.

### Predictors of response

The characteristics of patients with SVR and those without were compared (Table 2). By univariate analysis, time of viral clearance from blood ( $P = 0.018$ ) and

Table 2 Univariate logistic regression analysis for factors responsible for sustained virological response

	SVR	non-SVR	Univariate analysis <i>P</i>	Odds ratio
Age	51 (22-68)	52 (28-63)	0.805	0.992
Gender	21:17	7:2	0.195	0.329
Genotype (2a vs. 2b)	25:13	5:4	0.561	1.636
Histology of liver				
Grading (0/1/2/3)	0/21/16/2	0/8/1/0	0.086	6.438
Staging (0/1/2/3/4)	1/18/12/7/1	0/5/2/2/0	0.897	1.058
Pretreatment viral load (KIU/mL)	430 (8.7->850)	710 (480->850)	0.323	0.999
Time of viral clearance from blood (days)	14 (7-70)	52 (28-63)	0.018	0.649
Number of substituted amino acids in ISDR	1 (0-1)	0 (0-2)	0.048	3.716

SVR, sustained virological response.



Case No.		Number of substituted amino acids	Category (type)	Outcome
D10749	2219 PSLRATCTTHGKAYDVMVDANLFMGGDVTRIESES 2248	0		
2a-1		0	wild	ETR
2a-2		0	wild	ETR
2a-3		0	wild	ETR
2a-4		0	wild	SVR
2a-5		0	wild	SVR
2a-6		0	wild	SVR
2a-7		0	wild	SVR
2a-8		0	wild	SVR
2a-9		1	intermediate	ETR
2a-10	T	1	intermediate	SVR
2a-11	T	1	intermediate	SVR
2a-12	T	1	intermediate	SVR
2a-13	T	1	intermediate	SVR
2a-14	T	1	intermediate	SVR
2a-15	T	1	intermediate	SVR
2a-16		1	intermediate	SVR
2a-17		1	intermediate	SVR
2a-18	A M R	2	intermediate	SVR
2a-19	N V S	2	intermediate	SVR
2a-20	T E S	2	intermediate	SVR
2a-21	T E S	2	intermediate	SVR
2a-22	A N T S	3	intermediate	SVR
2a-23	S T S	3	intermediate	SVR
2a-24	S V S DY	4	mutant	SVR
2a-25	A L G-I	4	mutant	SVR
2a-26	YCR S	4	mutant	SVR
2a-27	YCR S	4	mutant	SVR
2a-28	YCR S	4	mutant	SVR
2a-29	YCR S	4	mutant	SVR
2a-30	A F R E K	5	mutant	SVR
2a-31	A ER V-LK-SG-I	9	mutant	SVR

Figure 1 Figures 1a and 1b show patients with genotypes 2a and 2b, respectively. The rate of sustained virological response (SVR) in patients with no amino acid substitutions in interferon sensitivity determining region (ISDR) sequence (wild type) was 57% (8/14). In patients with one to three amino acid substitutions (intermediate) and four or more substitutions (mutant) in the ISDR sequences, the rates of SVR were 85% (22/26) and 100% (8/8), respectively. ETR, end of treatment for virological response.

Case No.		Number of substituted amino acids	Category (type)	Outcome
D10988	2213 PSLKATCTTHKMAAYDCMVDANLFMGGDVTRIESES 2248	0		
2b-1		0	wild	ETR
2b-2		0	wild	ETR
2b-3		0	wild	SVR
2b-4		0	wild	SVR
2b-5		0	wild	SVR
2b-6		0	wild	SVR
2b-7	L	1	intermediate	SVR
2b-8	N	1	intermediate	SVR
2b-9	N	1	intermediate	SVR
2b-10	N	1	intermediate	SVR
2b-11	S	1	intermediate	SVR
2b-12	S	1	intermediate	SVR
2b-13	R T	2	intermediate	ETR
2b-14	T T	2	intermediate	SVR
2b-15	T E	2	intermediate	SVR
2b-16	T I	2	intermediate	SVR
2b-17	G V N	3	intermediate	SVR

amino acid mutations in the ISDR ( $P=0.048$ ) were found to be significantly linked to SVR. Because these variables were mutually correlated, multivariate analysis including histological grading was performed. In the final step, amino acid mutations in the ISDR (odds ratio [OR], 4.280; 95% confidence interval [CI], 1.139-16.038;  $P=0.031$ ) entered the model and could not be removed (Table 3). Therefore, amino acid mutations in ISDR are the only factor associated with SVR.

