

IV. 研究成果の刊行物・別刷

RESEARCH ARTICLE

Open Access

No association for Chinese HBV-related hepatocellular carcinoma susceptibility SNP in other East Asian populations

Hiromi Sawai^{1*}, Nao Nishida^{1,2}, Hamdi Mbarek³, Koichi Matsuda³, Yoriko Mawatari², Megumi Yamaoka¹, Shuhei Hige⁴, Jong-Hon Kang⁵, Koichi Abe⁶, Satoshi Mochida⁷, Masaaki Watanabe⁸, Masayuki Kurosaki⁹, Yasuhiro Asahina⁹, Namiki Izumi⁹, Masao Honda¹⁰, Shuichi Kaneko¹⁰, Eiji Tanaka¹¹, Kentaro Matsuura¹², Yoshito Itoh¹³, Eiji Mita¹⁴, Masaaki Korenaga¹⁵, Keisuke Hino¹⁵, Yoshikazu Murawaki¹⁶, Yoichi Hiasa¹⁷, Tatsuya Ide¹⁸, Kiyooki Ito², Masaya Sugiyama², Sang Hoon Ahn¹⁹, Kwang-Hyub Han¹⁹, Jun Yong Park¹⁹, Man-Fung Yuen²⁰, Yusuke Nakamura³, Yasuhito Tanaka¹², Masashi Mizokami² and Katsushi Tokunaga¹

Abstract

Background: A recent genome-wide association study (GWAS) using chronic HBV (hepatitis B virus) carriers with and without hepatocellular carcinoma (HCC) in five independent Chinese populations found that one SNP (rs17401966) in *KIF1B* was associated with susceptibility to HCC. In the present study, a total of 580 HBV-derived HCC cases and 1351 individuals with chronic hepatitis B (CHB) or asymptomatic carrier (ASC) were used for replication studies in order to evaluate the reported association with HBV-derived HCC in other East Asian populations.

Results: We did not detect any associations between rs17401966 and HCC in the Japanese cohorts (replication 1: OR = 1.09, 95 % CI = 0.82-1.43; replication 2: OR = 0.79, 95 % CI = 0.54-1.15), in the Korean cohort (replication 3: OR = 0.95, 95 % CI = 0.66-1.36), or in the Hong Kong Chinese cohort (replication 4: OR = 1.17, 95 % CI = 0.79-1.75). Meta-analysis using these cohorts also did not show any associations with $P = 0.97$.

Conclusions: None of the replication cohorts showed associations between rs17401966 and HBV-derived HCC. This may be due to differences in the genetic diversity among the Japanese, Korean and Chinese populations. Other reasons could be the high complexity of multivariate interactions between the genomic information and the phenotype that is manifesting. A much wider range of investigations is needed in order to elucidate the differences in HCC susceptibility among these Asian populations.

Keywords: Hepatitis B, hepatocellular carcinoma, candidate SNP, replication study, genome-wide association study

Background

Hepatitis B (HB) is a potentially life-threatening liver infection caused by the hepatitis B virus (HBV), and approximately 360 million people worldwide are thought to be chronically infected with HBV. The clinical course of HBV infection is variable, including acute self-limiting infection, fulminant hepatic failure, inactive carrier state and chronic hepatitis with progression to cirrhosis and

hepatocellular carcinoma (HCC). Although some HBV carriers spontaneously eliminate the virus, 2-10 % of individuals with chronic HB (CHB) develop liver cirrhosis every year, and a subset of these individuals suffer from liver failure or HCC. Around 600,000 new HCC cases are diagnosed annually worldwide, with HCC being relatively common in Asia-Pacific countries and sub-Saharan Africa; more than 70 % of HCC patients are diagnosed in Asia (with 55 % in China) [1]. However, HCC is relatively uncommon in the USA, Europe and Australia [1,2]. The majority of HCC develops in patients with cirrhosis, which is most often attributable

* Correspondence: sawai@m.u-tokyo.ac.jp

¹Department of Human Genetics, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
Full list of author information is available at the end of the article

to chronic HBV infection followed by chronic HCV in the Asia-Pacific region [3].

A recent genome-wide association study (GWAS) using Japanese CHB cases and controls confirmed that 11 SNPs in a region including *HLA-DPA1* and *-DPB1* were associated with CHB [4]. Moreover, a GWAS using chronic HBV carriers with and without HCC in five independent Chinese populations reported that one SNP (rs17401966) in *KIF1B* was associated with HCC susceptibility [5]. In the present study, we performed replication studies using Japanese, Korean and Hong Kong Chinese cases and controls in order to evaluate the reported association with HBV-derived HCC in other East Asian populations.

Results

We performed SNP genotyping of rs17401966 located in the *KIF1B* gene for the purpose of replication analysis of the previous GWAS report [5]. Four distinct cohorts were used for these replication analyses (Table 1). We first examined two independent Japanese case-control samples including 179 cases and 769 controls from Biobank Japan (replication 1), and 142 cases and 251 controls from various hospitals (replication 2). We did not detect any associations between rs17401966 and HCC in the Japanese cohorts (replication 1: OR = 1.09; 95 % CI = 0.82-1.43, replication 2: OR = 0.79; 95 % CI = 0.54-1.15). We further examined Korean case-control samples comprising 164 cases and 144 controls (replication 3) and Hongkongese 94 HCC cases and 187 CHB controls (replication 4), but again did not detect any association (replication 3: OR = 0.95; 95 % CI = 0.66-1.36, replication 4: OR = 1.17; 95 % CI = 0.79-1.75). Logistic regression analysis adjusted for age and gender also did not show any association ($P_{\log} = 0.65, 0.27, 0.11, 0.56$ for each replication

panel). Moreover, we conducted meta-analysis to combine these studies, also not detect any association ($P_{\text{meta}} = 0.97$).

Discussion and conclusions

Zhang et al. [5] reported that SNP rs17401966 was significantly associated with HBV-related HCC (joint OR = 0.61). They conducted a GWAS using 348 cases and 359 controls in a population in Guangxi in southern China, and selected 45 SNPs for the replication study based on the results ($P < 10^{-4}$). In the first replication study, they used 276 cases and 266 controls from Beijing in northern China, and 5 SNPs showed the same direction of association as in the GWAS ($P < 0.05$). They performed a further replication study (of 507 cases and 215 controls) in Jiangsu in eastern China and only one SNP showed the same trend ($P = 3.9 \times 10^{-5}$). Guangdong and Shanghai samples from southern and eastern China were used for further replication studies. The association yielded a p-value of 1.7×10^{-18} on meta-analysis.

We performed four replication analyses using Japanese, Korean and Hong Kong Chinese samples (Table 1). Although sample size of each cohort is smaller than that of the previous GWAS, we conducted meta-analysis of all our study. The result did not show any association between rs17401966 and HBV-derived HCC ($P_{\text{meta}} = 0.97$).

This may be due to differences in genetic diversity among Japanese, Korean and Chinese populations. A maximum-likelihood tree of 126 populations based on 19,934 SNPs showed that Japanese and Korean populations form a monophyletic clade with a 100 % bootstrap value [6]. However, Chinese populations form a paraphyletic clade with two other populations. This indicates that Japanese and Korean populations are genetically closer to one another than the Chinese population.

Table 1 Association between rs17401966 and HBV-derived HCC

cohort	sample size (cases/controls)	cases			controls			HWE p	OR (95 % CI)	P^a	P_{het}^b
		GG	AG	AA	GG	AG	AA				
replication 1	179/769	13	61	105	45	261	463	0.599	1.09	0.578	
(Japan 1)		(7.2)	(34.1)	(58.7)	(5.9)	(33.9)	(60.2)		(0.82-1.43)		
replication 2	142/251	5	46	91	14	91	146	1	0.79	0.212	
(Japan 2)		(3.5)	(32.4)	(64.1)	(5.6)	(36.2)	(58.2)		(0.54-1.15)		
replication 3	164/144	17	59	88	15	55	74	0.616	0.95	0.790	
(Korea)		(10.4)	(36.0)	(53.6)	(10.4)	(38.2)	(51.4)		(0.66-1.36)		
replication 4	94/187	10	39	44	13	80	94	0.767	1.17	0.432	
(Hong Kong)		(10.6)	(41.5)	(46.8)	(6.9)	(42.8)	(50.3)		(0.79-1.75)		
Meta-analysis ^c									0.996	0.965	0.423
									(0.84-1.18)		

^aP value of fisher's exact test for allele model.

