University, Graduate School of Medicine. Baseline characteristics are listed in Table 1. Sixty-seven and 30 patients were treatment-naïve and previously treated with interferon therapy, respectively. Previous relapse was defined as undetectable HCV RNA by the end of therapy [2], but then its reappearance after the end of therapy, and the definition of null response was less than 2 $\log_{10}$ decrease in HCV RNA from baseline after 12 weeks of therapy [2]. In 17 relapsers, 7, 3, 2, 2 and 3 received standard interferon monotherapy, standard interferon plus ribavirin, peginterferon monotherapy, peginterferon plus ribavirin and unknown, respectively. In 13 null-responders, 10, 1 and 2 received standard interferon monotherapy, standard interferon plus ribavirin and peginterferon plus ribavirin, respectively. Most patients were infected with HCV genotype 1 (83.5%) with high viral load (>5 log IU/mL) (97.9%). Ultrasound (US) findings showed cirrhosis of the liver in 12 cases (Table 1), 3 of which were also biopsy-proven.

3.2. Treatment

All 97 patients were treated with peginterferon-alfa once weekly and 400–1,000 mg of ribavirin daily [19–21]. Some of them stopped treatment at 12–16 weeks according to the early stopping rule.

3.3. HCV RNA Quantification

HCV RNA was determined by Amplicor HCV monitor assay, version 2.0 (range: 0.5–850 KIU/mL) (Roche Diagnostics, Tokyo, Japan), Amplicor HCV assay (Roche) or COBAS TaqMan HCV test (Roche) (range: 1.2–7.8 log IU/mL). The detection limit of this qualitative assay was 50 IU/mL, corresponding to 1.7 log IU/mL by COBAS TaqMan PCR assay [19]. We defined HCV RNA >5 log IU/mL and <5 log IU/mL as high and low viral titers of HCV RNA, respectively.

3.4. HCV Genotyping

HCV genotype was determined using the antibody-serotyping assay of Tsukiyama-Kohara *et al.* [22]. In this assay, HCV serotypes 1 and 2 correspond to genotypes 1a/1b and 2a/2b, respectively, according to Simmonds' classification [23].

3.5. Classification of Treatment Outcome

Patients were classified as having achieved RVR and early virological response (EVR) if HCV RNA was undetectable (<50 IU/mL) in serum at treatment week 4 and week 12, respectively, and as having SVR if HCV RNA was undetectable in serum 24 weeks after the completion of therapy.

3.6. DNA Extraction and TaqMan SNP Assay

To prepare the DNA sample from blood cells, we used DNA Extract All Lysis Reagents (Applied Biosystems Inc., Foster City, CA, USA). A specific TaqMan genotyping assay was performed for rs1127354, rs6051702 and rs8099917. Primers were manufactured by Applied Biosystems. Thermal cycling was performed with the ABI Step One real-time PCR system according to the manufacturer's protocol. Activation of TaqMan GTXpress Master Mix (Applied Biosystems) and the initial denaturation cycle was at 95 °C for 20 seconds, followed by 40 cycles at 95 °C for 3 seconds and 60 °C

for 20 seconds. We analyzed IL28B rs8099917 TT as major type and TG/GG as minor type, ITPA rs1127354 CC as major type and CA/AA as minor type, and ITPA rs6051702 AA as major type and AC/CC as minor type in the present study.

3.7. Statistical Analysis

Data were expressed as mean ± standard deviation. We used univariate analyses to compare patient characteristics and outcomes, applying Student's t-test or Chi-square test as appropriate. $P < 0.05$ was considered statistically significant.

4. Discussion and Conclusion

In the present study, we also observed that IL28B rs8099917 major genotype was useful for the prediction of treatment response, as in previous studies [4–9], which reported the association between IL28B genotypes and HCV eradication with peginterferon plus ribavirin therapies in chronic hepatitis C patients. SVR was strongly associated with IL28B major genotype (rs8099917 TT). Serum γGTP levels were significantly higher in IL28B rs8099917 minor-type patients, as we reported previously [8].

Previous studies [21,24] showed that HCV-infected patients who can be maintained on >80% of peginterferon and ribavirin dosage for the duration of treatment exhibit enhanced SVR rates. Adherence to therapy decreased over time with both antiviral medications, but more so with ribavirin [25]. Ribavirin could be associated with clinically significant hemolytic anemia, resulting in its necessary dose reduction or discontinuation [26,27]. However, we did not observe any association between ITPA genotypes and SVR.

We also observed that ribavirin-induced anemia is highly dependent on the ITPA rs1127354 genotypes between days 0 and 84, and ITPA rs1127354 major type has been reported to be associated with a reduction in hemoglobin between weeks 0 and 4 [28,29]. In the present study, we observed a difference in age between ITPA rs1127354 major and minor types (Table 2), albeit with a rather limited number of the latter patients. In this respect, further study will be needed, although our previous study showed that the SVR rate of patients aged ≤65 years was similar to that of patients aged >65 years [21]. Genetic variation of ITPA causing an accumulation of inosine triphosphate (ITP) could result in ribavirin-induced anemia. ITP confers protection against ribavirin-induced adenosine triphosphate (ATP) reduction by substituting for erythrocyte GTP, which is depleted by ribavirin, in the biosynthesis of ATP [30]. It is possible that ribavirin-induced anemia is due primarily to the effect of the drug on GTP and consequently ATP levels in erythrocytes [30].

Interestingly, we found that IL28B rs8099917 minor genotype was associated with greater reductions of neutrophils and platelets, although it was reported that IL28B polymorphisms were not associated with interferon-related cytopenia [31]. Our data support the previous reports that patients with ITPA rs1127354 major type had a higher degree of reactive increase in platelet count [32,33]. Further studies will be needed to investigate the potential underlying mechanism and to examine whether there is a synergistic effect of IL28B and ITPA. In the not-too-distant future, HCV therapy will likely move away from interferon-based regimens with increasing numbers of potent antiviral agents being approved, meaning that IL28B and/or ITPA genotyping would not play any additional role and be useful in clinical practice [34–36].

Recent studies revealed that IL28B is associated with hepatic interferon-stimulated gene (ISG) expression [10], hepatic STAT1-nuclear localization [9], hepatic suppressor of cytokine signal 3 (SOCS3) [37] and plasma interferon-gamma inducible protein-10 (IP-10) levels in chronic HCV infection [8]. It is possible that IL28B genotypes affect virus-host interaction through the interaction with interferon signaling pathways. IL28B major type also reported to be associated with a lower prevalence of hepatic steatosis and a less pronounced lipid metabolism, as reflected both by serum lipoprotein levels and hepatic steatosis in HCV infection [38–41]. Insulin resistance is more common in IL28B minor genotype than in major type in treatment-naïve patients with chronic hepatitis C [42,43]. Although there are contrary opinions [44,45], IL28B genotypes influence the stage of liver fibrosis [46,47] and HCV-related hepatocarcinogenesis [48]. Thus, IL28B genotypes play important roles in not only eradication of HCV but also HCV-related pathology.

In HCV infection, patients who developed HCC had lower platelet counts [49]. It is well known that the platelet count decreased with stage advancement of liver diseases in chronic hepatitis C patients [2,49–52]. Chronic hepatitis C is associated with variable degrees of anemia, neutropenia, and/or thrombocytopenia [52]. Multiple factors, including ITPA genotypes, might be involved in this phenomenon.

Our study showed that about 60% of Japanese patients infected with HCV have the preferable allele of IL28B rs8099917, but about 70% of patients also have the undesirable allele of ITPA rs1127354. There seem different distributions between IL28B and ITPA genotypes in the world [6,11]. In conclusion, ITPA rs1127354 is useful for the prediction of ribavirin-induced anemia in the earlier phase of peginterferon plus ribavirin treatment, and IL28B rs8099917 is useful for the prediction of SVR. Use of a combination of these genotypes could lead to a safe and effective treatment for chronic hepatitis C patients.

Acknowledgments

We thank Y. Tanaka, Nagoya City Graduate School of Medical Sciences, Nagoya, Japan, for technical advice on the TaqMan SNP assay, M. Omata, University of Tokyo, Tokyo, Japan for valuable discussions and S. Hasegawa for her excellent assistance. This work was supported by grants for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (TK), and a grant from Chiba University Young Research-Oriented Faculty Member Development Program in Bioscience Areas (TK).

Conflict of Interest

The authors declare no conflict of interest.

References and Notes

1. Di Bisceglie, A.M. Hepatitis C and hepatocellular carcinoma. *Hepatology* **1997**, *26*, 34S–38S.
2. Kanda, T.; Imazeki, F.; Yokosuka, O. New antiviral therapies for chronic hepatitis C. *Hepatol. Int.* **2010**, *4*, 548–561.
3. Jensen, D. A new era of hepatitis C therapy begins. *N. Engl. J. Med.* **2011**, *364*, 1272–1274.