## DISCUSSION

IN JAPAN, THE combination therapy of IFN and ribavirin for 24 weeks was approved in late 2001. It was shown that approximately 20% of patients infected with genotype 1b HCV with a high viral load attained SVR with this regimen.<sup>30</sup> Compared to those with genotype 1, patients with genotype 2 or 3 HCV infection are expected to achieve higher SVR rates.<sup>12-14</sup> However,

**Table 3** Multivariate logistic regression analysis for factors responsible for sustained virological response

	SVR	non-SVR	Multivariate analysis <i>P</i>	Odds ratio
Grading (0/1/2/3)	0/21/16/2	0/8/1/0	0.547	2.141 (0.180–25.463)
Time of viral clearance from blood (days)	14 (7–70)	52 (28–63)	0.091	0.552 (0.277–1.100)
Number of substituted amino acids in ISDR	1 (0–1)	0 (0–2)	0.031	4.280 (1.139–16.038)

ISDR, interferon sensitivity determining region; SVR, sustained virological response.

information on individual genotypes, in particular genotype 2, is quite limited,<sup>31</sup> which prompted us to conduct this study.

In this study the SVR rate of patients with genotype 2 was 82%, which is lower than that found in a previous report by Zeuzem *et al.*<sup>31</sup> According to the data of previous studies,<sup>32,33</sup> a high SVR rate may be expected in genotype 2 or 3 even if the treatment period is 24 weeks. One possible reason for the low SVR rate in this study is the use of conventional IFN- $\alpha$ . Pegylated IFN- $\alpha$  is superior to conventional IFN- $\alpha$  for inducing sustained viral clearance.<sup>33,34</sup> Another possible reason is ethnicity, because response to IFN-ribavirin combination therapy varies among races.<sup>35,36</sup>

The number of mutations in the ISDR of NS5A is variable and influences the efficacy of IFN-ribavirin combination therapy. Studies from Japan and Europe showed that the number of amino acid substitutions in ISDR influences the efficacy of IFN monotherapy in genotype 1 infection.<sup>16–20</sup> The efficacy of IFN-ribavirin combination therapy in genotype 1 infection is also influenced by the amino acid sequence of ISDR.<sup>37</sup> In genotype 2 infection, the amino acid sequence of ISDR has been reported to also correlate with the SVR to IFN monotherapy.<sup>22–24</sup> Our results suggest that the amino acid sequence of ISDR may also influence the efficacy of combination therapy in genotype 2 infection.

It is interesting that mutations in ISDR confer susceptibility to IFN-ribavirin combination therapy. It was reported that NS5A suppresses PKR protein kinase, a mediator of IFN-induced antiviral resistance<sup>38</sup> in genotype 1 infection. Multiple ISDR mutations probably abrogate this action of NS5A to inhibit PKR.<sup>39</sup> However, whether the mechanisms are also applicable to genotype 2 infection is still unclear and needs clarification.

Our study showed that about 20% of the patients with genotype 2 HCV infection were not cured by the combination therapy for 24 weeks. However, all of the uncured patients were relapsers, whose viral loads were cleared from the serum at the end of treatment. Therefore, it can be expected that these patients may be cured by a longer treatment, which should be studied further.

Figure 1a showed that cases 26, 27, 28 and 29, with no common infectious source, had the same mutations. Most of previous reported cases with mutant-type strains of ISDR had different amino acid sequences, which seems contradictory to our results.<sup>22–24</sup> However, one study showed that two of the four cases shared one mutant type sequence of ISDR.<sup>23</sup> These results imply that some viral strains with mutant type ISDR sequence are likely to be selected, which await further study.

To conclude, IFN-ribavirin combination therapy for 24 weeks cured 80% of the patients with genotype 2 HCV. Amino acid mutations in ISDR may determine the final outcome of the combination therapy.

#### ACKNOWLEDGMENTS

WE THANK MS Mie Kanke for her excellent technical assistance.