^bResult of Breslow-Day test.

^cResults of meta-analysis were calculated by the Mantel-Haenzel method.

We did not find any association with Hong Kong Chinese cohort ($P = 0.43$). Moreover, a study using 357 HCC cases and 354 HBV-positive non-HCC controls in Hong Kong Chinese did not show any significant difference ($P = 0.91$) [7]. Previous population studies have revealed that various Han Chinese populations show varying degrees of admixture between a northern Altaic cluster and a southern cluster of Sino-Tibetan/Tai-Kadai populations in southern China and northern Thailand [6]. Although Hong Kong is located close to the Guangdong (cohort 3 of Zhang et al study), there is great heterogeneity for rs17401966 between Hong Kong cohorts (our study and Chan's study [7]) and Guangdong cohort (our study versus Zhang's study: $P_{\text{het}} = 0.0066$; Chan's study versus Zhang's study: $P_{\text{het}} = 0.035$). This result suggests the existence of other confounding factors, which can differentiate the previous study in China and this study.

One of the possible reasons could be the high complexity of multivariate interactions between the genomic information and the phenotype that is manifesting. HCC development is a multiple process which links to causative factors such as age, gender, environmental toxins, alcohol and drug abuse, higher HBV DNA levels, and HBV genotype variations [8]. The eight HBV genotypes display distinct geographical and ethnic distributions. Genotypes B and C are prevalent in Asia. Specific variations in HBV have been associated with cirrhosis and HCC. These variations include in particular mutations in pre-core region (Pre-C), in basal core promoter (BCP) and in ORF encoding Pre-S1/Pre-S2/S and Pre-C/C. Because there is an overlap between Pre-C or BCP mutations and genotypes, these mutations appear to be more common in genotype C as compared to other genotypes [9].

Aflatoxins are a group of 20 related metabolites and Aflatoxin B1 is the most potent naturally occurring chemical liver carcinogen known. Aflatoxin exposures multiplicatively increase the risk of HCC in people chronically infected with HBV, which illustrates the deleterious impact that even low toxin levels in the diet can have on human health [10–12]. Liu and Wu estimated population risk for aflatoxin-induced HCC around the world [13]. Most cases occur in sub-Saharan Africa, Southeast Asia and China, where populations suffer from both high HBV prevalence and largely uncontrolled exposure to aflatoxin in food. But we could not obtain the information of these confounding factors from both of the previous GWAS study and this study. A much wider range of investigations is thus needed in order to elucidate the differences in HCC susceptibility among these Asian populations.

Methods

Samples

Case and control samples used in this study were collected from Japan, Korea and Hong Kong listed in supplementary

Additional file 1: Table S1. A total of 179 cases and 769 control subjects were analyzed in the first replication study. DNA samples from both CHB controls and HBV-related HCC cases used in this study were obtained from the BioBank Japan at the Institute of Medical Science, the University of Tokyo [14]. Among the BioBank Japan samples, we selected HBsAg-seropositive CHB patients with elevated serum aminotransferase levels for more than six months, according to the guidelines for diagnosis and treatment of chronic hepatitis from The Japan Society of Hepatology (<http://www.jsh.or.jp/medical/guidelines/index.html>). The mean (and standard deviation; SD) age was 62.0 (9.4) years for the cases and 54.7 (13.5) years for the controls. The second Japanese replication sample sets for the cases ($n = 142$) and controls ($n = 251$) study were obtained from 16 hospitals. The case samples for the second replication included 142 HCC patients and the controls included 135 CHB patients and 116 asymptomatic carriers (ASC). The mean (SD) age was 61.3 (10.2) years for the cases and 56.2 (10.9) years for the controls. The Korean replication samples were collected from Yonsei University College of Medicine. The third replication set was composed of 165 HCC patients and 144 CHB patients. The mean (SD) age was 52.2 (8.9) and 37.3 (11.3) years for the cases and controls, respectively. The samples in Hong Kong were collected from the University of Hong Kong, Queen Mary Hospital. The fourth replication set was composed of 94 HCC patients and 187 CHB patients. The mean (SD) age was 58.0 (10.5) and 56.9 (8.3) years for the cases and controls, respectively. All participants provided written informed consent. This research project was approved by the Research Ethics Committees at the Institute of Medical Science and the Graduate School of Medicine, the University of Tokyo, Yonsei University College of Medicine, the University of Hong Kong, National Center for Global Health and Medicine, Hokkaido University Graduate School of Medicine, Teine Keijinkai Hospital, Iwate Medical University, Saitama Medical University, Kitasato University School of Medicine, Musashino Red Cross Hospital, Kanazawa University Graduate School of Medicine, Shinshu University School of Medicine, Nagoya City University Graduate School of Medical Sciences, Kyoto Prefectural University of Medicine, National Hospital Organization Osaka National Hospital, Kawasaki Medical College, Tottori University, Ehime University Graduate School of Medicine, and Kurume University School of Medicine.

SNP Genotyping

For the first replication samples, we genotyped rs17401966 using PCR-based Invader assay (Third Wave Technologies, Madison, WI) [15], and for the second, third and fourth replication samples, we used TaqMan genotyping assay (Applied Biosystems, Carlsbad, CA). In the TaqMan SNP

genotyping assay, PCR amplification was performed in a 5- μ l reaction mixture containing 1 μ l of genomic DNA, 2.5 μ l of KAPA PROBE FAST qPCR Master Mix (Kapa Biosystems, Woburn, MA), and 40 x TaqMan SNP Genotyping Assay probe (ABI) for this SNP. QPCR thermal cycling was performed as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The SNP call rate of each replication panel was 100 %, 100 %, 99.7 % and 99.6 %.

Statistical analysis

We performed Hardy-Weinberg equilibrium test for the case and control samples in each replication study. Fisher's exact test was applied to two-by-two contingency tables for three different genetic models; allele frequency, dominant and recessive model. Odds ratios and confidence intervals were calculated using the major alleles as references. Meta-analysis was conducted using the Mantel-Haenszel method. Heterogeneity among studies was examined by using the Breslow-Day test. Genotype-phenotype association for the SNP rs17401966 was assessed using logistic regression analysis adjusted for age and gender in plink 1.07 (<http://pngu.mgh.harvard.edu/~purcell/plink/index.shtml>).

Additional file

Additional file 1: Table S1. Samples used in this study.

Abbreviations

HB: Hepatitis b; HBV: Hepatitis b virus; HCC: Hepatocellular carcinoma; CHB: Chronic hepatitis b; HCV: Hepatitis c virus; GWAS: Genome-wide association study; ASC: Asymptomatic carrier.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

This study was supported by a grant-in-aid from the Ministry of Health, Labour, and Welfare of Japan (H23-kanen-005), and Japan Science and Technology Agency (09038024). We thank Dr. Minae Kawashima to giving us technical advices.

Author details

¹Department of Human Genetics, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. ²The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa, Japan. ³Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, Tokyo, Japan. ⁴Department of Internal Medicine, Hokkaido University Graduate School of Medicine, Sapporo, Japan. ⁵Department of Internal Medicine, Teine Keijinkai Hospital, Sapporo, Japan. ⁶First Department of Internal Medicine, Iwate Medical University, Iwate, Japan. ⁷Division of Gastroenterology and Hepatology, Internal Medicine, Saitama Medical University, Saitama, Japan. ⁸Department of Gastroenterology, Kitasato University School of Medicine, Sagami-hara, Kanagawa, Japan. ⁹Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan. ¹⁰Department of Gastroenterology, Kanazawa University Graduate School of Medicine, Kanazawa, Japan. ¹¹Department of Medicine, Shinshu University School of Medicine, Matsumoto, Japan. ¹²Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan. ¹³Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Kyoto, Japan.

¹⁴National Hospital Organization Osaka National Hospital, Osaka, Japan.

¹⁵Division of Hepatology and Pancreatology, Kawasaki Medical College, Kurashiki, Japan.

¹⁶Second department of Internal Medicine, Faculty of Medicine, Tottori University, Yonago, Japan.

¹⁷Department of Gastroenterology and Metabolism, Ehime University Graduate School of Medicine, Ehime, Japan.

¹⁸Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, Fukuoka, Japan.

¹⁹Department of International Medicine, Yonsei University College of Medicine, Seoul, Korea.

²⁰Department of Medicine, the University of Hong Kong, Queen Mary Hospital, Hong Kong, China.