4. Suppiah, V.; Moldovan, M.; Ahlenstiel, G.; Berg, T.; Weltman, M.; Abate, M.L.; Bassendine, M.; Spengler, U.; Dore, G.J.; Powell, E.; *et al.* IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat. Genet.* **2009**, *41*, 1100–1104.
5. Tanaka, Y.; Nishida, N.; Sugiyama, M.; Kurosaki, M.; Matsuura, K.; Sakamoto, N.; Nakagawa, M.; Korenaga, M.; Hino, K.; Hige, S.; *et al.* Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat. Genet.* **2009**, *41*, 1105–1109.
6. Ge, D.; Fellay, J.; Thompson, A.J.; Simon, J.S.; Shianna, K.V.; Urban, T.J.; Heinzen, E.L.; Qiu, P.; Bertelsen, A.H.; Muir, A.J.; *et al.* Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* **2009**, *461*, 399–401.
7. Nakamoto, S.; Kanda, T.; Imazeki, F.; Wu, S.; Arai, M.; Fujiwara, K.; Yokosuka, O. Simple assay based on restriction fragment length polymorphism associated with IL28B in chronic hepatitis C patients. *Scand. J. Gastroenterol.* **2011**, *46*, 955–961.
8. Miyamura, T.; Kanda, T.; Nakamoto, S.; Wu, S.; Fujiwara, K.; Imazeki, F.; Yokosuka, O. Hepatic STAT1-nuclear translocation and interleukin 28B polymorphisms predict treatment outcomes in hepatitis C virus genotype 1-infected patients. *PLoS One* **2011**, *6*, e28617.
9. Lagging, M.; Askarieh, G.; Negro, F.; Bibert, S.; Soderholm, J.; Westin, J.; Lindh, M.; Romero, A.; Missale, G.; Ferrari, C.; *et al.* Response prediction in chronic hepatitis C by assessment of IP-10 and IL28B-related single nucleotide polymorphisms. *PLoS One.* **2011**, *6*, e17232.
10. Honda, M.; Sakai, A.; Yamashita, T.; Nakamoto, Y.; Mizukoshi, E.; Sakai, Y.; Yamashita, T.; Nakamura, M.; Shirasaki, T.; Horimoto, K.; *et al.* Hepatic ISG expression is associated with genetic variation in interleukin 28B and the outcome of IFN therapy for chronic hepatitis C. *Gastroenterology* **2010**, *139*, 499–509.
11. Fellay, J.; Thompson, A.J.; Ge, D.; Gumbs, C.E.; Urban, T.J.; Shianna, K.V.; Little, L.D.; Qui, P.; Bertelsen, A.H.; Watson, M.; *et al.* ITPA gene variants protect against anemia in patients treated for chronic hepatitis C. *Nature* **2010**, *464*, 40540–40548.
12. Magg, D.; Castro, C.; Hong, Z.; Cameron, C.E. Hepatitis C virus RNA-dependent RNA polymerase (NS5B) as a mediator of the antiviral activity of ribavirin. *J. Biol. Chem.* **2001**, *276*, 46094–46098.
13. Contreras, A.M.; Hiasa, Y.; He, W.; Terella, A.; Schmidt, E.V.; Chung, R.T. Viral RNA mutations are region specific and increased by ribavirin in a full-length hepatitis C virus replicon system. *J. Virol.* **2002**, *76*, 8505–8517.
14. Kanda, T.; Yokosuka, O.; Imazeki, F.; Tanaka, M.; Shino, Y.; Shimada, H.; Tomonaga, T.; Nomura, F.; Nagao, K.; Ochiai, T.; Saisho, H. Inhibition of subgenomic hepatitis C virus RNA in Huh-7 cells: Ribavirin induces mutagenesis in HCV RNA. *J. Viral Hepat.* **2004**, *11*, 479–487.
15. Zhou, S.; Liu, R.; Baroudy, B.M.; Malcolm, B.A.; Reyes, G.R. The effect of ribavirin and IMPDH inhibitors on hepatitis C virus subgenomic replicon RNA. *Virology* **2003**, *310*, 333–342.
16. Tam, R.C.; Pai, B.; Bard, J.; Lim, C.; Averett, D.R.; Phan, U.T.; Milovanovic, T. Ribavirin polarizes human T cell responses towards a Type 1 cytokine profile. *J. Hepatol.* **1999**, *30*, 376–382.
17. Thomas, E.; Feld, J.J.; Li, Q.; Hu, Z.; Fried, M.W.; Liang, T.J. Ribavirin potentiates interferon action by augmenting interferon-stimulated gene induction in hepatitis C virus cell culture models. *Hepatology* **2011**, *53*, 32–41.

18. Clark, V.; Nelson, D.R. The role of ribavirin in direct acting antiviral drug regimens for chronic hepatitis C. *Liver Int.* **2012**, *32*, 103–107.
19. Kanda, T.; Imazeki, F.; Yonemitsu, Y.; Mikami, S.; Takada, N.; Nishino, T.; Takashi, M.; Tsubota, A.; Kato, K.; Sugiura, N.; *et al.* Quantification of hepatitis C virus in patients treated with peginterfron-alfa 2a plus ribavirin treatment by COBAS TaqMan HCV test. *J. Viral Hepat.* **2011**, *18*, e292–e297.
20. Kanda, T.; Imazeki, F.; Azemoto, R.; Yonemitsu, Y.; Mikami, S.; Kita, K.; Takashi, M.; Sunaga, M.; Wu, S.; Nakamoto, S.; *et al.* Response to peginterferon-alfa 2b and ribavirin in Japanese patients with chronic hepatitis C genotype 2. *Dig. Dis. Sci.* **2011**, *56*, 3335–3342.
21. Miyauchi, T.; Kanda, T.; Imazeki, F.; Mikata, R.; Tawada, A.; Arai, M.; Fujiwara, K.; Nakamoto, S.; Wu, S.; Tanaka, T.; *et al.* Response to peginterferon-alpha 2b and ribavirin in Japanese patients with chronic hepatitis C genotype 1. *Hepatology Int.* **2012**, in press.
22. Tsukiyama-Kohara, K.; Yamaguchi, K.; Maki, N.; Ohta, Y.; Miki, K.; Mizokami, M.; Ohba, K.; Tanaka, S.; Hattori, N.; Nomoto, A.; Kohara, M. Antigenicities of group I and II hepatitis C virus polypeptides—Molecular basis of diagnosis. *Virology* **1993**, *192*, 430–437.
23. Simmonds, P.; Holmes, E.C.; Cha, T.A.; Chan, S.W.; McOmish, F.; Irvine, B.; Beall, E.; Yap, P.L.; Kolberg, J.; Urdea, M.S. Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. *J. Gen. Virol.* **1993**, *74*, 2391–2399.
24. McHutchison, J.G.; Manns, M.; Patel, K.; Poynard, T.; Lindsay, K.L.; Trepo, C.; Dienstag, J.; Lee, W.M.; Mak, C.; Garaud, J.J.; Albrecht, J.K. International Hepatitis Interventional Therapy Group. Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. *Gastroenterology* **2002**, *123*, 1061–1069.
25. Lo Re, V., 3rd.; Teal, V.; Localio, A.R.; Amorosa, V.K.; Kaplan, D.E.; Gross, R. Relationship between adherence to hepatitis C virus therapy and virologic outcomes: A cohort study. *Ann. Intern. Med.* **2011**, *156*, 353–360.
26. Reau, N.; Hadziyannis, S.J.; Messinger, D.; Fried, M.W.; Jensen, D.M. Early predictors of anemia in patients with hepatitis C genotype 1 treated with peginterferon alfa-2a (40KD) plus ribavirin. *Am. J. Gastroenterol.* **2008**, *103*, 1981–1988.
27. Sulkowski, M.S. Anemia in the treatment of hepatitis C virus infection. *Clin. Infect. Dis.* **2003**, *37*, S315–S322.
28. Ochi, H.; Maekawa, T.; Abe, H.; Hayashida, Y.; Nakano, R.; Kubo, M.; Tsunoda, T.; Hayes, C.N.; Kumada, H.; Nakamura, Y.; Chayama, K. ITPA polymorphism affects ribavirin-induced anemia and outcomes of therapy—A genome-wide study of Japanese HCV virus patients. *Gastroenterology* **2010**, *139*, 1190–1197.
29. Sakamoto, N.; Tanaka, Y.; Nakagawa, M.; Yatsuhashi, H.; Nishiguchi, S.; Enomoto, N.; Azuma, S.; Nishimura-Sakurai, Y.; Kakinuma, S.; Nishida, N.; *et al.* ITPA gene variant protects against anemia induced by pegylated interferon-α and ribavirin therapy for Japanese patients with chronic hepatitis C. *Hepatol. Res.* **2010**, *40*, 1063–1071.
30. Hitomi, Y.; Cirulli, E.T.; Fellay, J.; McHutchison, J.G.; Thompson, A.J.; Gumbs, C.E.; Shianna, K.V.; Urban, T.J.; Goldstein, D.B. Inosine triphosphate protects against ribavirin-induced adenosine triphosphate loss by adenylosuccinate synthase function. *Gastroenterology* **2011**, *140*, 1314–1321.

31. Thompson, A.J.; Clark, P.J.; Singh, A.; Ge, D.; Fellay, J.; Zhu, M.; Zhu, Q.; Urban, T.J.; Patel, K.; Tillmann, H.L.; *et al.* Genome-wide association study of interferon-related cytopenia in chronic hepatitis C patients. *J. Hepatol.* **2012**, *56*, 313–319.
32. Tanaka, Y.; Kurosaki, M.; Nishida, N.; Sugiyama, M.; Matsuura, K.; Sakamoto, N.; Enomoto, N.; Yatsuhashi, H.; Nishiguchi, S.; Hino, K.; *et al.* Genome-wide association study identified ITPA/DDRGK1 variants reflecting thrombocytopenia in pegylated interferon and ribavirin therapy for chronic hepatitis C. *Hum. Mol. Genet.* **2011**, *20*, 3507–3516.
33. Kurosaki, M.; Tanaka, Y.; Tanaka, K.; Suzuki, Y.; Hoshioka, Y.; Tamaki, N.; Kato, T.; Yasui, Y.; Hosokawa, T.; Ueda, K.; *et al.* Relationship between polymorphisms of the inosine triphosphatase gene and anemia or outcome after treatment with pegylated interferon and ribavirin. *Antivir. Ther.* **2011**, *16*, 689–694.
34. Jensen, D.; Pol, S. IL28B genetic polymorphism testing in the era of direct acting antivirals therapy for chronic hepatitis C: Ten years too late? *Liver Int.* **2012**, *32*, 74–78.
35. Lok, A.S.; Gardiner, D.F.; Lawitz, E.; Martorell, C.; Everson, G.T.; Ghalib, R.; Reindollar, R.; Rustgi, V.; McPhee, F.; Wind-Rotolo, M.; *et al.* Preliminary study of two antiviral agents for hepatitis C genotype 1. *N. Engl. J. Med.* **2012**, *366*, 216–224.
36. Omata, M.; Kanda, T.; Yu, M.L.; Yokosuka, O.; Lim, S.G.; Jafri, W.; Tateishi, R.; Hamid, S.S.; Chuang, W.L.; Chutaputti, A.; *et al.* APASL consensus statements and management algorithms for hepatitis C virus infection. *Hepatol. Int.* **2012**, *6*, 409–435.
37. Miyaaki, H.; Ichikawa, T.; Yatsuhashi, H.; Taura, N.; Miura, S.; Usui, T.; Mori, S.; Kamihira, S.; Tanaka, Y.; Mizokami, M.; Nakao, K. Suppressor of cytokine signal 3 and IL28 genetic variation predict the viral response to peginterferon and ribavirin. *Hepatol. Res.* **2011**, *41*, 1216–1222.
38. Tillmann, H.L.; Patel, K.; Muir, A.J.; Guy, C.D.; Li, J.H.; Lao, X.Q.; Thompson, A.; Clark, P.J.; Gardner, S.D.; McHutchison, J.G.; McCarthy, J.J. Beneficial IL28B genotype associated with lower frequency of hepatic steatosis in patients with chronic hepatitis C. *J. Hepatol.* **2011**, *55*, 1195–1200.
39. Toyoda, H.; Kumada, H. Favorable association between genetic polymorphisms near the IL28B gene and hepatic steatosis: Direct or indirect? *J. Hepatol.* **2012**, *56*, 738–739.
40. Clark, P.J.; Thompson, A.J.; Zhu, M.; Vock, D.M.; Zhu, Q.; Ge, D.; Patel, K.; Harrison, S.A.; Urban, T.J.; Naggie, S.; *et al.* Interleukin 28B polymorphisms are the only common genetic variants associated with low-density lipoprotein cholesterol (LDL-C) in genotype-1 chronic hepatitis C and determine the association between LDL-C and treatment response. *J. Viral Hepat.* **2012**, *19*, 332–340.
41. Rembeck, K.; Alsio, A.; Christensen, P.B.; Farkkila, M.; Langeland, N.; Buhl, M.R.; Pedersen, C.; Morch, K.; Westin, J.; Lindh, M.; *et al.* Impact of IL28B-related single nucleotide polymorphisms on liver histopathology in chronic hepatitis C genotype 2 and 3. *PLoS One* **2012**, *7*, e29370.
42. Stattermayer, A.F.; Rutter, K.; Beinhardt, S.; Scherzer, T.M.; Stadlmayr, A.; Hofer, H.; Wrba, F.; Steindl-Munda, P.; Krebs, M.; Datz, C.; *et al.* Association of the IL28B genotype with insulin resistance in patients with chronic hepatitis C. *J. Hepatol.* **2012**, in press.