#### REFERENCES

- 1 Tong MJ, el-Farra NS, Reikes AR, Co RL. Clinical outcomes after transfusion-associated hepatitis C. *N Engl J Med* 1995; **332**: 1463–6.
- 2 Takahashi M, Yamada G, Miyamoto R, Doi T, Endo H, Tsuji T. Natural course of chronic hepatitis C. *Am J Gastroenterol* 1993; **88**: 240–3.
- 3 Yano M, Kumada H, Kage M *et al.* The long-term pathological evolution of chronic hepatitis C. *Hepatology* 1996; **23**: 1334–40.
- 4 Kiyosawa K, Umemura T, Ichijo T *et al.* Hepatocellular carcinoma: recent trends in Japan. *Gastroenterology* 2004; **127**: S17–26.
- 5 Iino S. Natural history of hepatitis B and C virus infections. *Oncology* 2002; **62** (Suppl 1): 18–23.
- 6 Hu KQ, Tong MJ. The long-term outcomes of patients with compensated hepatitis C virus-related cirrhosis and history of parenteral exposure in the United States. *Hepatology* 1999; **29**: 1311–16.
- 7 Camma C, Giunta M, Pinzello G, Morabito A, Verderio P, Pagliaro L. Chronic hepatitis C and interferon alpha: conventional and cumulative meta-analyses of randomized controlled trials. *Am J Gastroenterol* 1999; **94**: 581–95.

- 8 Poynard T, Leroy V, Cohard M *et al.* Meta-analysis of interferon randomized trials in the treatment of viral hepatitis C: effects of dose and duration. *Hepatology* 1996; 24: 778-89.
- 9 Niederau C, Heintges T, Haussinger D. Treatment of chronic hepatitis C with  $\alpha$ -interferon: an analysis of the literature. *Hepatogastroenterology* 1996; 43: 1544-56.
- 10 Lai MY, Kao JH, Yang PM *et al.* Long-term efficacy of ribavirin plus interferon alfa in the treatment of chronic hepatitis C. *Gastroenterology* 1996; 111: 1307-12.
- 11 Reichard O, Norkrans G, Fryden A, Braconier JH, Sonnerborg A, Weiland O. Randomised, double-blind, placebo-controlled trial of interferon alpha-2b with and without ribavirin for chronic hepatitis C. The Swedish Study Group. *Lancet* 1998; 351: 83-7.
- 12 Poynard T, Marcellin P, Lee SS *et al.* Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). *Lancet* 1998; 352: 1426-32.
- 13 McHutchison JG, Gordon SC, Schiff ER *et al.* Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; 339: 1485-92.
- 14 Davis GL, Esteban-Mur R, Rustgi V *et al.* Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. International Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; 339: 1493-9.
- 15 Zeuzem S. Heterogeneous virologic response rates to interferon-based therapy in patients with chronic hepatitis C: who responds less well? *Ann Intern Med* 2004; 140: 370-81.
- 16 Enomoto N, Sakuma I, Asahina Y *et al.* Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 1996; 334: 77-81.
- 17 Kurosaki M, Enomoto N, Murakami T *et al.* Analysis of genotypes and amino acid residues 2209-2248 of the NS5A region of hepatitis C virus in relation to the response to interferon-beta therapy. *Hepatology* 1997; 25: 750-3.
- 18 Saiz JC, Lopez-Labrador FX, Ampurdanes S *et al.* The prognostic relevance of the nonstructural 5A gene interferon sensitivity determining region is different in infections with genotype 1b and 3a isolates of hepatitis C virus. *J Infect Dis* 1998; 177: 839-47.
- 19 Chayama K, Tsubota A, Kobayashi M *et al.* Pretreatment virus load and multiple amino acid substitutions in the interferon sensitivity-determining region predict the outcome of interferon treatment in patients with chronic genotype 1b hepatitis C virus infection. *Hepatology* 1997; 25: 745-9.
- 20 Yoshioka K, Kobayashi M, Orito E *et al.* Biochemical response to interferon therapy correlates with interferon sensitivity-determining region in hepatitis C virus genotype 1b infection. *J Viral Hepat* 2001; 8: 421-9.
- 21 Schinkel J, Spaan WJ, Kroes AC. Meta-analysis of mutations in the NS5A gene and hepatitis C virus resistance to interferon therapy: uniting discordant conclusions. *Antivir Ther* 2004; 9: 275-86.
- 22 Murakami T, Enomoto N, Kurosaki M, Izumi N, Marumo F, Sato C. Mutations in nonstructural protein 5A gene and response to interferon in hepatitis C virus genotype 2 infection. *Hepatology* 1999; 30: 1045-53.
- 23 Kobayashi M, Watanabe K, Ishigami M *et al.* Amino acid substitutions in the nonstructural region 5A of hepatitis C virus genotypes 2a and 2b and its relation to viral load and response to interferon. *Am J Gastroenterol* 2002; 97: 988-98.
- 24 Akuta N, Suzuki F, Tsubota A *et al.* Association of amino acid substitution pattern in nonstructural protein 5A of hepatitis C virus genotype 2a low viral load and response to interferon monotherapy. *J Med Virol* 2003; 69: 376-83.
- 25 Lee SC, Antony A, Lee N *et al.* Improved version 2.0 qualitative and quantitative AMPLICOR reverse transcription-PCR tests for hepatitis C virus RNA: calibration to international units, enhanced genotype reactivity, and performance characteristics. *J Clin Microbiol* 2000; 38: 4171-9.
- 26 Mukaide M, Tanaka Y, Kakuda H *et al.* New combination test for hepatitis C virus genotype and viral load determination using Amplicor GT HCV MONITOR test v2.0. *World J Gastroenterol* 2005; 11: 469-75.
- 27 Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* 1994; 19: 1513-20.
- 28 Okamoto H, Okada S, Sugiyama Y *et al.* Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier: comparison with reported isolates for conserved and divergent regions. *J Gen Virol* 1991; 72: 2697-704.
- 29 Okamoto H, Kuri K, Okada S *et al.* Full-length sequence of a hepatitis C virus genome having poor homology to reported isolates: comparative study of four distinct genotypes. *Virology* 1992; 188: 331-41.
- 30 Tsubota A, Arase Y, Suzuki F *et al.* High-dose interferon alpha-2b induction therapy in combination with ribavirin for Japanese patients infected with hepatitis C virus genotype 1b with a high baseline viral load. *J Gastroenterol* 2004; 39: 155-61.
- 31 Zeuzem S, Hultcrantz R, Bourliere M *et al.* Peginterferon alfa-2b plus ribavirin for treatment of chronic hepatitis C in previously untreated patients infected with HCV genotypes 2 or 3. *J Hepatol* 2004; 40: 993-9.
- 32 Cornberg M, Huppe D, Wiegand J *et al.* Treatment of chronic hepatitis C with PEG-interferon alpha-2b and ribavirin: 24 weeks of therapy are sufficient for HCV genotype 2 and 3. *Z Gastroenterol* 2003; 41: 517-22.
- 33 Manns MP, McHutchison JG, Gordon SC *et al.* Peginterferon alfa-2b plus ribavirin compared with interferon

- alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; 358: 958-65.
- 34 Lee SD, Yu ML, Cheng PN *et al.* Comparison of a 6-month course peginterferon alpha-2b plus ribavirin and interferon alpha-2b plus ribavirin in treating Chinese patients with chronic hepatitis C in Taiwan. *J Viral Hepat* 2005; 12: 283-91.
- 35 McHutchison JG, Poynard T, Pianko S *et al.* The impact of interferon plus ribavirin on response to therapy in black patients with chronic hepatitis C. The International Hepatitis Interventional Therapy Group. *Gastroenterology* 2000; 119: 1317-23.
- 36 Hepburn MJ, Hepburn LM, Cantu NS, Lapeer MG, Lawitz EI. Differences in treatment outcome for hepatitis C among ethnic groups. *Am J Med* 2004; 117: 163-8.
- 37 Hung CH, Lee CM, Lu SN *et al.* Mutations in the NS5A and E2-PePHD region of hepatitis C virus type 1b and correlation with the response to combination therapy with interferon and ribavirin. *J Viral Hepat* 2003; 10: 87-94.
- 38 Gale MJ Jr, Korth MJ, Tang NM *et al.* Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. *Virology* 1997; 230: 217-27.
- 39 Noguchi T, Satoh S, Noshi T *et al.* Effects of mutation in hepatitis C virus nonstructural protein 5A on interferon resistance mediated by inhibition of PKR kinase activity in mammalian cells. *Microbiol Immunol* 2001; 45: 829-40.