Author contributions

Study design and discussion: H.S., N.N., Y.T., Ko.M., M.M., K.T.; sample collection: Y.T., Ko.M., Y.N., S.H.A., K.H.H., J.Y.P., M.F.Y., S.H., J.H.K., K.A., S.M., M.W., M.Ku., Y.A., N. I., M.H., S.K., E.T., Ke.M., Y.I., E.M., M.Ko., K.H., Y.Mu., Y.H., T.I., K.I., M.S., M.M.; genotyping: H.S., Y.M., M.Y., H.M.; statistical analysis: H.S.; manuscript writing: H.S., N.N., Y.T., M.M., K.T. All authors read and approved the final manuscript.

Received: 2 March 2012 Accepted: 19 June 2012

Published: 19 June 2012

References

1. Parkin DM, Bray F, Ferlay J, Pisani P: **Global cancer statistics, 2002.** *CA: a cancer journal for clinicians* 2005, **55**(2):74–108.
2. Parkin DM: **Global cancer statistics in the year 2000.** *The lancet oncology* 2001, **2**(9):533–543.
3. Marrero CR, Marrero JA: **Viral hepatitis and hepatocellular carcinoma.** *Archives of medical research* 2007, **38**(6):612–620.
4. Kamatani Y, Wattanapokayakit S, Ochi H, Kawaguchi T, Takahashi A, Hosono N, Kubo M, Tsunoda T, Kamatani N, Kumada H, et al: **A genome-wide association study identifies variants in the HLA-DP locus associated with chronic hepatitis B in Asians.** *Nature genetics* 2009, **41**(5):591–595.
5. Zhang H, Zhai Y, Hu Z, Wu C, Qian J, Jia W, Ma F, Huang W, Yu L, Yue W, et al: **Genome-wide association study identifies 1p36.22 as a new susceptibility locus for hepatocellular carcinoma in chronic hepatitis B virus carriers.** *Nature genetics* 2010, **42**(9):755–758.
6. Abdulla MA, Ahmed I, Assawamakin A, Bhak J, Brahmachari SK, Calacal GC, Chaurasia A, Chen CH, Chen J, Chen YT et al: **Mapping human genetic diversity in Asia.** *Science (New York, NY)* 2009, **326**(5959):1541–1545.
7. Chan KY, Wong CM, Kwan JS, Lee JM, Cheung KW, Yuen MF, Lai CL, Poon RT, Sham PC, Ng IO: **Genome-wide association study of hepatocellular carcinoma in Southern Chinese patients with chronic hepatitis B virus infection.** *PLoS One* 2011, **6**(12):e28798.
8. Sherman M: **Hepatocellular carcinoma: epidemiology, surveillance, and diagnosis.** *Semin Liver Dis* 2010, **30**(1):3–16.
9. Yang HJ, Yeh SH, Chen PJ, Iloeje UH, Jen CL, Su J, Wang LY, Lu SN, You SL, Chen DS, et al: **Associations between hepatitis B virus genotype and mutants and the risk of hepatocellular carcinoma.** *J Natl Cancer Inst* 2008, **100**(16):1134–1143.
10. Qian GS, Ross RK, Yu MC, Wogan GN, Qian GS, Tu JT, Groopman JD, Gao YT, Groopman JD: **A follow-up study of urinary markers of aflatoxin exposure and liver cancer risk in Shanghai, People's Republic of China.** *Cancer Epidemiol Biomarkers Prev* 1994, **3**(1):3–10.
11. Ross RK, Yuan JM, Yu MC, Wogan GN, Qian GS, Tu JT, Groopman JD, Gao YT, Henderson BE: **Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma.** *Lancet* 1992, **339**(8799):943–946.
12. Wang LY, Hatch M, Chen CJ, Levin B, You SL, Lu SN, Wu MH, Wu WP, Wang LW, Wang Q, et al: **Aflatoxin exposure and risk of hepatocellular carcinoma in Taiwan.** *International journal of cancer* 1996, **67**(5):620–625.
13. Liu Y, Wu F: **Global burden of aflatoxin-induced hepatocellular carcinoma: a risk assessment.** *Environmental health perspectives* 2010, **118**(6):818–824.
14. Nakamura Y: **The BioBank Japan Project.** *Clin Adv Hematol Oncol* 2007, **5**(9):696–697.
15. Ohnishi Y, Tanaka T, Ozaki K, Yamada R, Suzuki H, Nakamura Y: **A high-throughput SNP typing system for genome-wide association studies.** *Journal of human genetics* 2001, **46**(8):471–477.

doi:10.1186/1471-2350-13-47

Cite this article as: Sawai et al.: No association for Chinese HBV-related hepatocellular carcinoma susceptibility SNP in other East Asian populations. *BMC Medical Genetics* 2012 **13**:47.

Genome-Wide Association Study Confirming Association of HLA-DP with Protection against Chronic Hepatitis B and Viral Clearance in Japanese and Korean

Nao Nishida^{1,2*}, Hiromi Sawai², Kentaro Matsuura³, Masaya Sugiyama¹, Sang Hoon Ahn⁴, Jun Yong Park⁴, Shuhei Hige⁵, Jong-Hon Kang⁶, Kazuyuki Suzuki⁷, Masayuki Kurosaki⁸, Yasuhiro Asahina⁸, Satoshi Mochida⁹, Masaaki Watanabe¹⁰, Eiji Tanaka¹¹, Masao Honda¹², Shuichi Kaneko¹², Etsuro Orito¹³, Yoshito Itoh¹⁴, Eiji Mita¹⁵, Akihiro Tamori¹⁶, Yoshikazu Murawaki¹⁷, Yoichi Hiasa¹⁸, Isao Sakaida¹⁹, Masaaki Korenaga²⁰, Keisuke Hino²⁰, Tatsuya Ide²¹, Minae Kawashima², Yoriko Mawatari^{1,2}, Megumi Sageshima², Yuko Ogasawara², Asako Koike²², Namiki Izumi⁸, Kwang-Hyub Han⁴, Yasuhito Tanaka³, Katsushi Tokunaga², Masashi Mizokami¹

1 Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa, Chiba, Japan, **2** Department of Human Genetics, The University of Tokyo, Bunkyo-ku, Tokyo, Japan, **3** Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Aichi, Japan, **4** Department of Internal Medicine, Yonsei University College of Medicine, Seoul, South Korea, **5** Department of Internal Medicine, Hokkaido University Graduate School of Medicine, Sapporo, Japan, **6** Department of Internal Medicine, Teine Keijinkai Hospital, Sapporo, Japan, **7** Department of Gastroenterology and Hepatology, Iwate Medical University, Morioka, Japan, **8** Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan, **9** Division of Gastroenterology and Hepatology, Saitama Medical University, Saitama, Japan, **10** Department of Gastroenterology, Kitasato University School of Medicine, Sagami-hara, Kanagawa, Japan, **11** Department of Medicine, Shinshu University School of Medicine, Matsumoto, Japan, **12** Department of Gastroenterology, Kanazawa University Graduate School of Medicine, Kanazawa, Japan, **13** Department of Gastroenterology, Nagoya Daini Red Cross Hospital, Nagoya, Japan, **14** Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Kyoto, Japan, **15** Department of Gastroenterology and Hepatology, National Hospital Organization Osaka National Hospital, Osaka, Japan, **16** Department of Hepatology, Osaka City University Graduate School of Medicine, Osaka, Japan, **17** Second Department of Internal Medicine, Faculty of Medicine, Tottori University, Yonago, Japan, **18** Department of Gastroenterology and Metabolism, Ehime University Graduate School of Medicine, Ehime, Japan, **19** Gastroenterology and Hepatology, Yamaguchi University Graduate School of Medicine, Yamaguchi, Japan, **20** Division of Hepatology and Pancreatology, Kawasaki Medical College, Kurashiki, Japan, **21** Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, Fukuoka, Japan, **22** Central Research Laboratory, Hitachi Ltd., Kokubunji, Tokyo, Japan

Abstract

Hepatitis B virus (HBV) infection can lead to serious liver diseases, including liver cirrhosis (LC) and hepatocellular carcinoma (HCC); however, about 85–90% of infected individuals become inactive carriers with sustained biochemical remission and very low risk of LC or HCC. To identify host genetic factors contributing to HBV clearance, we conducted genome-wide association studies (GWAS) and replication analysis using samples from HBV carriers and spontaneously HBV-resolved Japanese and Korean individuals. Association analysis in the Japanese and Korean data identified the *HLA-DPA1* and *HLA-DPB1* genes with $P_{meta} = 1.89 \times 10^{-12}$ for rs3077 and $P_{meta} = 9.69 \times 10^{-10}$ for rs9277542. We also found that the *HLA-DPA1* and *HLA-DPB1* genes were significantly associated with protective effects against chronic hepatitis B (CHB) in Japanese, Korean and other Asian populations, including Chinese and Thai individuals ($P_{meta} = 4.40 \times 10^{-19}$ for rs3077 and $P_{meta} = 1.28 \times 10^{-15}$ for rs9277542). These results suggest that the associations between the *HLA-DP* locus and the protective effects against persistent HBV infection and with clearance of HBV were replicated widely in East Asian populations; however, there are no reports of GWAS in Caucasian or African populations. Based on the GWAS in this study, there were no significant SNPs associated with HCC development. To clarify the pathogenesis of CHB and the mechanisms of HBV clearance, further studies are necessary, including functional analyses of the *HLA-DP* molecule.