43. Veldt, B.J.; Duarte-Rojo, A.; Thompson, A.J.; Watt, K.D.; Heimbach, J.K.; Tillmann, H.L.; Goldstein, D.D.; McHutchison, J.G.; Charlton, M.R. Recipient IL28B polymorphism is an important independent predictor of posttransplant diabetes mellitus in liver transplant patients with chronic hepatitis C. *Am. J. Transplant.* **2012**, *12*, 737–744.
44. Marabita, F.; Aghemo, A.; Nicola, S.D.; Rumi, M.G.; Cheroni, C.; Scavelli, R.; Crimi, M.; Soffredini, R.; Abrignani, S.; De Francesco, R.; Colombo, M. Genetic variation in the interleukin-28B gene is not associated with fibrosis progression in patients with chronic hepatitis C and known date of infection. *Hepatology* **2011**, *54*, 1127–1134.
45. Joshita, S.; Umemura, T.; Katsuyama, Y.; Ichikawa, Y.; Kimura, T.; Morita, S.; Kamijo, A.; Komatsu, M.; Ichijo, T.; Matsumoto, A.; *et al.* Association of IL28B gene polymorphism with development of hepatocellular carcinoma in Japanese patients with chronic hepatitis C virus infection. *Hum. Immunol.* **2012**, *73*, 298–300.
46. Bochud, P.Y.; Bibert, S.; Kutalik, Z.; Patin, E.; Guergnon, J.; Nalpas, B.; Goossens, N.; Kuske, L.; Mullhaupt, B.; Gerlach, T.; *et al.* IL28B alleles associated with poor hepatitis C virus (HCV) clearance protect against inflammation and fibrosis in patients infected with non-1 HCV genotypes. *Hepatology* **2012**, *55*, 384–394.
47. Di Marco, V.; Bronte, F.; Calvaruso, V.; Capra, M.; Borsellino, Z.; Maggio, A.; Renda, M.C.; Pitrolo, L.; Pinto, M.C.L.; Rizzo, M.; *et al.* IL28B polymorphisms influence stage of the liver fibrosis and spontaneous or interferon-induced viral clearance in thalassemia patients with hepatitis C virus infection. *Haematologica* **2012**, in press.
48. Eurich, D.; Boas-Knoop, S.; Bahra, M.; Neuhaus, R.; Somasundaram, R.; Neuhaus, P.; Neumann, U.; Seehofer, D. Role of IL28B polymorphism in the development of hepatitis C virus-induced hepatocellular carcinoma, graft fibrosis, and posttransplant antiviral therapy. *Transplantation* **2012**, *93*, 644–649.
49. Masuzaki, R.; Tateishi, R.; Yoshida, H.; Arano, T.; Uchino, K.; Enooku, K.; Goto, E.; Nakagawa, H.; Asaoka, Y.; Kondo, Y.; *et al.* Assessment of disease progression in patients with transfusion-associated chronic hepatitis C using transient elastography. *World J. Gastroenterol.* **2012**, *18*, 1385–1390.
50. Osada, M.; Kaneko, M.; Sakamoto, M.; Endoh, M.; Takigawa, K.; Suzuki-Inoue, O.; Satoh, K.; Enomoto, N.; Yatomi, Y.; Ozaki, Y. Causes of thrombocytopenia in chronic hepatitis C viral infection. *Clin. Appl. Thromb. Hemost.* **2012**, *18*, 272–280.
51. Ohira, M.; Ishifuro, M.; Ide, K.; Irei, T.; Tashiro, H.; Itamoto, T.; Ito, K.; Chayama, K.; Asahara, T.; Ohdan, H. Significant correlation between spleen volume and thrombocytopenia in liver transplant patients: A concept for predicting persistent thrombocytopenia. *Liver Transpl.* **2009**, *15*, 208–215.
52. Olariu, M.; Olariu, C.; Olteanu, D. Thrombocytopenia in chronic hepatitis C. *J. Gastrointestin. Liver Dis.* **2010**, *19*, 381–385.

Hepatitis B Virus e Antigen Physically Associates With Receptor-Interacting Serine/Threonine Protein Kinase 2 and Regulates *IL-6* Gene Expression

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We previously reported that hepatitis B virus (HBV) e antigen (HBeAg) inhibits production of interleukin 6 by suppressing NF-κB activation. NF-κB is known to be activated through receptor-interacting serine/threonine protein kinase 2 (RIPK2), and we examined the mechanisms of interleukin 6 regulation by HBeAg. HBeAg inhibits RIPK2 expression and interacts with RIPK2, which may represent 2 mechanisms through which HBeAg blocks nucleotide-binding oligomerization domain-containing protein 1 ligand–induced NF-κB activation in HepG2 cells. Our findings identified novel molecular mechanisms whereby HBeAg modulates intracellular signaling pathways by targeting RIPK2, supporting the concept that HBeAg could impair both innate and adaptive immune responses to promote chronic HBV infection.

Hepatitis B virus (HBV) nucleoprotein exists in 2 forms [1, 2]. Nucleocapsid, designated HBV core antigen (HBcAg), is an intracellular, 21-kDa protein that self-assembles into particles that encapsidate viral genome and polymerase and is essential for function and maturation of virion. HBV also secretes a nonparticle second form of the nucleoprotein, designated precore or HBV e antigen (HBeAg) [1, 2]. Precore and core proteins are translated from 2 RNA species that have different 5′ initiation sites. Precore messenger RNA (mRNA) encodes a hydrophobic signal sequence that directs precore protein to the endoplasmic reticulum, where it undergoes N- and C-terminal cleavage within the secretory pathway and is secreted as an 18-kDa monomeric protein [3–5].

Nucleotide-binding oligomerization domain–containing protein 1 (NOD1) and NOD2 are cytosolic pattern-recognition receptors involved in the sensing of bacterial peptidoglycan subcomponents [6]. NOD1 and NOD2 stimulation activates NF-κB through receptor-interacting serine/threonine protein kinase 2 (RIPK2; also known as RIP2, RICK, or CARDIAK), a caspase-recruitment domain-containing kinase. RIPK2 is also involved in Toll-like receptor (TLR)–signaling pathway and plays an important role in the production of inflammatory cytokines through NF-κB activation [6, 7].

We previously reported that HBeAg inhibits the production of interleukin 6 (IL-6) through suppression of NF-κB activation [4]. In the present study, we investigated the molecular mechanism of HBeAg functions for the requirement of RIPK2 in NF-κB transcriptional regulation.

METHODS

Cell Culture and Plasmids

HepG2, Huh7, HT1080, COS7, and HEK293T cells were used in the present study. Stable cell lines were obtained as previously described [4]. Briefly, HepG2, Huh7, and HT1080 were transfected with pCXN2-HBeAg(+) or pCXN2-HBeAg(−) in Effectene (Qiagen). After G418 screening, HBeAg-positive and -negative HepG2/Huh7/HT1080 cell lines were collected for further analysis [4]. The plasmid pCXN2-HBeAg(+), which can produce both HBeAg and core peptides, and the plasmid pCXN2-HBeAg(−), which can produce only core peptides, were obtained as described previously [4]. pNF-κB–luc, which expresses luciferase upon promoter activation by NF-κB, was purchased from Stratagene [4]. pGFP–human RIPK2 (kindly provided by Prof John C. Reed, Sanford-Burnham Institute for Medical Research) can express GFP-human $RIP2^{WT}$ [8].

HepG2 cells were transfected with plasmid control–small hairpin RNA (shRNA) or with RIPK2-shRNA (Santa Cruz). After puromycin screening, individual colonies were picked up and examined for expression of endogenous RIPK2, and clones HepG2-shC and HepG2-shRIPK2-3 were selected for subsequent studies.

Received 1 January 2012; accepted 3 February 2012; electronically published 21 May 2012.
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The Journal of Infectious Diseases 2012;206:415–20

DOI: 10.1093/infdis/jis363

Luciferase Assays and Treatment of Cells With NOD Ligands

Around 1.0×10^5 HepG2 and Huh7 cells were plated in 6-well plates (Iwaki Glass, Tokyo, Japan) for 24 hours and transfected with 0.4 μg of pNF-κB–luc. For luciferase assay of NF-κB activation, cells were treated for 4 hours with or without NOD1 ligand (C12-iEDAP, 2.5 μg/mL) and NOD2 ligand (muramyl dipeptide [MDP], 10 μg/mL) (InvivoGen) at 44 hours after transfection [9]. After 48 hours, cells were lysed with reporter lysis buffer (Promega), and luciferase activity was determined as described previously [4].

RNA Extraction, Complementary DNA (cDNA) Synthesis, Real-Time Polymerase Chain Reaction (PCR) Analysis, and PCR Array

Total RNA was isolated by RNeasy Mini Kit (Qiagen). A total of 5 μg of RNA was reverse transcribed using the First Strand cDNA Synthesis Kit (Qiagen) [4]. Quantitative amplification of cDNA was monitored with SYBR Green by real-time PCR in a 7300 Real-Time PCR system (Applied Biosystems). Gene expression profiling of 84 TLR-related genes was performed using RT^2 profiler PCR arrays (Qiagen) in accordance with the manufacturer's instructions [4].

Gene expression was normalized to 2 internal controls (GAPDH and/or β-actin) to determine the fold-change in gene expression between the test sample (HBeAg-positive HepG2/Huh7/HT1080) and the control sample (HBeAg-negative HepG2/Huh7/HT1080) by the 2^{-ddCT} (comparative cycle threshold) method [4]. Three sets of real-time PCR arrays were performed. Some results of HepG2 cells were previously reported [4].

Coimmunoprecipitation

Cells were cotransfected with 2.5 μg pCXN2-HBeAg(+) or 2.5 μg pCXN2-HBeAg(−), as well as with 2.5 μg pGFP–human RIPK2, and cell lysates were prepared after 48 hours, using lysis buffer containing a cocktail of protease inhibitors. Cell lysates were incubated with anti-GFP rabbit polyclonal antibody (Santa Cruz) or anti-HBe mouse monoclonal antibody (Institute of Immunology, Tokyo, Japan) for 3 hours at 4°C, followed by overnight incubation with protein G–Sepharose beads (Santa Cruz). Immunoprecipitates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose membrane. Immunoblotting was performed by incubating the membrane for 1 hour with anti-HBe antibody. Proteins were detected by enhanced chemiluminescence (GE Healthcare), using an image analyzer (LAS-4000, Fuji Film). The membrane was reprobed with a monoclonal antibody to GFP or RIPK2 (Cell Signaling).

