

the SMAD mutant models have developed HCC, however. SMAD function is dependent upon adaptor proteins such as embryonic liver fodrin (ELF), a β -spectrin protein. ELF associates with SMAD3, SMAD 4, and the TGF- β receptor complex, and ultimately leads to their translocation to the nucleus. Mishra et al. report that ELF^{+/-} knockout mice develop steatosis and spontaneous HCC. Loss of ELF in these mice results in cell cycle disruption with significant increases in Cdk4, cyclin D1 and pRb hyperphosphorylation [157].

In addition to TGF- β , activated stellate cells produce a number of other profibrotic cytokines such as platelet derived growth factor (PDGF). Induction of PDGF receptor mRNA is one of the earliest events in stellate cell activation, and its over-expression has been linked to fibrosis [158]. Kanzler's group developed a model in which the PDGF-B ligand is inducibly over-expressed in the liver. They found that PDGF-B expression caused hepatic stellate cell activation and collagen deposition [159]. Campbell et al. have described a PDGF-C transgenic model expressing human PDGF-C driven by the albumin promoter. These mice develop fibrosis and steatosis, and 80% develop HCC by 12 months of age [160]. Interestingly, no cirrhosis or regenerating nodules were observed in either of these models.

Interleukin-6 (IL-6) is the cytokine largely responsible for hepatic response to infections and inflammation. IL-6 serum concentrations are increased in patients with HBV and HCV infections and with HCC [161]. Naugler et al. induced liver disease with DEN in IL-6 knockout (IL-6^{-/-}) mice to determine whether gender bias in IL-6 production accounts for the sex difference seen in HCC development in both humans and in rodent models [162]. The carcinogenic effects of DEN were suppressed in IL-6^{-/-} male mice: <10% developed HCC by 8 months of age, compared to 100% in wild-type male mice. No difference was seen in IL-6^{-/-} versus WT female mice. Estrogens inhibit IL-6 promoter activity by decreasing activity of the transcription factors NF- κ B and C/EBP β , a process dependent on IKK β and toll-like receptor (TLR) adaptor Myd-88. In the same study, Myd-88 was found to be required for IL-6 induction by necrotic hepatocyte debris, and Myd-88 knockout (Myd-88^{-/-}) male mice developed fewer and smaller HCCs in response to injury by DEN than did WT male mice. The results of this experiment provide a potential explanation for the gender differences in the incidence of liver cancer, which ranges between 2:1 and 4:1 male to female ratio [163].

Alpha-1-antitrypsin (AAT)-deficient transgenic mice express the transport-impaired Z variant of the human disease. These mice accumulate AAT and form foci of hyperplasia surrounded by inflammatory infiltrates [41], developing hepatitis, adenomas after 12 months, and HCC after 16–20 months [164].

The Mdr-2 gene encodes a protein involved in transport of phosphatidylcholine into the bile. Mdr-2 knock-

out mice accumulate toxic bile salts in their intrahepatic biliary system, which causes a non-suppurative inflammatory cholangitis and ductular proliferation and eventually nodules and HCC at 6–12 months [165,166]. A similar pathogenesis occurs in acyl-CoA oxidase (AOX) knockout mice, which develop steatohepatitis followed by a complete liver regeneration; this sequence of inflammation followed by proliferation results in the formation of HCCs by the age of 15 months [167].

4. Integrating functional genomics in HCC: from mice to humans

The progression from dysplastic foci to HCC involves the accumulation of genetic changes which can be monitored with cytogenetic studies that show karyotypic alterations in various chromosomes [137]. This type of chromosomal gains and losses are particularly numerous in lesions from rodents subjected to the carcinogen initiator-promoter protocol, or in SV40/T antigen transgenic mice. Various genes involved in hepatocarcinogenesis such as c-H-ras, met, HGF, myc, and p53 are located on rat chromosomes exhibiting frequent aberrations [41].

Thorgeirsson et al. applied a genome-wide microarray analysis to three transgenic mouse models of HCC, and found that although gene expression profiles in tumors derived from the three transgenic lines were highly similar, it was possible to identify oncogene-specific gene expression signatures at an early dysplastic stage of hepatocarcinogenesis [168]. In a related study, gene expression patterns of HCC tumors from seven different mouse models and 91 human HCCs from predefined subclasses were measured to compare the molecular features of mouse and human HCCs [90]. The authors found that gene expression patterns in tumors from Myc, E2f1 and Myc/E2f1 transgenic mice were similar to those of the better survival group of human HCC, whereas the expression patterns in HCCs from Myc/Tgf α transgenic mice and from DEN-treated mice were most similar to those of the poorer survival group of human HCC. Gene expression patterns in HCC from Acox1^{-/-} mice and in ciprofibrate-induced HCCs were least similar to those observed in human HCCs. This study supports the notion that comparison of gene expression between the two species can be used to identify the mouse models of HCC that most closely mimic the tumors in humans.