Citation: Nishida N, Sawai H, Matsuura K, Sugiyama M, Ahn SH, et al. (2012) Genome-Wide Association Study Confirming Association of HLA-DP with Protection against Chronic Hepatitis B and Viral Clearance in Japanese and Korean. PLoS ONE 7(6): e39175. doi:10.1371/journal.pone.0039175

Editor: Anand S. Mehta, Drexel University College of Medicine, United States of America

Received: February 1, 2012; **Accepted:** May 16, 2012; **Published:** June 21, 2012

Copyright: © 2012 Nishida et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by Grants-in-Aid from the Ministry of Health, Labour, and Welfare of Japan (H22-kanen-005, H23-kanen-005), the Japan Science and Technology Agency (09038024), and the Miyakawa Memorial Research Foundation. Partial support by Grant-in-Aid for Young Scientists (B) (22710191) from the Ministry of Education, Culture, Sports, Science, and Technology is also acknowledged. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: AK is an employee of the Central Research Laboratory, Hitachi Ltd. There are no patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials, as detailed online in the guide for authors.

* E-mail: nishida-75@umin.ac.jp

Introduction

Overall, one-third of the world's population (2.2 billion) is infected with hepatitis B virus (HBV), and about 15% of these are chronic carriers. About 75% of the chronic carriers live in the east-south Asia and east pacific area, and there are 1.3–1.5 million chronic carriers living in Japan [1]. Of chronic carriers, 10–15% develop liver cirrhosis (LC), liver failure and hepatocellular carcinoma (HCC), and the remaining individuals eventually achieve a state of nonreplicative infection, resulting in hepatitis B surface antigen (HBsAg) negative and hepatitis B core antibody (anti-HBc) positive, i.e. HBV-resolved individuals [2–3]. In Japan, although the major route of HBV transmission was perinatal transmission and horizontal transmission in early childhood, infant HBV carriers have successfully been reduced since 1986 through a selective vaccination policy by the Japanese government [4–7]. However, the prevalence of HBV genotype A in acute HBV (AHB) infection has increased markedly since 2000, reaching approximately 52% in 2008 due to the lack of a universal HB vaccination, and around 10% of AHB cases could be persistent infection [8–9]. Viral factors, as well as host factors, are thought to be associated with persistent HB infection.

In 2009, significant associations between chronic hepatitis B (CHB) and a region including *HLA-DPA1* and *HLA-DPB1* were identified using 786 Japanese individuals having CHB and 2,201 control individuals through a two-stage genome-wide association study (GWAS) [10]. The same group was also subjected to a second GWAS using a total of 2,667 Japanese persistent HBV infection cases and 6,496 controls, which confirmed significant associations between the *HLA-DP* locus and CHB, in addition to associations with another two SNPs located in the genetic region including the *HLA-DQ* gene [11]. The associations between *HLA-DP* variants with HBV infection were replicated in other Asian populations, including Thai and Han Chinese individuals [10,12–13]. With regard to HBV clearance, the association between the human leukocyte antigen (HLA) class II allele and clearance of HBV was confirmed by the candidate gene approach in African, Caucasian and Asian populations [14–18]. However, in a previous GWAS using samples of Japanese CHB and control individuals, the clinical data on HBV exposure in the control individuals were unknown, and this may have led to bias. Moreover, there have been no reports of GWAS using samples from HBV carriers and HBV-resolved individuals to identify host genetic factors associated with HBV clearance other than HLA class II molecules.

Here, we performed a GWAS using samples from Japanese HBV carriers, healthy controls and spontaneously HBV-resolved individuals in order to confirm or identify the host genetic factors related to CHB and viral clearance. In the subsequent replication analysis, we validated the associated SNPs in the GWAS using two independent sets of Japanese and Korean individuals. In our study, healthy controls were randomly selected with clinically no evidence of HBV exposure, therefore, HBV-resolved individuals were prepared to clearly identify the host genetic factors related with CHB or HBV clearance.

Results

Protective Effects Against Chronic Hepatitis B in Japanese and Korean Individuals

In this study, we conducted a GWAS using samples from 181 Japanese HBV carriers (including asymptomatic carriers (ASC)), CHB cases, LC cases and HCC cases, based on the criteria described in Materials and Methods) and 184 healthy controls in

order to identify the host genetic factors related to progression of CHB. All samples were genotyped using a genome-wide SNP typing array (Affymetrix Genome-Wide Human SNP Array 6.0 for 900 K SNPs). Figure 1a shows a genome-wide view of the single point association data based on allele frequencies using the SNPs that met the following filtering criteria: (i) SNP call rate $\geq 95\%$; (ii) minor allele frequency (MAF) $\geq 1\%$ for HBV carriers and healthy controls; and (iii) no deviation from Hardy-Weinberg equilibrium (HWE) $P \geq 0.001$ in healthy controls. We identified significant associations of protective effects against CHB with two SNPs (rs3077 and rs9277542) using the allele frequency model, both of which are located in the 3' UTR of *HLA-DPA1* and in the sixth exon of *HLA-DPB1*, respectively (rs3077, $P = 1.14 \times 10^{-7}$, and rs9277542, $P = 5.32 \times 10^{-8}$, respectively). The association for rs9277542 reached a genome-wide level of significance in the GWAS panel (Bonferroni criterion $P < 8.36 \times 10^{-8}$ (0.05/597,789)).

In order to validate the results of GWAS, a total of 32 SNPs, including the associated two SNPs (rs3077 and rs9277542), were selected for replication in two independent sets of HBV carriers and healthy controls (replication-1:256 Japanese HBV carriers and 236 Japanese healthy controls; and replication-2:344 Korean HBV carriers and 151 Korean healthy controls; Table 1). The associations for the original significant SNP (rs9277542) and marginal SNP (rs3077) on GWAS were replicated in both replication sets [replication-1 (Japanese); rs3077, $P = 2.70 \times 10^{-8}$, OR = 0.48 and rs9277542, $P = 3.33 \times 10^{-6}$, OR = 0.54; replication-2 (Korean); rs3077, $P = 2.08 \times 10^{-6}$, OR = 0.47 and rs9277542, $P = 8.29 \times 10^{-5}$, OR = 0.54, Table 2]. We conducted meta-analysis to combine these studies using the DerSimonian Laird method (random effects model) to incorporate variation among studies. As shown in Table 2, the odds ratios were quite similar across the three studies (GWAS and two replication studies) and no heterogeneity was observed ($P_{het} = 0.80$ for rs3077 and 0.40 for rs9277542). P_{meta} values were 4.40×10^{-19} for rs3077 (OR = 0.46, 95% confidence interval (CI) = 0.39–0.54), and 1.28×10^{-15} for rs9277542 (OR = 0.50, 95% CI = 0.43–0.60). Among the remaining 30 SNPs in the replication study, 27 SNPs were successfully genotyped by the DigiTag2 assay with SNP call rate $\geq 95\%$ and HWE p -value ≥ 0.01 . Two SNPs (rs9276431 and rs7768538), located in the genetic region including the *HLA-DQ* gene, were marginally replicated in the two sets of HBV carriers and healthy controls with Mantel-Haenszel P values of 2.80×10^{-7} (OR = 0.56, 95% CI = 0.45–0.70) and 1.09×10^{-7} (OR = 0.53, 95% CI = 0.42–0.67), respectively, when using additive, two-tailed Cochran Mantel-Haenszel (CMH) fixed-effects model with no evidence of heterogeneity ($P_{het} = 0.67$ for rs9276431 and 0.70 for rs7768538) (Table S1).