Transfection of pGFP–Human RIPK2 and Confocal Microscopy

Formaldehyde (3.7%)–fixed cells were incubated with anti-HBe antibody and stained with fluorochrome-conjugated secondary antibody (Alexa Fluor 555 conjugate, Cell Signaling). Cells were mounted for confocal microscopy (ECLIPSE TE 2000-U, Nikon). Whenever necessary, images were merged digitally to monitor colocalization. Cotransfection of 0.1 μg pCXN2-HBeAg(+) or 0.1 μg pCXN2-HBeAg(−) with 0.3 μg pGFP–human RIPK2 into the cells was performed. After 48 hours, intracellular localization of RIPK2 was visualized by confocal microscopy.

Enzyme-Linked Immunosorbent Assay (ELISA) for IL-6

Cell culture fluid was analyzed for IL-6 by ELISA (KOMA-BIOTECH, Seoul, Korea), in accordance with the manufacturer's protocol [4].

Small Interfering RNA (siRNA) Transfection and Wound-Healing Assay

Control siRNA (siC) and siRNA specific for RIPK2 (siRIPK2) were purchased from Thermo Fisher Scientific. Cells were transfected with siRNA by electroporation. After 48 hours, cells were treated with 10 ng/mL tumor necrosis factor α (TNF-α) (Wako Pure Chemical, Osaka, Japan), while the wound-healing (ie, scratch) assay was performed using a p-200 pipette tip to induce RIPK2 [10]. Up to 12 hours after scratching, the cells were observed by microscopy. Cell migration was measured using Scion Images (SAS). Migration by siC-transfected cells was set at 1.

Statistical Analysis

Results are expressed as mean values ± SD. The Student *t* test was used to determine statistical significance.

RESULTS

HBeAg Downregulates RIPK2 Expression

To explore the effect of HBeAg on TLR-related gene expression, we generated HepG2, Huh7, and HT1080 cell lines that stably expressed HBV core region with or without precore region. HT1080, a primate fibrosarcoma cell line, is useful for the study of interferon signaling. HBeAg and HBV core–related antigen (HBcrAg) levels of these cell lines demonstrated that expression of HBV core region without HBV precore region did not allow HBeAg secretion by cells (data are cited elsewhere [4] or not shown). First, we performed real-time RT-PCR analysis of these cell lines, using focused gene arrays (Figure 1*A*). We observed that, in 3 cell lines, 5 genes (*RIPK2, TLR9, TNF, CD180,* and *IL1A*) were downregulated ≥1.3-fold in HBeAg-positive cells than in HBeAg-negative cells. We chose to focus our investigation on RIPK2 because HBeAg inhibits the production of IL-6 through the suppression of NF-κB activation [4], and NF-κB is known to be activated through RIPK2 [4]. RIPK2 expression was >100-, 1.41-, and 1.45-fold lower in HBeAg-positive HepG2, Huh7, and HT1080 cells, respectively, compared with their HBeAg-negative counterparts

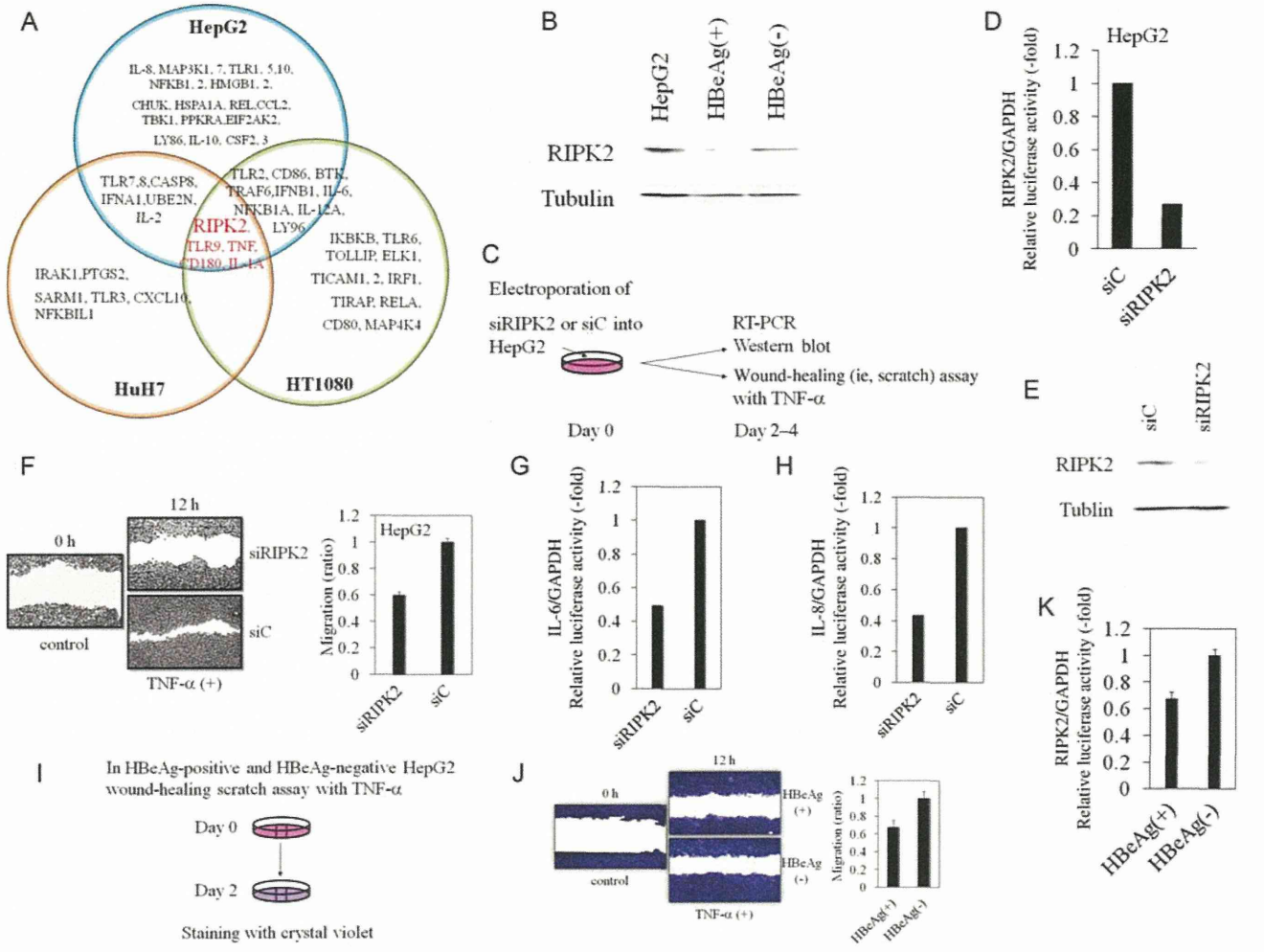

Figure 1. Receptor-interacting serine/threonine protein kinase 2 (RIPK2) expression is downregulated by hepatitis B virus e antigen (HBeAg), and knockdown of RIPK2 and HBeAg impairs hepatic wound repair. *A*, Venn diagram representing Toll-like receptor (TLR)–related genes downregulated ≥1.3-fold in HBeAg-positive HepG2/Huh7/HT1080 cells, compared with HBeAg-negative cells. Cellular RNA was extracted and analyzed with focused array, quantifying 84 genes. Gene expression levels were normalized to actin and GAPDH expression levels. *B*, HBeAg downregulates RIPK2 expression in HepG2 cells. Western blot analysis of RIPK2 and tubulin expression in HepG2, HBeAg(+) HepG2, and HBeAg(−) HepG2. *C*, Experimental protocol of electroporation of control (siC) and RIPK2 (siRIPK2) small interfering RNA (siRNA) into HepG2 cells. *D* and *E*, Real-time polymerase chain reaction (PCR; *D*) and Western blot (*E*) analyses of RIPK2 expression in siC- or siRIPK2-expressing HepG2 cells. RIPK2 messenger RNA (mRNA) levels were normalized to GAPDH levels. *F–H*, siC- and siRIPK2-transfected HepG2 cells were scratch wounded and incubated with 10 ng/mL tumor necrosis factor α (TNF-α), and cell migration was analyzed after 12 hours and quantified using Scion Image (*F*). Interleukin 6 (IL-6; *G*) and interleukin 8 (IL-8; *H*) mRNA expression are quantified by real-time reverse transcription–PCR (RT-PCR) and expressed relative to GAPDH mRNA expression. *I*, Protocol of wound-healing (ie, scratch) assay in HBeAg(+) and HBeAg(−) HepG2 cells. TNF-α was used at 10 ng/mL. *J*, Cell migration was analyzed using Scion Image. *K*, RIPK2 mRNA expression was quantified by real-time RT-PCR and expressed relative to GAPDH mRNA expression. Primers specific for RIPK2 were 5′-AGACAC-TACTGACATCCAAG-3′ (sense) and 5′-CACAAGTATTTCCGGGTAAG-3′ (antisense), and primers for other genes were as described previously [4]. Data are mean values ± SD of 3 independent experiments.

(Figure 1*A*). Western blot analyses confirmed lower levels of RIPK2 in HBeAg-positive HepG2 than in HBe-negative HepG2 or parental HepG2 (Figure 1*B*). The fact that RIPK2 is one of the targets for the ubiquitin proteasome system and uses a ubiquitin-dependent mechanism to achieve NF-κB activation [6] might be a reason for the differences between RIPK2 mRNA and protein expression status. We also observed lower levels of RIPK2 mRNA expression (0.18-fold) in HepG2.2.15 cells, which secrete complete HBV virion and HBeAg, compared with expression in HepG2 cells (data not shown).

Knockdown of RIPK2 and HBeAg Impairs Hepatic Cell Migration

It has recently been reported that RIPK2 expression is induced by TNF-α plus scratch wounding in keratinocytes [10]. Therefore, we next examined whether RIPK2 affected hepatic

wound healing in the presence of TNF-α in vitro (Figure 1*C*). As shown in Figure 1*D* and 1E, RIPK2 mRNA and protein expression were efficiently decreased in HepG2 cells transfected with RIPK2 siRNA (siRIPK2), but not control (siC). RIPK2 silencing reduced hepatic wound closure 1.8-fold, which was associated with a 2-fold decrease in IL-6 production, known to be an important cytokine for the regeneration of liver [11], and a 2.3-fold decrease in interleukin 8 production (Figure 1*F–H*). Importantly, RIPK2 silencing did not affect cell viability (data not shown).