5. Conclusion

We have described both traditional models of carcinogenesis in which the expression of oncogenes and tumor suppressor genes is genetically altered to produce

HCC, and other models in which tumor formation is dependent on inflammation. The natural history of HCC development in humans, combined with the evidence that genetic mutations alone sometimes do not generate tumors unless initiated by a proinflammatory agent, underscore the need to develop new models in which HCCs develop spontaneously in an environment of fibrosis, in order to best recapitulate the human disease process. In addition, integrative functional genomic studies have suggested that human HCCs can be classified into subgroups based on molecular pathway activation. Comparison of gene expression between mouse models and human HCC may allow us to create mouse models in future which recapitulate the various subgroups, which would make ideal models for preclinical studies.

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METABOLISM, CANCER AND GENETICS

Molecular basis for the synergy between alcohol and hepatitis C virus in hepatocarcinogenesis

Kazuhiro Koike, Takeya Tsutsumi, Hideyuki Miyoshi, Seiko Shinzawa, Yoshizumi Shintani, Hajime Fujie, Hiroshi Yotsuyanagi and Kyoji Moriya

Department of Internal Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan



Kazuhiro Koike

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Correspondence

Professor Kazuhiro Koike, Department of Infectious Diseases, Internal Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Email: kkoike-ty@umin.ac.jp

Introduction

Hepatitis C virus (HCV) infects approximately 170 million people persistently worldwide, and induces a spectrum of chronic liver diseases, from chronic hepatitis, cirrhosis to hepatocellular carcinoma (HCC).¹ HCV has been given increasing attention because of its wide and deep penetration in the community, coupled with a very high incidence of HCC in persistent HCV infection. It impacts the medical, sociological and economic domains of society. Once liver cirrhosis is established in hosts infected with HCV, HCC develops at a yearly rate of 5–7%,² resulting in the development of HCC in nearly 90% of HCV-associated cirrhosis patients in 15 years. In addition, the outstanding features in the mode of hepatocarcinogenesis in HCV infection (i.e. development of HCC in a multicentric fashion and a very high incidence), are not common in other

Abstract

Overwhelming lines of epidemiological evidence have indicated that persistent infection with hepatitis C virus (HCV) is a major risk for the development of hepatocellular carcinoma (HCC). In addition, heavy alcohol use has been linked with earlier progression to HCC in chronic hepatitis C patients. However, in the pathogenesis of HCV-associated HCC, it still remains controversial as to whether the virus plays a direct or an indirect role, and as to how alcohol operates in the acceleration of HCC development. Several studies using transgenic mouse models, in which the core protein of HCV has an oncogenic potential, indicate that HCV is directly involved in hepatocarcinogenesis, although other factors such as continuous inflammation or environmental factors seem also to play a role. The downstream events of the HCV core protein expression in the transgenic mouse HCC model are segregated into two pathways. One is the augmented production of oxidative stress in the absence of inflammation along with the attenuation of some scavenging systems in the putative preneoplastic stage with steatosis in the liver. The other pathway is the alteration in cellular gene expression and intracellular signaling, including the mitogen-activated protein kinase cascade. The combination of these pathways would explain the unusually high incidence and multicentric nature of HCC development in HCV infection. In addition, alcohol feeding in this animal model further activated the two pathways synergistically with HCV, leading to an earlier development of HCC. Such a synergy would reveal the molecular basis for the acceleration of HCC development by alcohol in HCV infection.

malignancies except for hereditary cancers such as familial polyposis of the colon. Knowledge of the mechanism underlying HCC development in persistent HCV infection is therefore imminently required for the prevention of HCC.

However, alcohol has been known as an accelerating factor in the development of HCC in persistent HCV infection.^{3–5} The pattern of the risk for HCC due to alcohol intake shows a continuous dose-effect curve without a definite threshold, although most studies have found that HCC risk increased only for alcohol consumption above 40–60 g of ethanol per day. Some evidence supports a positive interaction of alcohol intake, probably with HCV infection and possibly with HBV infection.³ Synergistic interactions on the additive model were observed between heavy alcohol consumption and chronic hepatitis virus infection and diabetes mellitus.⁴ However, it is unclear how alcohol causes the acceleration of HCC development in HCV infection.