Meta-analysis using the random effects model across 6 independent studies, including 5 additional published data, showed $P_{meta} = 3.94 \times 10^{-45}$, OR = 0.55 for rs3077, $P_{meta} = 1.74 \times 10^{-21}$, OR = 0.61 for rs9277535 and $P_{meta} = 1.69 \times 10^{-15}$, OR = 0.51 for rs9277542, with the SNP rs9277535 being located about 4-kb upstream from rs9277542 and showing strong linkage disequilibrium of $r^2 = 0.955$ on the HapMap JPT (Table S2). As shown in Table S2, the odds ratio was very similar among the 6 studies, and heterogeneity was negligible with $P_{het} > 0.01$.

Moreover, based on GWAS using samples from 94 chronic HBV carriers with LC or HCC and 87 chronic HBV carriers without LC and HCC, we found no significant SNPs associated with CHB progression (Figure S1).

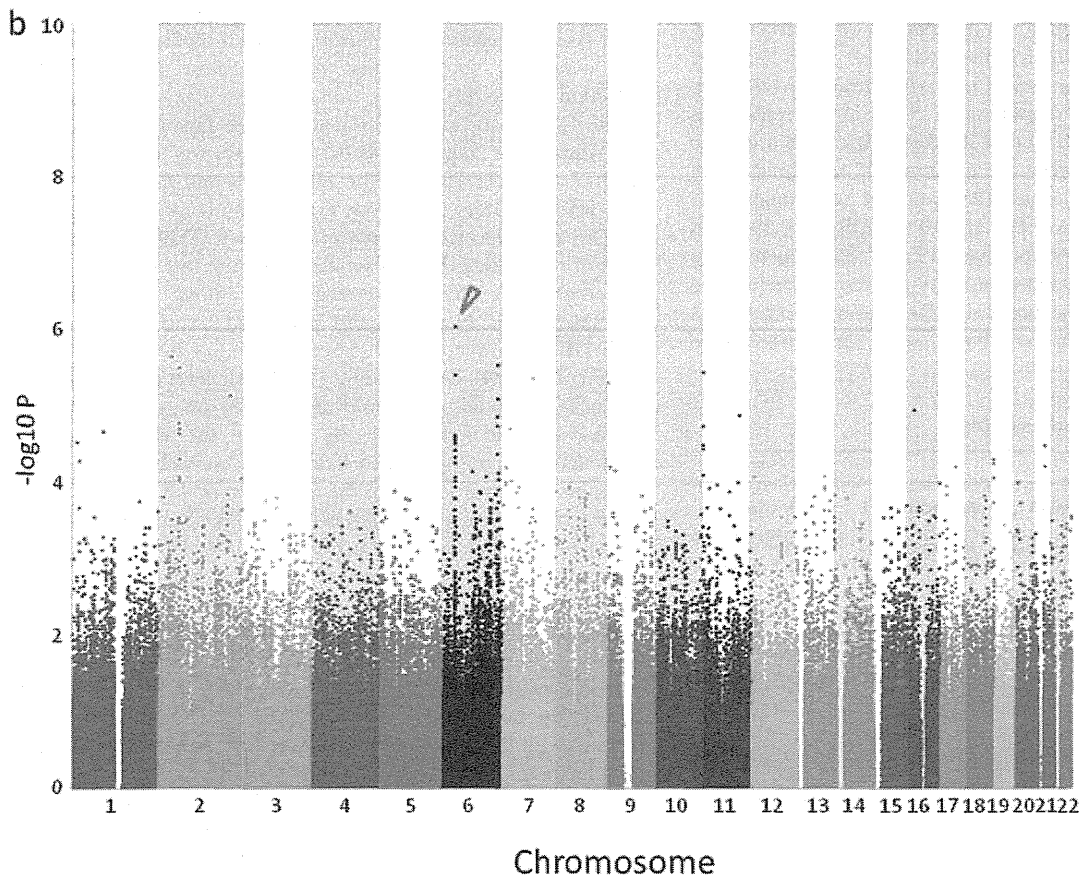
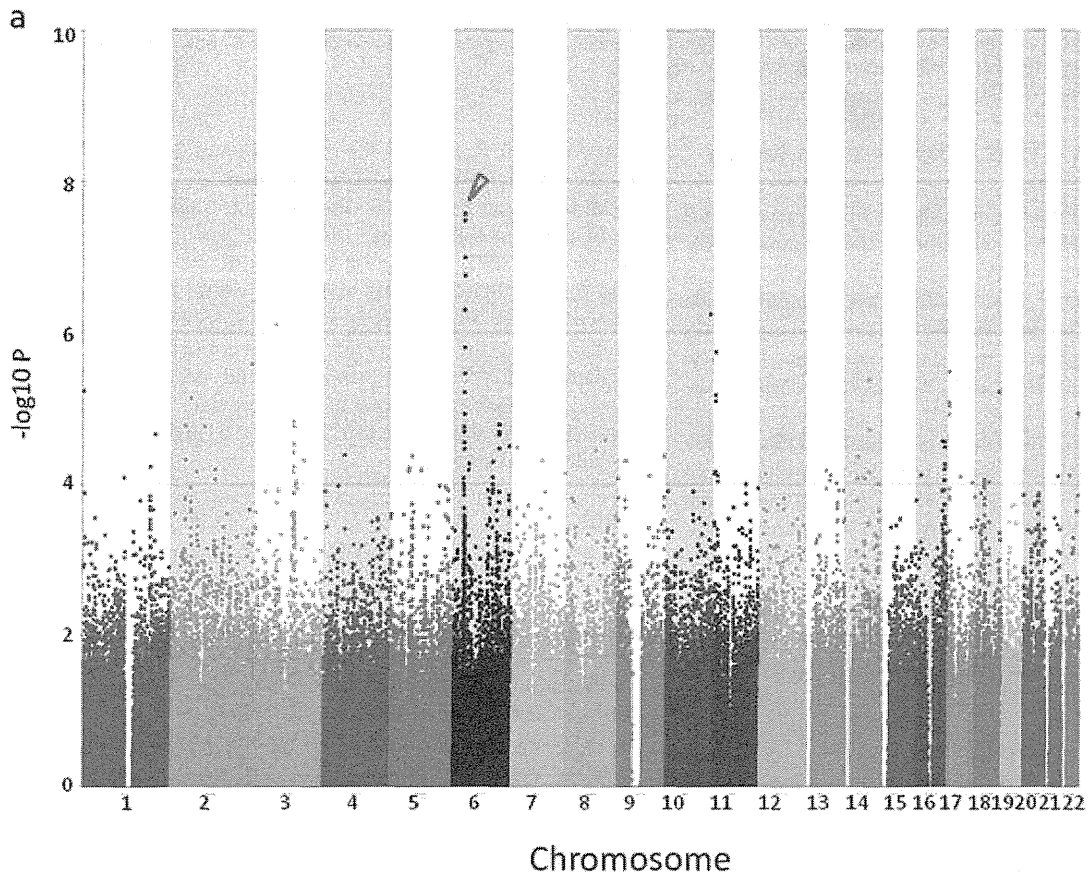


Figure 1. Results of genome-wide association studies. a) HBV carriers and healthy controls, and b) HBV carriers and HBV-resolved individuals were compared. *P* values were calculated by chi-squared test for allele frequencies. Dots with arrows on chromosome 6 show strong associations with protective effects against persistent HB infection and with HBV clearance.
doi:10.1371/journal.pone.0039175.g001

Clearance of Hepatitis B virus in Japanese and Korean Individuals

We also conducted a GWAS to identify the host genetic factors related to clearance of HBV in the above 181 Japanese HBV carriers and 185 Japanese HBV-resolved individuals using a genome-wide SNP typing array (Affymetrix Genome-Wide Human SNP Array 6.0 for 900 K SNPs). The same two SNPs (rs3077 and rs9277542) showed strong associations in the allele frequency model ($P=9.24\times 10^{-7}$ and $P=3.15\times 10^{-5}$) with clearance of HBV (Figure 1b).