Given that HBeAg downregulates RIPK2 expression (Figure 1*A* and 1*B*), we examined whether HBeAg has an effect on hepatic wound healing in the presence of TNF-α (Figure 1*I*). As expected, we observed that both cell migration

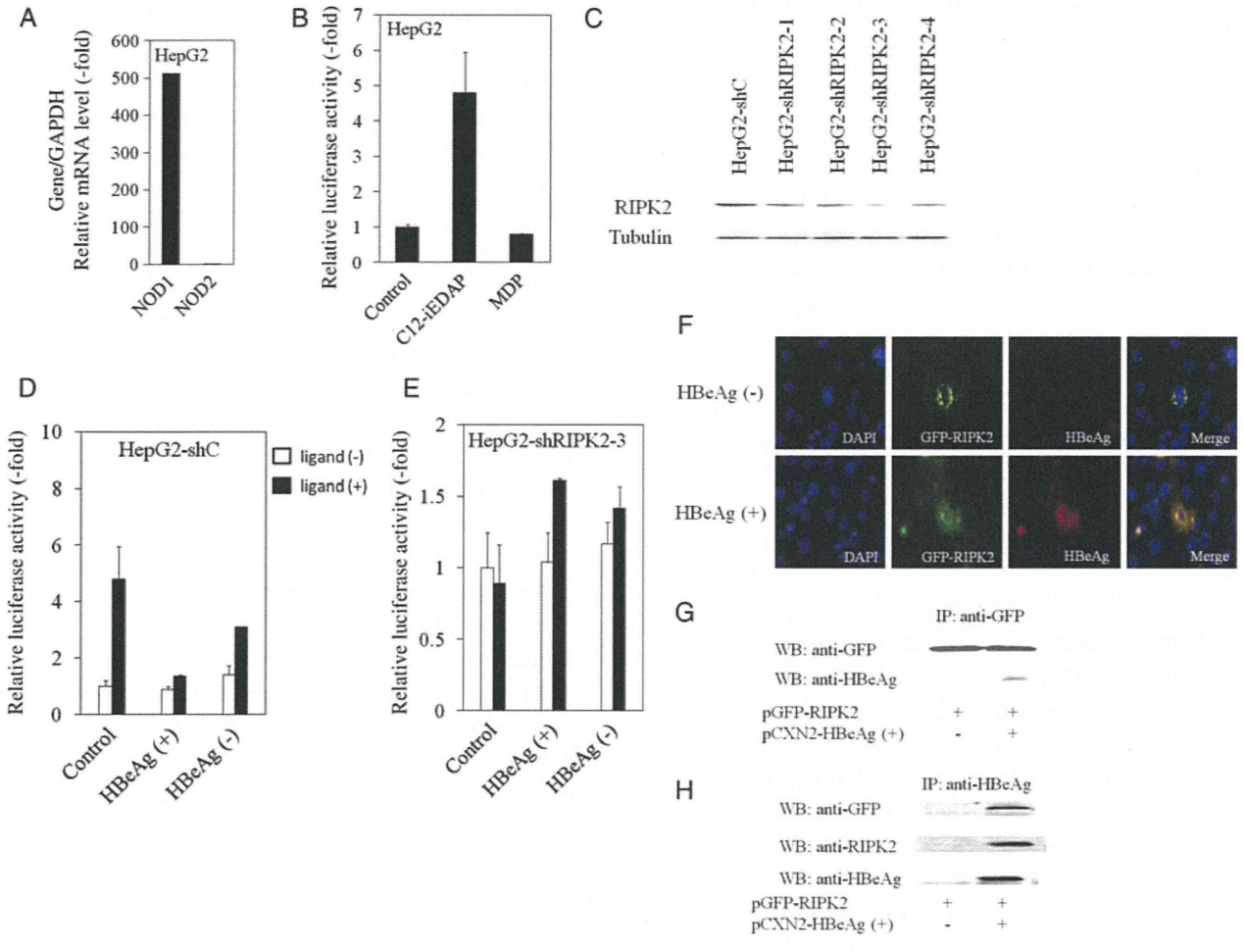

Figure 2. The nucleotide-binding oligomerization domain–containing protein 1 (NOD1) ligand C12-iEDAP induces NF-κB activation, knockdown of receptor-interacting serine/threonine protein kinase 2 (RIPK2) inhibits NOD1 ligand–induced NF-κB activation in HepG2 cells, and hepatitis B virus e antigen (HBeAg) interacts with RIPK2. *A*, Real-time reverse transcription–polymerase chain reaction analysis of NOD1 and NOD2 messenger RNA expression in HepG2. NOD1 and NOD2 expression levels were normalized to GAPDH expression levels. *B*, NF-κB–driven luciferase activity in HepG2 cells stimulated with the NOD1 ligand C12-iEDAP or the NOD2 ligand muramyl dipeptide (MDP) in HepG2. *C*, Western blot analysis of RIPK2 and tubulin expression in HepG2 cells stably transfected with control small hairpin RNA (shRNA; HepG2-shC) or with RIPK2 shRNA (HepG2–shRIPK2-1/2-4) expressing plasmids. *D* and *E*, HepG2-shC (*D*) and HepG2–shRIPK2-3 (*E*) cell lines were transiently transfected with pCXN2, pCXN2-HBeAg(+), or pCXN2-HBeAg(−) plasmids together with pNF-κB–luc. Cells were treated for 4 hours, with or without NOD1 ligand C12-iEDAP (2.5 μg/mL), and luciferase activity was determined. Primers specific for NOD1 (sense primer: 5′-ACTACCTCAAGCTGACCTAC-3′; antisense primer: 5′-CTGGTTTACGCTGAGTCTG-3′), for NOD2 (sense primer: 5′-CCTTGCATGCAGGCAGAAC-3′; antisense primer: 5′-TCTGTTGCCCCAGAATCCC-3′), and for other genes as described previously were purchased from Sigma [4]. *F*, HBeAg specifically colocalizes with RIPK2. COS7 cells were transiently cotransfected with 0.1 μg pCXN2-HBeAg(+) or pCXN2-HBeAg(−) together with 0.3 μg pGFP–human RIPK2. HBeAg was revealed with anti-HBeAg primary antibody and Alexa-Fluor-548 secondary antibody. *G* and *H*, HEK293T cells were transiently transfected with or without GFP-RIPK2 and HBeAg-expressing plasmids. Cellular extracts were precleared with protein G–Sepharose, and interacting complexes were immunoprecipitated (IP) with either anti-GFP (*G*) or anti-HBeAg (*H*) antibodies. Immunocomplexes were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and proteins were visualized by immunoblotting (WB) with indicated antibodies. Results are representative of 3 independent experiments.

and RIPK2 mRNA expression were reduced in HBeAg-positive HepG2 cells, compared with HBeAg-negative cells (1.5-fold decrease; Figure 1*J* and 1*K*). These results suggest that HBeAg impairs hepatic cell migration–dependent RIPK2 expression. Among NF-κB–targeting genes, expression of vimentin mRNA was impaired in HepG2-shRIP2 and in HBeAg-positive HepG2 (data not shown), and vimentin might be one of the candidates for impairment of their migrations [12].

RIPK2 Plays an Important Role in NF-κB Activation Induced by NOD1 Ligand, and HBeAg Blocks This Pathway

HepG2 cells express NOD1 but not NOD2 at the mRNA level (Figure 2*A*). In agreement with this finding, NF-κB was activated in HepG2 cells exposed to NOD1 ligand C12-iEDAP (level of activation, 4.8-fold, compared with untreated control) but not in those exposed to NOD2 ligand MDP (Figure 2*B*). As for Huh7 cells, activation of NF-κB was not detected following exposure to C12-iEDAP or MDP (data not shown). These results suggest that C12-iEDAP triggered NF-κB activation through NOD1 in HepG2 cells, which is consistent with findings from a previous study [9].

We examined whether knockdown of RIPK2 has an effect on NOD1-induced NF-κB activation in HepG2 cells. First, we established HepG2 cell lines that constitutively expressed RIPK2-shRNA (HepG2-shRIPK2-1/2-4) or control-shRNA (HepG2-shC) (Figure 2*C*). The HepG2–shRIPK2-3 cell line, which expresses the lowest levels of RIPK2, and the HepG2-shC cell line were treated for 4 hours, with or without C12-iE-DAP, before measurement by the NF-κB–driven luciferase assay (Figure 2*D* and 2*E*). C12-iEDAP triggered NF-κB activation in HepG2-shC (Figure 2*D*) but not in HepG2–shRIPK2-3 (Figure 2*E*), indicating that RIPK2 plays an important role in NF-κB activation induced through NOD1 triggering.

To assess the influence of HBeAg in that pathway, we measured NOD1-mediated NF-κB activity in HepG2-shC and HepG2–shRIPK2-3 cell lines transiently transfected with HBeAg-expressing plasmids. As shown in Figure 2*D*, HBeAg expression in HepG2-shC abolished C12-iEDAP–induced NF-κB activation.

HBeAg Interacts With RIPK2 and Colocalizes With RIPK2

RIPK2 mediates activation of transcription factors, such as NF-κB, following its activation, which is initiated by membrane-bound or intracytosolic receptors, such as TLR, NOD1, and NOD2 [7, 13, 14]. Confocal microscopy analysis of cells transfected with GFP-RIPK2 revealed subcellular localization of RIPK2 (data not shown). To compare the localization of RIPK2 with that of HBeAg, cells were cotransfected with pGFP–human RIPK2 with pCXN2-HBeAg(+) or pCXN2-HBeAg(−). After 48 hours, cells were stained with mouse monoclonal anti-HBe antibody. Confocal microscopy suggested subcellular colocalization of RIPK2 with HBeAg (Figure 2*F*). Reinforcing this assumption, GFP-RIPK2 coimmunoprecipitated with HBeAg (Figure 2*G*), while HBeAg coimmunoprecipitated with RIPK2 (Figure 2*H*) in transiently transfected cells with RIPK2- and HBeAg-expressing plasmids.

DISCUSSION

In the present study, we have shown the expression of NOD1 and NOD1 ligand–induced NF-κB activation in HepG2 cells and that RIPK2 plays an important role in NOD1 ligand–induced NF-κB activation. NF-κB activation plays an essential role in the production of inflammatory cytokines such as IL-6, which HBeAg could suppress in hepatocytes [4]. We have also shown that HBeAg inhibits RIPK2 expression and interacts with RIPK2, which may represent 2 mechanisms through which HBeAg blocks NOD1 ligand–induced NF-κB activation, thus contributing to the pathogenesis of chronic HBV infection and establishing viral persistence, although further studies including clinical situations might be needed.

HBeAg can be secreted by hepatocytes. Yet, it has been reported that as much as 80% of the precore protein p22 remains localized to the cytoplasm rather than undergoing further cleavage that allows its secretion as mature HBeAg [15]. Our present study showed subcellular colocalization of HBeAg with RIPK2 (Figure 2*F*). In addition to HBeAg protein in cell culture medium, we observed similar inhibition of NF-κB activation (data not shown).