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.^{1,6,7} 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.^{8,9} 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.⁹ The envelope gene transgenic mice do not develop HCC, despite high expression levels of both E1 and E2 proteins.^{10,11} 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.¹² 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

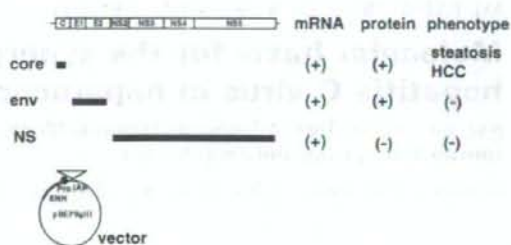


Figure 1 Transgenic mouse lines carrying the hepatitis C virus (HCV) genome. Three different types of transgenic mouse lines, carrying the core gene, envelope genes or non-structural genes of HCV, respectively, were established under the control of the same regulatory elements. Among these mouse strains, 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.^{13–15} 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.^{9,16} 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.^{17,18} 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 underlying-

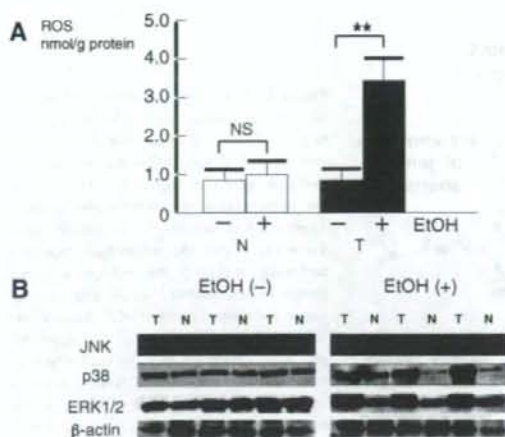


Figure 2 Alcohol administration enhances oxidative stress production and mitogen-activated protein kinase (MAPK) pathway activation in a synergistic fashion with hepatitis C virus (HCV) core protein. Administration of 5% alcohol for 3 weeks provoked an induction of reactive oxygen species (ROS) in HCV 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.^{16,17} The dysfunction of the electron transfer system of the mitochondrion is suggested in association with the presence of the HCV core protein.¹⁹ Hepatic steatosis in hepatitis C, which is also attributed to the action of the core protein,⁸ may work as fuel for oxidative stress overproduction.^{18,20,21}

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)- α and interleukin-1 β have been found transcriptionally activated.²² The core protein has also been found to interact with some cellular proteins, such as retinoid X receptor (RXR)- α , that play pivotal roles in cell proliferation and lipid metabolism.²³ 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.^{22,24} 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 28y (PA28y), is indispensable for the core protein to exert its function for the development of steatosis, insulin resistance and HCC.^{25,26}

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).¹⁷ 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.²⁴

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- α or suppressor of cytokine signaling-1, and the

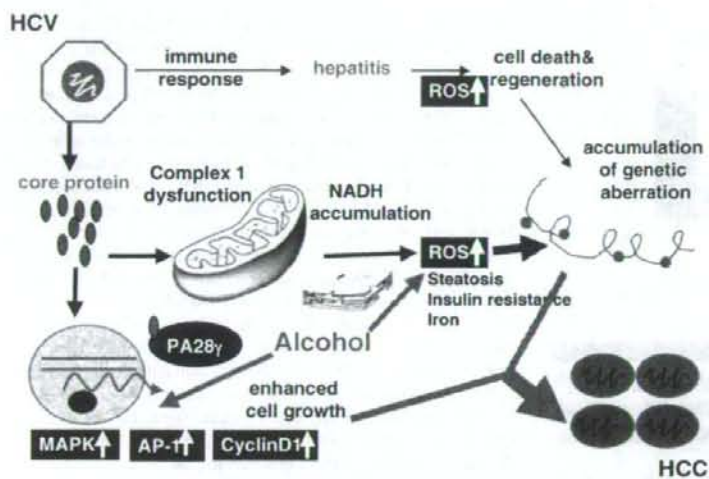


Figure 3 Molecular pathogenesis of hepatocellular carcinoma (HCC) development in hepatitis C virus (HCV) infection in association with alcohol. We postulate that induction of oxidative stress through the dysfunction in the mitochondrial electron transfer system, together with alterations in cellular 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 γ , proteasome activator 28 γ ; ROS, reactive oxygen species; SOCS-1, suppressor of cytokine signaling-1; TNF- α , tumor necrosis factor- α .

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.