The above 32 SNPs, including the two associated SNPs (rs3077 and rs9277542), were selected for a replication study in two independent sets of HBV carriers and HBV resolved individuals (replication-1:256 Japanese HBV carriers and 150 Japanese HBV resolved individuals; and replication-2:344 Korean HBV carriers and 106 Korean HBV resolved individuals; Table 1). All 32 SNPs were genotyped using the DigiTag2 assay and 29 of 32 SNPs were successfully genotyped (Table S3). The associations of the original SNPs were replicated in both replication sets [replication-1 (Japanese): rs3077, $P=3.32\times 10^{-2}$, OR = 0.72 and rs9277542, $P=1.25\times 10^{-2}$, OR = 0.68; replication-2 (Korean): rs3077, $P=2.35\times 10^{-7}$, OR = 0.41 and rs9277542, $P=4.97\times 10^{-6}$, OR = 0.46; Table 3]. Meta-analysis using random effects model showed $P_{meta}=1.56\times 10^{-4}$ for rs3077 (OR = 0.51, 95% CI = 0.36–0.72), and 5.91×10^{-7} for rs9277542 (OR = 0.55, 95% CI = 0.43–0.69). While there was evidence of heterogeneity between these studies for rs3077 ($P_{het}=0.03$) and no evidence for rs9277542 ($P_{het}=0.19$), significant associations with HBV clearance were observed with Mantel-Haenszel $P_{meta}=3.28\times 10^{-12}$ for rs3077 and 1.42×10^{-10} for rs9277542, when using CMH fixed-effects model. Among the remaining 27 SNPs in the replication study, two SNPs (rs9276431 and rs7768538), located in a genetic region including *HLA-DQ* gene, were marginally replicated in the two sets of HBV carriers and HBV resolved individuals with Mantel-Haenszel *P* values of 2.10×10^{-5} (OR = 0.59) and 1.10×10^{-5} (OR = 0.56), respectively (Table S3), when using CMH fixed-effect model. Due to the existing heterogeneity among three groups (GWAS, Replication-1 and Replication-2) ($P_{het}=0.03$ for rs9276431 and 0.04 for rs7768538), weak associations were

observed with $P_{meta}=0.03$ for rs9276431 and 0.02 for rs7768538 by the random effects model meta-analysis.

Meta-analysis across 6 independent studies, including 5 additional published data, showed $P_{meta}=1.48\times 10^{-9}$, OR = 0.60 for rs3077, $P_{meta}=1.08\times 10^{-17}$, OR = 0.66 for rs9277535 and $P_{meta}=5.14\times 10^{-5}$, OR = 0.55 for rs9277542 (Table S4). As shown in Table S4, the OR for the rs9277535 and rs9277542 were similar among the 6 independent studies, and heterogeneity was negligible ($P_{het}=0.03$ for rs9277535 and 0.14 for rs9277542). However, significant level of heterogeneity for rs3077 was observed with $P_{het}=9.57\times 10^{-6}$ across 5 independent studies, including our study.

URLs

The results of the present GWAS are registered at a public database: https://gwas.lifesciencedb.jp/cgi-bin/gwasdb/gwas_top.cgi.

Discussion

The recent genome-wide association study showed that the SNPs located in a genetic region including *HLA-DPA1* and *HLA-DPB1* genes were associated with chronic HBV infection in the Japanese and Thai population [10,11]. In this study, we confirmed a significant association between SNPs (rs3077 and rs9277542) located in the same genetic region as *HLA-DPA1* and *HLA-DPB1* and protective effects against CHB in Korean and Japanese individuals. Meta-analysis using the random effects model across 6 independent studies including our study suggested that, widely in East Asian populations, variants in antigen binding sites of *HLA-DP* contribute to protective effects against persistent HBV infection (Table S2).

On GWAS and replication analysis with Japanese and Korean individuals, we identified associations between the same SNPs (rs3077 and rs9277542) in the *HLA-DPA1* and *HLA-DPB1* genes and HBV clearance; however, no new candidate SNPs from the GWAS were detected on replication analysis (Table S3). When the data of reference#18 was excluded from the meta-analysis across 6 independent studies, heterogeneity among 4 studies was estimated to be $P_{het}=0.15$ and significant association of rs3077 with HBV clearance was observed with $P_{meta}=5.88\times 10^{-24}$, OR = 0.56 (Table S4). In our study, a negligible level of heterogeneity for rs3077 was also observed ($P_{het}=0.03$) on meta-analysis by adding replication-1 (Table 3). Despite the heterogeneity in replication-1, a marginal association was observed for rs3077 with the same downward trend in the odds ratio ($P=3.32\times 10^{-2}$, OR = 0.72). Moreover, meta-analysis using GWAS and replication-2 showed significant association of $P_{meta}=1.89\times 10^{-12}$, OR = 0.43 for rs3077 with no evidence of heterogeneity ($P_{het}=0.75$). Although the reason why heterogeneity was observed in replication-1 is unclear, one possible reason is the clinical heterogeneity due to different kits being used for antibody testing. The associations of *HLA-DPA1*/*-DPB1* with CHB and HBV clearance showed the same level of significance in the comparison of HBV patients with HBV resolved individuals (OR = 0.43 for rs3077 and 0.49 for rs9277542) as the one with healthy controls (OR = 0.46 for rs3077 and 0.50 for rs9277542), when the replication-1 was excluded in the analysis (Table 2 and Table 3). The results of meta-analysis across 6 independent studies including our study also showed the same or slightly weaker associations in the

Table 1. Number of study samples.

		GWAS	Replication-1	Replication-2
population		Japanese	Japanese	Korean
HBV carriers	Total	181	256	344
	IC	20	94	–
	CH	67	101	177
	LC	3	10	–
	HCC	91	51	167
Healthy controls		184	236	151
Resolved individuals		185	150	106

Abbreviation: IC, Inactive Carrier; CH, Chronic Hepatitis; LC, Liver Cirrhosis; HCC, Hepatocellular Carcinoma.

doi:10.1371/journal.pone.0039175.t001

Table 2. Results of replication study for protective effects against CHB.

dbSNP rsID	Position		MAF ^a (allele)	Allele (1/2)	Stage (population)	HBV carriers			Healthy controls			OR ^b		P-value ^c	P _{het} ^d	
	Chr	Buld 36.3				Nearest Gene	11	12	22	11	12	22	HWEp			95% CI
rs3077	6	33141000	HLA-DPA1	0.44 (T)	T/C (Japanese)	GWAS	13	51	117	28	88	67	0.919	0.42	1.14 × 10 ⁻⁷	
						Replication-1	26	95	134	46	125	65	0.309	0.48	2.70 × 10 ⁻⁸	
						(Japanese)	(10.2)	(37.3)	(52.5)	(19.5)	(53.0)	(27.5)		(0.37–0.62)		
						Replication-2	23	81	111	31	74	40	0.767	0.47	2.08 × 10 ⁻⁶	
						(Korean)	(10.7)	(37.7)	(51.6)	(21.4)	(51.0)	(27.6)		(0.35–0.65)		
					Meta-analysis ^e							0.46	4.40 × 10 ⁻¹⁹	0.80		
												(0.39–0.54)				
rs9277542	6	33163225	HLA-DPB1	0.45 (T)	T/C (Japanese)	GWAS	18	53	110	29	102	52	0.073	0.42	5.32 × 10 ⁻⁸	
						Replication-1	30	106	118	54	114	67	0.681	0.54	3.33 × 10 ⁻⁶	
						(Japanese)	(11.8)	(41.7)	(46.5)	(23.0)	(48.5)	(28.5)		(0.42–0.70)		
						Replication-2	30	87	94	35	72	36	0.933	0.54	8.29 × 10 ⁻⁵	
						(Korean)	(14.2)	(41.2)	(44.5)	(24.5)	(50.3)	(25.2)		(0.40–0.74)		
					Meta-analysis ^e							0.50	1.28 × 10 ⁻¹⁵	0.40		
												(0.43–0.60)				

^aMinor allele frequency and minor allele in 198 healthy Japanese (ref#19).

^bOdds ratio of minor allele from two-by-two allele frequency table.

^cP value of Pearson's chi-square test for allelic model.

^dHeterogeneity was tested using general variance-based method.

^eMeta-analysis was tested using the random effects model.

doi:10.1371/journal.pone.0039175.t002

comparison of HBV patients with HBV resolved individuals (OR = 0.56 for rs3077, 0.66 for rs9277535 and 0.55 for rs9277542) than in the one with healthy controls (OR = 0.55 for rs3077, 0.61 for rs9277535 and 0.51 for rs9277542), which was the opposite result as we expected (Table S2 and Table S4). These results may suggest that other unknown immune system(s) exist to eliminate the HBV in the HBV resolved individuals.