Overall, we provided a novel molecular mechanism whereby HBeAg modulates innate immune signal-transduction pathways through RIPK2. Elsewhere, it was also reported that HBeAg impairs cytotoxic T-lymphocyte activity [2]. HBeAg inhibits RIPK2 expression and interacts with RIPK2, decreasing NF-κB activation and inflammatory cytokine production in hepatocytes. Taken together, HBeAg could impair both innate and adaptive immune responses to promote chronic HBV infection.

Notes

Acknowledgments. We thank Prof John C. Reed and Prof Junichi Miyazaki, for providing the plasmids, and Ms. Satomi Hasegawa, for providing technical assistance.

Financial support. This work was supported by the Japan Science and Technology Agency, Ministry of Education, Culture, Sports, Science, and Technology, Japan (21590829 to T. K. and 21590828 to F. I.); the Japan Society of Hepatology (T. K.); the Chiba University Young Research-Oriented Faculty Member Development Program in Bioscience Areas (T. K.); and the Research Grant-in-Aid from Miyakawa Memorial Research Foundation (W. S.).

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Ait-Goughoulte M, Lucifora J, Zoulim F, Durantel D. Innate antiviral immune responses to hepatitis B virus. Viruses **2010**; 2:1394–410.
2. Chen M, Sallberg M, Hughes J, et al. Immune tolerance split between hepatitis B virus precore and core proteins. J Virol **2005**; 79:3016–27.
3. Ou JH, Laub O, Rutter WJ. Hepatitis B virus gene function: the precore region targets the core antigen to cellular membranes and causes the secretion of the e antigen. Proc Natl Acad Sci U S A **1986**; 83:1578–82.
4. Wu S, Kanda T, Imazeki F, et al. Hepatitis B virus e antigen down-regulates cytokine production in human hepatoma cell lines. Viral Immunol **2010**; 23:467–76.
5. Lang T, Lo C, Skinner N, Locarnini S, Visvanathan K, Mansell A. The hepatitis B e antigen (HBeAg) targets and suppresses activation of the Toll-like receptor signaling pathway. J Hepatol **2011**; 55:762–9.
6. Hasegawa M, Fujimoto Y, Lucas PC, et al. A critical role of RICK/RIP2 polyubiquitination in Nod-induced NF-kappaB activation. EMBO J **2008**; 27:373–83.
7. Kobayashi K, Inohara N, Hernandez LD, et al. RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. Nature **2002**; 416:194–9.
8. Krieg A, Correa RG, Garrison JB, et al. XIAP mediates NOD signaling via interaction with RIP2. Proc Natl Acad Sci U S A **2009**; 106:14524–9.
9. Scott MJ, Chen C, Sun Q, Billiar TR. Hepatocytes express functional NOD1 and NOD2 receptors: a role for NOD1 in hepatocyte CC and CXC chemokine production. J Hepatol **2010**; 53:693–701.
10. Adams S, Valchanova RS, Munz B. RIP2: a novel player in the regulation of keratinocyte proliferation and cutaneous wound repair? Exp Cell Res **2010**; 316:728–36.
11. Cressman DE, Greenbaum LE, DeAngelis RA, et al. Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice. Science **1996**; 274:1379–83.
12. Moura-Neto V, Kryszke MH, Li Z, Vicart P, Lilienbaum A, Paulin D. A 28-bp negative element with multiple factor-binding activity controls expression of the vimentin-encoding gene. Gene **1996**; 168:261–6.
13. Meylan E, Tschopp J. The RIP kinases: crucial integrators of cellular stress. Trends Biochem Sci **2005**; 30:151–9.
14. Chin AI, Dempsey PW, Bruhn K, Miller JF, Xu Y, Cheng G. Involvement of receptor-interacting protein 2 in innate and adaptive immune responses. Nature **2002**; 416:190–4.
15. Garcia PD, Ou JH, Rutter WJ, Walter P. Targeting of the hepatitis B virus precore protein to the endoplasmic reticulum membrane: after signal peptide cleavage translocation can be aborted and the product released into the cytoplasm. J Cell Biol **1988**; 106:1093–104.

Hepatology Research

Hepatology Research 2012; **42**: 974–981 doi: 10.1111/j.1872-034X.2012.01009.x

Original Article

Hepatitis A, B, C and E virus markers in Chinese residing in Tokyo, Japan

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Aim: Recently, the number of foreigners living in Japan has been increasing, with the majority originating from China. It is important for us to know the prevalence of hepatitis virus markers among them, as proper medical practices and vaccinations should be prepared when seeing them and their offspring.

Methods: We examined the relationship between the prevalence of hepatitis virus markers: hepatitis B surface antigen (HBsAg), anti-HBs, anti-hepatitis C virus (HCV), anti-hepatitis A virus (HAV) and anti-hepatitis E virus immunoglobulin (Ig)G, and background such as age, birthplace and length of stay in Japan, of 568 Chinese residing in Tokyo, and also of 55 indigenous Japanese.

Results: The prevalence of HBV and HAV markers in Chinese staying in Tokyo is higher than in indigenous Japanese (HBsAg, 10% vs 1.8%; anti-HBs, 45% vs 9.0%; anti-HAV, 90% vs 14%). There were no differences in anti-HCV and anti-HEV IgG between the two groups.

Conclusion: Indigenous Japanese subjects have less immunity against HAV and HBV. The HBV carrier rate is higher in Chinese subjects, and attention should be paid to this issue in clinical practice. It might be important to control hepatitis viruses in Chinese subjects when doctors see them in Japan.

Key words: Chinese, HAV, HBV, HCV, HEV, Tokyo

INTRODUCTION

HEPATITIS A, B, C and E virus (HAV, HBV, HCV and HEV, respectively) cause acute hepatitis, and occasionally fulminant hepatitis, and HBV and HCV also lead to chronic hepatitis, cirrhosis and hepatocellular carcinoma in Japan as well as throughout the world.[1–6] In general, the prevalence of hepatitis viruses follows a wide range of diverse patterns, being dependent on different areas and countries.[7–10]

In Japan, hepatitis B surface antigen (HBsAg) and antibody to HCV (anti-HCV), respectively, were detected in 0.63% and 0.49% in sera from first-time blood donors aged 16–64 years.[5] It was also reported that only fewer than 50% of people have immunity against HAV, estimated from anti-HAV prevalence.[11] Of qualified blood donors, 3.4% were regarded as positive for anti-HEV immunoglobulin (Ig)G.[12] On the other hand, as an example, in China, the prevalence of HBsAg, anti-HBs, anti-HCV, anti-HAV and anti-HEV IgG was reported to be 5.84%, 41.3%, 0.58%, 72.8% and 17.66%, respectively, although this prevalence pattern is well known to differ among different areas in China.[13]

By the end of 2009, 2 186 121 foreigners were living in Japan, and the largest proportion, 31.6%, was born in China, Taiwan and Hong Kong.[14] With increasing numbers of foreigners living in Japan, we will have more opportunities to see them as patients in clinical practice. It is important for us to know, among other things, their prevalence of hepatitis virus markers, as vaccinations and appropriate medical practices should be provided when seeing them and their offspring.

Therefore, in the present study, we examined the relationship between the prevalence of hepatitis virus markers and background such as age, birthplace and their duration of domicile in Japan among Chinese living in Tokyo, Japan.

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Received 31 December 2011; revision 27 February 2012; accepted 18 March 2012.

METHODS

Study subjects and serum collection

THE SUBJECTS IN this study were 623 consecutive outpatients attending the Kyowa Clinic in Tokyo. Of these patients, 568 (80%) were Chinese who were staying in Japan. The others were 55 indigenous Japanese, and all patients were seen between August 2010 and January 2011 (Table 1). The duration of the Chinese subjects' stay in Japan was 103 ± 76 days. There were no differences in age, sex or alanine aminotransferase (ALT) levels between the two groups, but the platelet counts of the Chinese were lower than those of the Japanese subjects (Table 1). Chinese patients were divided into eight groups according to their birthplace in China, as follows: 32, nine, one, 180, 331, 10, zero and five were from North China (Beijing, Tianjing, Hebei, Shanxi and Inner Mongolia), Central China (Henan, Hunan and Hubei), South China (Guangdong, Guangxi and Hainan), East China (Shanghai, Jiangsu, Zhejiang, Fujian, Shandong, Jiangxi and Anhui), North-East China (Heilongjiang, Liaoning and Jilin), South-West China (Sichuan, Chongqing, Yunnan, Guizhou and Tibet), North-West China (Xinjiang, Shanxi, Gansu, Ningxia and Qinghai) and Hong Kong, Macao and Taiwan, respectively. All patients were adults and the most common symptoms were other than liver diseases. Family history of liver diseases, history of surgeries, blood transfusion, drug abuse and tattoo were investigated from patients' interviews and medical records.

Serological diagnosis

All patients were screened by serological tools for hepatitis A, B, C and E virus infections. HBsAg, anti-HBs, anti-HCV, anti-HAV and anti-HEV IgG were tested in each sample by magnetizing particle aggregation (MAT; Shino-Test Tokyo, Japan), particle agglutination (PA; Fujirebio, Tokyo, Japan), chemiluminescent enzyme immunoassay (CLEIA; Fujirebio), chemiluminescent immunoassay (CLIA; Abbott Laboratories, North Chicago, IL, USA) and enzyme immune assay (EIA; Institute of Immunology, Tokyo, Japan), respectively. A positive reaction was indicated when the cut-off index (COI) exceeded 1.0 in anti-HCV, anti-HAV and anti-HEV IgG. The lower detection limit for HBsAg tested by MAT was 8 IU/mL, corresponding to approximately 10 COI measured by CLEIA method. The lower detection limit for anti-HBs examined by PA corresponded to 30 mIU/mL measured by CLEIA.

Hepatitis B virus genotype of patient sera was determined by ELISA (Institute of Immunology) based on the methodology described by Usuda *et al.*[7,15] Informed consent was obtained at the time of blood sampling from each patient included in the study. This study was approved by the ethics committee of Chiba University, Japan, and that of Kyowa Clinic, and conformed to the Declaration of Helsinki. Sera were collected as part of clinical practice and stored at −20°C until laboratory testing was performed.

Table 1 Background of study patients and hepatitis virus markers

	Total subjects	Chinese staying in Japan	Indigenous Japanese	*P*-value*
No. of patients	623	568	55	
Age, years	47 ± 14	47 ± 14	45 ± 15	NS
Sex (M/F)	292/331	264/304	28/27	NS
ALT (IU/L)	26 ± 44	26 ± 46	25 ± 19	NS
Platelets ($\times 10^4/mm^3$)	22 ± 5.4	22 ± 5.8	24 ± 5.1	0.013
HBsAg (+/−)	63/556	62/502	1/54	0.031
Anti-HBs (+/−)	258/362	259/305	5/50	<0.0001
Anti-HCV (+/−)	11/607	10/553	1/54	NS
Anti-HAV (+/−)	518/100	510/53	8/47	<0.0001
Anti-HEV IgG (+/−)	128/493	120/446	8/47	NS

**P*-value between Chinese subjects staying in Japan and indigenous Japanese subjects.
+, Positive; −, negative; ALT, alanine aminotransferase; HAV, hepatitis A virus; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; HEV, hepatitis E virus; IgG, immunoglobulin G; NS, not significant.