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Original Article

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

Nobukazu Ishizaka,¹ Yuko Ishizaka,² George Seki,³ Ryozi Nagai,¹ Minoru Yamakado² and Kazuhiko Koike⁴Departments of ¹Cardiovascular Medicine, ⁴Infectious Diseases and ³Nephrology, University of Tokyo Graduate School of Medicine, and ²Center 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);¹ HBV infection has been reported to be found in 0.8% and HCV infection in 0.5% of Japanese workers.² 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.³ 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.⁴ HBV infection may also be associated with MGN and MPGN,^{5,6} and about 3% of HBV-infected patients were reported to have glomerulonephritis.⁷

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-ty@umin.ac.jp

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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.⁸ 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: $HOMA-IR = (\text{fasting immunoreactive insulin } [\mu\text{U/mL}] \times \text{fasting plasma glucose } [\text{mg/dL}]) / 405$. 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),⁹ as follows: estimated GFR (eGFR; mL/min/1.73 m²) = 186.3 × (serum creatinine)^{-1.154} × (age)^{-0.203} × 0.881 × 0.742 (if female). In this MDRD formula, 0.881 is a coefficient for eGFR specific to the Japanese population.¹⁰ For the diagnosis of albuminuria, spot urine samples were collected and expressed as urine albumin excretion ratio (UAER), which was expressed per g-creatinine. CKD was diagnosed when individuals had an eGFR of <60 mL/min/1.73 m², designated as low eGFR, and/or UAER of ≥30 mg/g, designated as albuminuria.¹¹

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),¹² with body mass index (BMI) used as a surrogate for waist circumference.¹³ Metabolic syndrome was said to be present when three or more of the following conditions were met: TG levels ≥150 mg/d; HDL-C levels <40 mg/dL (men), <50 mg/dL (women); FPG levels ≥110 mg/dL or taking antidiabetic medication; systolic blood pressure (SBP) ≥130 mmHg or diastolic blood pressure (DBP) ≥85 mmHg or taking an antihypertensive medication; BMI ≥25 kg/m².

Statistical analysis

The data in this study were analyzed by one-way ANOVA with Bonferroni post hoc test, χ^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 ± 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 ²	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 ³ cells/μL	5.3 ± 1.4	5.0 ± 1.2	5.0 ± 1.7	0.025
RBC count, ×10 ⁴ /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 ⁴ /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 creatine, mg/dL	0.78 ± 0.26	0.78 ± 0.14	0.81 ± 0.28	0.65
eGFR, mL/min/1.73m ²	70 ± 10	70 ± 9	67 ± 13	0.087
Low eGFR, n (%)	1887 (15)	13 (10)	22 (31)	<0.001
UAE, 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; γGTP, gamma-glutamyltransferase; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment-insulin resistance; U/AER, 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, 524×10^4 (cells/microL); Plt 25.4×10^4 (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

	CKD Odds ratio (95% CI)	P-value	Dependent variables			
			Low eGFR		Albuminuria	
			Odds ratio (95% CI)	P-value	Odds ratio (95% CI)	P-value
Unadjusted						
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
Adjusted for age and sex						
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
Adjusted for age, sex, SBP and FPG						
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.^{14,15} 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 ≥ 300 mg/g (macroalbuminuria). The median (interquartile range) of eGFR (mL/min/1.73 m²) 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 χ^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
HCCaAg 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
HCCaAg 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/HCCaAg 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 ± 3.1 and 1.4 ± 1.0 , respectively ($P < 0.001$). Age and sex-adjusted logistic regression analysis showed that HCCaAg 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 HCCaAg 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 HCCaAg

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 HCCaAg 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 HCCaAg 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 HCCaAg 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 HCCaAg 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

	CKD Odds ratio (95% CI)	P-value	Dependent variables			
			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

	CKD Odds ratio (95% CI)	P-value	Dependent variables			
			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 (0.20-1.53)	<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,¹⁶ 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.¹⁷ 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.⁸ 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.^{18,19} We have shown previously that HCV infection induces insulin resistance by the virus itself, which may influence the progression of chronic liver disease.^{20,21} Compared to HCV infection, the relationship between HBsAg and insulin resistance has been less extensively studied. Castro *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.²² 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.²³ 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.²³ 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,²⁴ and albuminuria is one of the diagnostic components of metabolic syndrome in WHO criteria.¹² 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.^{25,26} 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 m² in Japanese.¹⁰ Second, we could not assess data of anti-HBe positivity, which might affect the prevalence of extrahepatic manifestations in HBV infection.⁷ 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,²⁷ 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.²⁸⁻³⁰ 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

IN 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.

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