Among the HLA class II loci (*HLA-DPA1*, *HLA-DPB1* and *HLA-DQB2*), which were associated with CHB and HBV clearance, a weak linkage disequilibrium ($r^2 < 0.1$) was observed between *HLA-DQB2* locus and *HLA-DPA1*/*-DPB1* loci in Japanese and Korean populations (Figure S2). We also found that similar linkage disequilibrium blocks (r^2) were observed among three subgroups (HBV carriers, HBV resolved individuals and Healthy controls). Moreover, logistic regression analysis of *HLA-DP* (rs3077 and rs9277542) with use of *HLA-DQ* (rs9276431 and rs768538) as covariates showed that the same level of significant associations of *HLA-DP* with CHB and HBV clearance as shown in the single-point association analysis, while no associations of *HLA-DQ* with $P_{log} > 0.05$ were detected both in Japanese and in Korean (Table S5). These results show that *HLA-DP* is the main genetic factor for susceptibility to CHB and HBV clearance, and the associations of *HLA-DQB2* would result from linkage disequilibrium of *HLA-DPA1*/*-DPB1*.

In this study, we confirmed the significant associations between *HLA-DPA1* and *HLA-DPB1*, and protective effects against CHB and HBV clearance in Japanese and Korean individuals. These results suggest that the associations between the *HLA-DP* locus, CHB and HBV clearance are widely replicated in East Asian populations, including Chinese, Thai, Japanese and Korean individuals; however, there have been no similar GWAS performed in Caucasian and African populations. Moreover,

there were no significant SNPs associated with HCC development in this study, thus suggesting that it is necessary to increase the sample size. To clarify the pathogenesis of CHB or the mechanisms of HBV clearance, further studies are necessary, including a functional study of the *HLA-DP* molecule, identification of novel host genetic factors other than *HLA-DP*, and variation analysis of HBV.

Materials and Methods

Ethics Statement

All study protocols conform to the relevant ethical guidelines, as reflected in the *a priori* approval by the ethics committees of all participating universities and hospitals. The written informed consent was obtained from each patient who participated in this study and all samples were anonymized.

Genomic DNA Samples and Clinical Data

All of the 1,793 Japanese and Korean samples, including individuals with CHB, healthy controls and HBV-resolved individuals (HBsAg-negative and anti-HBc-positive), were collected at 20 multi-center hospitals (liver units with hepatologists) throughout Japan and Korea. The 19 hospitals in Japan were grouped into the following 8 areas: Hokkaido area (Hokkaido University Hospital, Teine Keijinkai Hospital), Tohoku area (Iwate Medical University Hospital), Kanto area (Musashino Red Cross Hospital, Saitama Medical University, Kitasato University Hospital, University of Tokyo), Koshin area (Shinshu University Hospital, Kanazawa University Hospital), Tokai area (Nagoya City University Hospital, Nagoya Daini Red Cross Hospital), Kinki area (Kyoto Prefectural University of Medicine Hospital, National Hospital Organization Osaka National Hospital, Osaka

Table 3. Results of replication study for clearance of hepatitis B virus.

dbSNP rsID	Position		Nearest Gene	MAF ^a (allele)	Allele (1/2)	Stage (population)	HBV carriers			Resolved individuals			OR ^b 95% CI	P-value ^c	P _{het} ^d
	Chr	Buld 36.3					11	12	22	11	12	22			
rs3077	6	33141000	HLA-DPA1	0.44	T/C	GWAS	13	51	117	29	82	74	0.44	9.24 × 10 ⁻⁷	
							(7.2)	(28.2)	(64.6)	(15.7)	(44.3)	(40.0)	(0.32–0.61)		
						Replication-1	26	95	134	20	64	60	0.72	3.32 × 10 ⁻²	
							(10.2)	(37.3)	(52.5)	(13.9)	(44.4)	(41.7)	(0.53–0.97)		
						Replication-2	23	81	111	29	48	28	0.41	2.35 × 10 ⁻⁷	
							(10.7)	(37.7)	(51.6)	(27.6)	(45.7)	(26.7)	(0.29–0.58)		
			Meta-analysis ^e								0.51	1.56 × 10 ⁻⁴	0.03		
												(0.36–0.72)			
												0.43	1.89 × 10 ⁻¹²	0.75	
													(0.34–0.54)		
rs9277542	6	33163225	HLA-DPB1	0.45	T/C	GWAS	18	53	110	28	88	69	0.51	3.15 × 10 ⁻⁵	
							(9.9)	(29.3)	(60.8)	(15.1)	(47.6)	(37.3)	(0.37–0.70)		
						Replication-1	30	106	118	28	62	52	0.68	1.25 × 10 ⁻²	
							(11.8)	(41.7)	(46.5)	(19.7)	(43.7)	(36.6)	(0.51–0.92)		
						Replication-2	30	87	94	30	53	22	0.46	4.97 × 10 ⁻⁶	
							(14.2)	(41.2)	(44.5)	(28.6)	(50.5)	(21.0)	(0.33–0.64)		
			Meta-analysis ^e									0.55	5.91 × 10 ⁻⁷	0.19	
													(0.43–0.69)		
													0.49	9.69 × 10 ⁻¹⁰	0.65
														(0.39–0.61)	

^aMinor allele frequency and minor allele in 198 healthy Japanese (ref#19).

^bOdds ratio of minor allele from two-by-two allele frequency table.

^cP value of Pearson's chi-square test for allelic model.

^dHeterogeneity was tested using general variance-based method.

^eMeta-analysis was tested using the random effects model.

doi:10.1371/journal.pone.0039175.t003

City University), Chugoku/Shikoku area (Tottori University Hospital, Ehime University Hospital, Yamaguchi University Hospital, Kawasaki Medical College Hospital) and Kyushu area (Kurume University Hospital). Korean samples were collected at Yonsei University College of Medicine.

HBV status was measured based on serological results for HBsAg and anti-HBc with a fully automated chemiluminescent enzyme immunoassay system (Abbott ARCHITECT; Abbott Japan, Tokyo, Japan, or LUMIPULSE f or G1200; Fujirebio, Inc., Tokyo, Japan). For clinical staging, inactive carrier (IC) state was defined by the presence of HBsAg with normal ALT levels over 1 year (examined at least four times at 3-month intervals) and without evidence of portal hypertension. Chronic hepatitis (CH) was defined by elevated ALT levels (>1.5 times the upper limit of normal [35 IU/L]) persisting over 6 months (at least by 3 bimonthly tests). Liver cirrhosis (LC) was diagnosed principally by ultrasonography (coarse liver architecture, nodular liver surface, blunt liver edges and hypersplenism), platelet counts <100,000/cm³, or a combination thereof. Histological confirmation by fine-needle biopsy of the liver was performed as required. Hepatocellular carcinoma (HCC) was diagnosed by ultrasonography, computerized tomography, magnetic resonance imaging, angiography, tumor biopsy or a combination thereof.

The Japanese control samples from HBV-resolved subjects (HBsAg-negative and anti-HBc-positive) at Nagoya City University-affiliated healthcare center were used by comprehensive agree-

ment (anonymization in an unlinkable manner) in this study. Some of the unrelated Japanese healthy controls were obtained from the Japan Health Science Research Resources Bank (Osaka, Japan). One microgram of purified genomic DNA was dissolved in 100 µl of TE buffer (pH 8.0) (Wako, Osaka, Japan), followed by storage at -20°C until use.

SNP Genotyping and Data Cleaning

For GWAS, we genotyped a total of 550 individuals, including 181 Japanese HBV carriers, 184 Japanese healthy controls and 185 spontaneously HBV-resolved Japanese individuals (HBsAg-negative and anti-HBc-positive), using the Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix, Inc., Santa Clara, CA), in accordance with the manufacturer's instructions. The average QC call rate for 550 samples reached 98.47% (95.00–99.92%), which had an average sample call rate of 98.91% (93.55–99.74%) by determining the genotype calls of over 900 K SNPs using the Genotyping Console v4.1 software (with Birdseed v1 algorithm) provided by the manufacturer [19]. We then applied the following thresholds for SNP quality control in data cleaning: SNP call rate ≥95% and MAF ≥1% for three groups (HBV carriers, healthy controls and HBV-resolved individuals), and HWE P-value ≥0.001 for healthy controls [20]. Here, SNP call rate is defined for each SNP as the number of successfully genotyped samples divided by the number of total samples genotyped. A total of 597,789 SNPs and 590,278 SNPs on autosomal chromosomes

passed the quality control filters in the genome-wide association analysis using HBV carriers and healthy controls, and using HBV carriers and HBV-resolved individuals, respectively (Figure 1). All cluster plots for the SNPs showing $P < 0.0001$ on association analyses in the allele frequency model were confirmed by visual inspection, and SNPs with ambiguous cluster plots were excluded.