Data analysis

Data were expressed as mean ± standard deviation. Differences were evaluated by Student *t*-test or χ^2-test. $P < 0.05$ was considered statistically significant. For all tests, two-sided *P*-values were calculated and the results were considered statistically significant at $P < 0.05$. Statistical analysis was performed using the Excel statistics program for Windows ver. 7 (SSRI, Tokyo, Japan) and DA Stats software (O. Nagata, Nifty Serve: PAF01644).

RESULTS

Chinese subjects staying in Tokyo have more immunity against HAV and HBV

AMONG 623 STUDY subjects, 549 (88%) had normal ALT levels (ALT ≤40 IU/L). HBsAg, anti-HBs, anti-HCV, anti-HAV and anti-HEV IgG were determined in 619 (99%), 620 (99%), 618 (99%), 618 (99%) and 621 (99%), respectively. The overall prevalence of HBsAg, anti-HBs, anti-HCV, anti-HAV and anti-HEV IgG in the present study was 10%, 45%, 1.7%, 90% and 21%, respectively (Table 1). The prevalence of HBV and HAV markers of Chinese staying in Japan was higher than that of indigenous Japanese (HBsAg, 10% vs 1.8%; anti-HBs, 45% vs 9.0%; anti-HAV, 90% vs 14%), but there were no differences in anti-HCV and anti-HEV IgG between the two groups (Table 1). These results suggest that Chinese have more immunity against HAV and HBV than indigenous Japanese. A greater proportion of Chinese subjects was HBsAg positive compared to indigenous Japanese subjects.

Sex differences in hepatitis virus markers

Next, we examined the sex differences in the two groups (Table 2). There were no sex differences concerning HBsAg, anti-HBs, anti-HCV and anti-HAV in each of the two groups. Among Chinese subjects staying in Japan, men with anti-HEV IgG were predominant, but this predominance was not seen in the Japanese group (Table 2).

Age differences in relation to prevalence of hepatitis virus markers

Among Chinese subjects staying in Japan, the HBsAg positive rate under 30 years was higher than in those in their 30s ($P = 0.0018$), 40s ($P < 0.0001$) and over 50 years ($P < 0.0001$), and the HBsAg positive rate of those in their 30s was also higher than those over 50 years ($P < 0.053$) (Fig. 1a). Only one HBsAg positive Japanese subject was a 53-year-old man. There were no differences in each age group between Chinese and Japanese subjects (Fig. 1a).

Positive rates of anti-HBs in those under 30 years, in their 30s, 40s and over 50 years in Chinese subjects staying in Japan were higher than those in indigenous Japanese ($P = 0.0037$, 0.0020, 0.0065 and 0.0034,

Table 2 Background of study patients and hepatitis virus markers according to sex differences

	Chinese staying in Japan			Indigenous Japanese		
	Male ($n = 264$)	*P*	Female ($n = 304$)	Male ($n = 28$)	*P*	Female ($n = 27$)
Age, years	47 ± 14	NS	47 ± 13	46 ± 13	NS	43 ± 18
ALT (IU/L)	29 ± 43	NS	23 ± 49	31 ± 19	0.0083	18 ± 16
Platelets ($\times 10^4/mm^3$)	21 ± 5.7	<0.0001	23 ± 5.9	24 ± 5.4	NS	24 ± 4.9
Length of stay (days)	103 ± 78	NS	104 ± 75			
Family of liver diseases (+/−)	13/248	NS	24/273	2/26	NS	0/27
Transfusion (+/−)	5/259	NS	6/297	1/27	NS	1/26
Surgery (+/−)	24/240	0.017	49/254	4/24		4/23
Drug abuse (+/−)	0/264	NA	0/303	0/28	NA	0/27
Tattoo (+/−)	0/264	NS	1/302	0/28	NA	0/27
HBsAg (+/−)	30/232	NS	32/270	1/27	NS	0/27
Anti-HBs (+/−)	119/144	NS	140/162	2/26	NS	3/24
Anti-HCV (+/−)	3/259	NS	7/292	1/27	NS	0/27
Anti-HAV (+/−)	235/28	NS	275/25	3/25	NS	5/22
Anti-HEV IgG (+/−)	68/195	0.015	52/251	2/26	NS	6/21

+, Positive; −, negative; ALT, alanine aminotransferase; HAV, hepatitis A virus; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; HEV, hepatitis E virus; IgG, immunoglobulin G; NS, not significant.

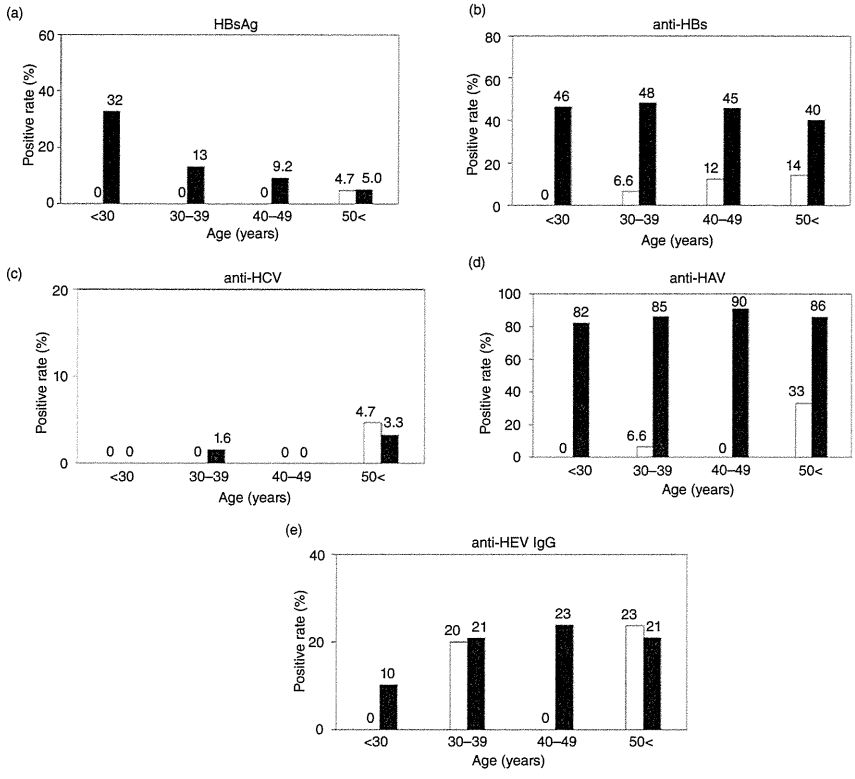

Figure 1 Hepatitis virus markers in study subjects according to age. (a) Hepatitis B surface antigen (HBsAg); (b) anti-HBs antibody; (c) anti-hepatitis C virus (HCV) antibody; (d) anti-hepatitis A virus (HAV) antibody; and (e) anti-hepatitis E virus (HEV) immunoglobulin (Ig)G antibody. White bar, indigenous Japanese; black bar, Chinese staying in Japan. Positive rates (%) are indicated.

respectively). There were no differences between each age group of Chinese subjects and also no differences between each age group of indigenous Japanese subjects in the present study (Fig. 1b).

There were no significant differences in anti-HCV positive rates in each age group of Chinese subjects or in each age group of Japanese indigenous subjects. There were also no significant differences in anti-HCV positive rates of each age group between Chinese and Japanese groups (Fig. 1c).

The positive rate of anti-HAV in subjects under 30 years was lower than in those over 50 years in the indigenous Japanese group ($P = 0.030$) (Fig. 1d). There were no differences among the respective age groups in Chinese subjects. Among the same age groups, the positive rates of anti-HAV in Chinese subjects were higher than those in indigenous Japanese subjects ($P < 0.0001$, each) (Fig. 1d).

There were no significant differences of anti-HEV IgG positive rates in each age group of Japanese indigenous subjects. As for Chinese subjects, there was a difference in anti-HEV IgG positive rate between the groups under 30 years and those in their 40s ($P = 0.029$). There were no significant differences in anti-HEV positive rates of each age group between the Chinese and Japanese groups (Fig. 1e).

Prevalence of hepatitis virus markers in Chinese subjects according to birthplace

Next, we examined the prevalence of hepatitis virus markers in Chinese subjects according to birthplace (Tables 3,4). Although the number of study subjects was limited, the prevalence of anti-HBs, anti-HCV, anti-HAV and anti-HEV IgG was quite similar in Chinese subjects independent of their place of birth. Interestingly, the HBsAg carrier rate was higher in the patients from East China than in those from North-East China (Table 4, $P < 0.0001$). In the background between these two areas (Table 3), young age, male dominance and longer term stays from East China were more than those from North-East China ($P < 0.0001$, $P = 0.029$ and $P < 0.0001$, respectively). As for risk factors of hepatitis virus infection, a history of surgery was seen more frequently in those from North China ($P = 0.023$, Table 3). We determined HBV genotypes in 57 of 63 HBsAg positive subjects and revealed that HBV genotype B was more common in those from East China than in those from North-East China ($P = 0.013$, Table 4). We also

Table 3 Background and risk factors of hepatitis virus infection in Chinese subjects staying in Japan: comparison with indigenous Japanese subjects

Birthplace	Chinese staying in Japan							Japanese
	North China	Central China	South China	East China	North-East China	South-West China	Hong Kong and Taiwan	Indigenous
No. of patients	32	9	1	180	331	10	5	55
Age, years	53 ± 13	40 ± 14	39	41 ± 12	50 ± 13	42 ± 11	60 ± 13	45 ± 15
Sex (M/F)	14/18	8/1	0/1	95/85	140/191	4/6	3/2	28/27
ALT (IU/L)	29 ± 53	22 ± 12	10	26 ± 46	26 ± 47	20 ± 9.8	20 ± 9.8	25 ± 19
Platelets ($\times 10^4/mm^3$)	22 ± 5.2	22 ± 7.3	27	22 ± 5.5	22 ± 6	21 ± 6.5	21 ± 6.5	24 ± 5
Length of their stay (days)	137 ± 90	66 ± 71	46	80 ± 68	113 ± 75	94 ± 76	192 ± 103	
Family history of liver diseases (+/−)	2/30	0/9	0/1	14/162	21/304	0/10	0/5	2/53
History (+/−)								
Transfusion	0/32	1/8	0/1	3/177	6/324	1/9	0/5	2/53
Surgery	7/25	1/8	0/1	14/166	50/280	1/9	0/5	8/43
Drug abuse	0/32	0/9	0/1	0/180	0/330	0/10	0/5	0/54
Tattoo	0/32	0/9	0/1	0/18	1/329	0/10	0/5	0/5

+, Positive; −, negative; ALT, alanine aminotransferase.

Table 4 Hepatitis virus markers in study subjects according to birthplace in Chinese subjects staying in Japan: comparison with indigenous Japanese subjects

Birthplace	Chinese staying in Japan							Japanese
	North China	Central China	South China	East China	North-East China	South-West China	Hong Kong and Taiwan	Indigenous
No. of patients	32	9	1	180	331	10	5	55
Hepatitis virus markers: +/−								
HBsAg	5/27	1/8	0/1	37/142	17/311	1/9	1/4	1/54
HBV genotype (B/C)	1/4	0/1	0/0	12/20	0/16	1/0	0/1	0/1
Anti-HBs	10/22	4/5	0/1	83/95	158/172	5/5	0/5	5/50
Anti-HCV	0/32	0/9	0/1	3/177	7/320	0/10	0/5	1/54
Anti-HAV	25/7	8/1	1/0	147/29	315/15	9/1	5/0	8/47
Anti-HEV IgG	10/22	2/7	0/1	41/138	60/270	5/5	2/3	8/47

+, Positive; −, negative; ALT, alanine aminotransferase; HAV, hepatitis A virus; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; HEV, hepatitis E virus; IgG, immunoglobulin G; NS, not significant.

determined HCV genotype by direct sequencing HCV core region in two cases from North-East China and one indigenous Japanese, with all three showing genotype 2a (data not shown).

DISCUSSION

THE PRESENT STUDY revealed that Chinese subjects staying in Tokyo have more immunity against HAV and HBV than indigenous Japanese subjects and that approximately 10% of Chinese subjects staying in Tokyo are HBsAg carriers. The HBsAg carrier rate seems to be higher in patients from East China than those from North-East China (Table 4). This might be useful to see the Chinese patients from these areas in clinical practices. There have been several reports about the HBsAg carrier rates of East China[16,17] and North-East China.[13,18] Hepatitis B vaccine was first recommended for routine vaccination of infants in China in 1992.[19] Because of high vaccine prices and user fees charged to parents by local health departments for vaccine purchase and administration, until 2002, infant hepatitis vaccination occurred primarily in large cities of wealthier eastern provinces.[19] In the 2004 survey, estimated vaccine coverage was higher in East China than in North-East China.[19] It is a possible reason why the difference in HBsAg prevalence between these areas was observed in the present study. We do not know the exact reason for this difference, and we consider that further studies will be needed.

Several medical institutes at which mostly Chinese gather have existed in Japan. Kyowa Clinic, one such facility, is located in Okachimachi, Tokyo, an important juncture of traffic networks. Because Japanese newspapers advertise this clinic, and the doctor sees the patients using both Chinese and Japanese languages, this outpatient-only clinic is known to Chinese subjects' staying in Japan. The patients of this clinic consist of 90% Chinese and 10% Japanese. Most of the less than 65-year-old male Chinese patients are cooks in Chinese restaurants, interior decorators and students, most of the less than 65-year-old female Chinese patients are housewives and students, and most of the Chinese patients 65 years or older are unemployed. Most of the Japanese patients are employees of small businesses and residents near this clinic. The present study has an authentic potential in terms of the clinical practice being different from previous studies, such as those concerning blood donors, in spite of the population selection of the present study seeming unnatural. Although selection biasness of patients with Japanese and Chinese background might exist, we included these Japanese patients, who come to the same clinic as controls to compare with Chinese in the present study. Although the number of hepatitis cases is decreasing, hepatitis is still a major health problem in Japan.[5,8,10,20] In China as well, hepatitis is a major public health burden.[13] As more foreigners take up residence in Japan, we are likely to see more Chinese patients in clinical practice, as approximately one-third of such foreigners come from China.[14] The present study might provide us with important information.

The number of cases of adult hepatitis A has been decreasing in Japan in accordance with socioeconomic and sanitation improvements.[9,10] In 1986, a national prevention program was launched in Japan with selective vaccination of babies born to carrier mothers with hepatitis B e antigen (HBeAg).[21] In 1995, this was extended to babies born to HBeAg negative carrier mothers. As a result, the prevalence of HBsAg among younger people born since 1986 has decreased dramatically.[21,22] Because there are no universal vaccination programs against HAV or HBV in Japan, HAV and HBV infections are still seen as important issues.[10]

Hepatitis A virus is a single-stranded RNA virus and usually spreads via the fecal–oral route, similarly to HEV. Of interest is the fact that the distribution of anti-HEV IgG among Chinese subjects staying in Tokyo is similar to that of indigenous Japanese subjects, although the prevalence of anti-HAV in Chinese staying in Tokyo is higher than that of indigenous Japanese (Fig. 1d,e). This may be related to differences in infectious routes of transmission of these two viruses or in differences of HAV vaccination between the two countries, as a certain number of HAV-vaccinated young Chinese adults seemed to be included in the present study.[23,24] In any event, a large proportion of Chinese adults seem to be protected by latent infection or immunization against HAV.[13,25]

The positive rate of anti-HEV IgG in the Kanto metropolitan area of Japan was previously reported as 8.6% in qualified blood donors[12] and 6.5% in health checkups.[26] In general, the positive rate of anti-HEV IgG in China has been recognized to be higher than that in Japan,[27] and the same report described a positive rate of anti-HEV IgG of more than 20% in indigenous Japanese aged 70 years or older. In the present study, the mean age of indigenous Japanese was 45 years (Table 1), and anti-HEV IgG positive indigenous Japanese numbered three in their 30s, one in their 50s, three in their 60s and one in their 70s, with the anti-HEV IgG positive rate being higher than in previous reports.[12,25,27] In most areas of

Japan, the positive rate of anti-HEV IgG in males was higher than that in females. We do not know the exact reasons why our anti-HEV IgG patients were not male-dominant (Table 2). The population selection of the present study may not be unbiased. However, as it seems that Japanese females have in recent years developed a taste for broiled pig innards on skewers compared to before, the potential of HEV infection is likely to grow, and greater attention should also be paid to Japanese females.

As HCV is a blood-borne RNA virus, and blood screening for HCV is a standard procedure in Japan, the distribution of anti-HCV of indigenous Japanese subjects is similar to that of Chinese subjects staying in Tokyo. HBV is an incomplete double-stranded DNA virus that infects through blood products and sexual contact as well as mother-to-baby transmission. The differences in the distribution of anti-HBs may be dependent on a different HBV vaccination status or different past HBV infection.

In conclusion, indigenous Japanese subjects have less immunity against HAV and HBV. As the HBV carrier rate is higher in Chinese subjects, this should receive some attention in clinical practice, and it might be important to control hepatitis viruses in Chinese subjects when they are seen by doctors in Japan.

ACKNOWLEDGMENTS

THIS WORK WAS supported by a grant from the Japan Society of Hepatology (T. K.), a grant from Chiba University Young Research-Oriented Faculty Member Development Program in Bioscience Areas (T. K.) and a grant from the Ministry of Health, Labor and Welfare of Japan (O. Y.).

REFERENCES

1 Dagan R, Leventhal A, Anis E, Slater P, Ashur Y, Shouval D. Incidence of hepatitis A in Israel following universal immunization of toddlers. *JAMA* 2005; **294**: 202–10.
2 Huang MA, Lok AS. Natural history of hepatitis B and outcomes after liver transplantation. *Clin Liver Dis* 2003; **7**: 521–36.
3 Saito I, Miyamura T, Ohbayashi A *et al.* Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc Natl Acad Sci U S A* 1990; **87**: 6547–9.
4 Kanda T, Yokosuka O, Imazeki F, Saisho H. Acute hepatitis C virus infection, 1986–2001: a rare cause of fulminant hepatitis in Chiba, Japan. *Hepatogastroenterology* 2004; **51**: 556–8.
5 Tanaka J, Kumagai J, Katayama K *et al.* Sex- and age-specific carriers of hepatitis B and C viruses in Japan estimated by the prevalence in the 3,485,648 first-time blood donors during 1995–2000. *Intervirology* 2004; **47**: 32–40.
6 FitzSimons D, Hendrickx G, Vorsters A, Damme PV. Hepatitis A and E: update on prevention and epidemiology. *Vaccine* 2010; **28**: 583–8.
7 Sumi H, Yokosuka O, Seki N *et al.* Influence of hepatitis B virus genotypes on the progression of chronic type B liver disease. *Hepatology* 2003; **37**: 19–26.
8 Kanda T, Imazeki F, Yokosuka O. New antiviral therapies for chronic hepatitis C. *Hepatol Int* 2010; **19**: 548–61.
9 Kanda T, Jeong SH, Imazeki F, Fujiwara K, Yokosuka O. Analysis of 5′ nontranslated region of hepatitis A viral RNA genotype I from South Korea: comparison with disease severities. *PLoS ONE* 2010; **5**: e15139.
10 Miyamura T, Ishii K, Kanda T *et al.* Possible widespread presence of hepatitis A subgenotype IIIA in Japan: recent trend of hepatitis A causing acute liver failure. *Hepatol Res* 2012; **42**: 248–53.
11 Jacobsen KH, Wiersma ST. Hepatitis A virus seroprevalence by age and world region, 1990 and 2005. *Vaccine* 2010; **28**: 6653–7.
12 Takeda H, Matsubayashi K, Sakata H *et al.* A nationwide survey for prevalence of hepatitis E virus antibody in qualified blood donors in Japan. *Vox Sang* 2010; **99**: 307–13.
13 Lu J, Zhou Y, Lin X *et al.* General epidemiological parameters of viral hepatitis A, B, C, and E in six regions of China: a cross-sectional study in 2007. *PLoS ONE* 2009; **4**: e8467.
14 The Ministry of Justice. [Cited 21 Dec 2011.] Available from URL: http://www.moj.go.jp/nyuukokukanri/kouhou/nyuukokukanri04_00005.html
15 Usuda S, Okamoto H, Iwanari H *et al.* Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. *J Virol Methods* 1999; **80**: 97–112.
16 Zhou YM, Yin ZF, Yang JM *et al.* Risk factors for intrahepatic cholangiocarcinoma: a case-control study in China. *World J Gastroenterol* 2008; **14**: 632–5.
17 Yuan Q, Ou SH, Chen CR *et al.* Molecular characteristics of occult hepatitis B virus from blood donors in southeast China. *J Clin Microbiol* 2010; **48**: 357–62.
18 Chen SJ, Zhao YX, Fang Y *et al.* Viral deletions among healthy young Chinese adults with occult hepatitis B virus infection. *Virus Res* 2012; **163**: 197–201.
19 Centers for Disease Control and Prevention (CDC). Progress in hepatitis B prevention through universal infant vaccination – China, 1997–2006. *MMWR Morb Mortal Wkly Rep* 2007; **56**: 441–5.
20 Tanaka H, Imai Y, Hiramatsu N *et al.* Declining incidence of hepatocellular carcinoma in Osaka, Japan, from 1990 to 2003. *Ann Intern Med* 2008; **148**: 820–6.
21 Tamada Y, Yatsuhashi H, Masaki N *et al.* Hepatitis B virus strains of subgenotype A2 with an identical sequence spreading rapidly from the capital region to all over Japan