In the following replication stage, we selected a set of 32 SNPs with $P < 0.0001$ in the GWAS using HBV carriers and HBV-resolved individuals. SNP genotyping in two independent sets of 256 Japanese HBV carriers, 236 Japanese healthy controls and 150 Japanese HBV-resolved individuals (Table 1, replication-1), and 344 Korean HBV carriers, 151 Korean healthy controls and 106 Korean HBV-resolved individuals (Table 1, replication-2) was completed for the selected 32 SNPs using the DigiTag2 assay [21,22] and custom TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA) on the LightCycler 480 Real-Time PCR System (Roche, Mannheim, Germany).

Statistical Analysis

The observed associations between SNPs and the protective effects on chronic hepatitis B or clearance of hepatitis virus B were assessed by chi-squared test with a two-by-two contingency table in allele frequency model. SNPs on chromosome X were removed because gender was not matched among HBV carriers, healthy controls and HBV-resolved individuals. A total of 597,789 SNPs and 590,278 SNPs passed the quality control filters in the GWAS stage; therefore, significance levels after Bonferroni correction for multiple testing were $P = 8.36 \times 10^{-8}$ ($0.05/597,789$) and $P = 8.47 \times 10^{-8}$ ($0.05/590,278$), respectively. For the replication study, 29 of 32 SNPs were successfully genotyped; therefore, we applied $P = 0.0017$ ($0.05/29$) as a significance level, and none of the 29 markers genotyped in the replication stage showed deviations from the Hardy-Weinberg equilibrium in healthy controls ($P > 0.01$).

The genetic inflation factor λ was estimated by applying the Cochran-Armitage test on all SNPs and was found to be 1.056 and 1.030 in the GWAS using HBV carriers and healthy controls, and using HBV carriers and HBV-resolved individuals, respectively (Figure S3). These results suggest that the population substructure should not have any substantial effect on statistical analysis. In addition, the principal component analysis in a total of 550 individuals in the GWAS stage together with the HapMap samples also revealed that the effect of population stratification was negligible (Figure S4).

Based on the genotype data of a total of 1,793 samples including 1,192 Japanese samples and 601 Korean samples in both GWAS and replication stages, haplotype blocks were estimated using the Gabriel's algorithm using the Haploview software (v4.2) (Figure S2). In the logistic regression analysis, two SNPs (rs9276431 and rs7768538) within the HLA-DQ locus were individually involved as a covariate (Table S5). Statistical analyses were performed using the SNP & Variation Suite 7 software (Golden Helix, MT, USA).

Supporting Information

Figure S1 GWAS using samples from HBV carriers with LC or HCC, and HBV carriers without LC and HCC. P values were calculated using chi-squared test for allele frequencies. (PPTX)

Figure S2 Estimation of linkage disequilibrium blocks in HBV patients, HBV resolved individuals and healthy controls in Japanese and Korean. The LD blocks (r^2) were analyzed using the Gabriel's algorithm. (PPTX)

Figure S3 Quantile-quantile plot for test statistics (allele-based chi-squared tests) for GWAS results. Dots represent P values of each SNP that passed the quality control filters. Inflation factor λ was estimated to be: a) 1.056 in the analysis with HBV carriers and healthy controls; and b) 1.030 with HBV carriers and HBV-resolved individuals. (PPTX)

Figure S4 Principal component analysis on a total of 550 individuals in GWAS, together with HapMap samples (CEU, YRI and JPT). (PPTX)

Table S1 Results for 29 SNPs selected in replication study using samples of HBV carriers and healthy controls. ^a P values by chi-squared test for allelic model. ^bOdds ratio of minor allele from two-by-two allele frequency table. ^cMeta-analysis was tested using additive, two-tailed CMH fixed-effects model. (XLSX)

Table S2 Results of meta-analysis for protective effects against persistent HB infection across 6 independent studies, including this study. ^aMinor allele frequency and minor allele in 198 healthy Japanese (ref#19). ^bOdds ratio of minor allele from two-by-two allele frequency table. ^c P value of Pearson's chi-squared test for allele model. ^dHeterogeneity was tested using general variance-based method. ^eMeta-analysis was tested using the random effects model. (XLSX)

Table S3 Results for 29 SNPs selected in replication study using samples from HBV carriers and HBV-resolved individuals. ^a P values by chi-squared test for allelic model. ^bOdds ratio of minor allele from two-by-two allele frequency table. ^cMeta-analysis was tested using additive, two-tailed CMH fixed-effects model. (XLSX)

Table S4 Results of meta-analysis for clearance of HBV across 6 independent studies, including this study. ^aMinor allele frequency and minor allele in 198 healthy Japanese (ref#19). ^bOdds ratio of minor allele from two-by-two allele frequency table. ^c P value of Pearson's chi-squared test for allele model. ^dHeterogeneity was tested using general variance-based method. ^eMeta-analysis was tested using the random effects model. (XLSX)

Table S5 Logistic regression analysis of HLA-DP (rs3077 and rs9277542) and HLA-DQ (rs9276431 and rs7768538) with susceptibility to CHB and HBV clearance using the HLA-DQ genotypes individually as a covariate. (XLSX)

Acknowledgments

We thank all the patients and families who contributed to the study and Ms. Yasuka Uehara-Shibata and Ms. Yoshimi Ishibashi for technical assistance.

Author Contributions

Conceived and designed the experiments: NN HS YT. Performed the experiments: HS Y. Mawatari M. Sageshima YO. Analyzed the data: NN MK AK. Contributed reagents/materials/analysis tools: KM M. Sugiyama SHA JYP SH JHK KS M. Kurosaki YA SM MW ET MH SK EO YI EM AT Y. Murawaki YH IS M. Korenaga KH TI NI KHH YT MM. Wrote the paper: NN M. Kawashima YT KT MM.

References

1. Arauz-Ruiz P, Norder H, Robertson BH, Magnus LO (2002) Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* 83: 2059–2073.
2. Hoofnagle JH, Doo E, Liang TJ, Fleischer R, Lok ASF (2007) Management of hepatitis B: Summary of a clinical research workshop. *Hepatology* 45: 1056–1075.
3. Yokosuka O, Kurosaki M, Imazeki F, Arase Y, Tanaka Y, et al. (2011) Management of hepatitis B: Consensus of the Japan society of Hepatology 2009. *Hepatol Res* 41: 1–21.
4. Tada H, Uga N, Fuse Y, Shimizu M, Nemoto Y, et al. (1992) Prevention of perinatal transmission of hepatitis B virus carrier state. *Acta Paediatr Jpn* 34: 656–659.
5. Stevens CE, Toy PT, Taylor PE, Lee T, Yip HY (1992) Prospects for control of hepatitis B virus infection: implications of childhood vaccination and long-term protection. *Pediatrics* 90: 170–173.
6. Szmunn W (1979) Large-scale efficacy trials of hepatitis B vaccines in the USA: baseline data and protocols. *J Med Virol* 4: 327–340.
7. Kwon H, Lok AS (2011) Hepatitis B therapy. *Nat Rev Gastroenterol Hepatol* 8: 275–284.
8. Kobayashi M, Ikeda K, Arase Y, Suzuki F, Akuta N, et al. (2008) Change of Hepatitis B virus genotypes in acute and chronic infections in Japan. *J Med Virol* 80: 1880–1884.
9. Yano K, Tamada Y, Yatsubashi H, Komori A, Abiru S, et al. (2010) Dynamic epidemiology of acute viral hepatitis in Japan. *Intervirology* 53: 70–75.
10. Kamatani Y, Watanapokayakit S, Ochi H, Kawaguchi T, Takahashi A, et al. (2009) A genome-wide association study identifies variants in the HLA-DP locus associated with chronic hepatitis B in Asians. *Nat Genet* 41: 591–595.
11. Mbarek H, Ochi H, Urabe Y, Kumar V, Kubo M, et al. (2011) A genome-wide association study of chronic hepatitis B identified novel risk locus in a Japanese population. *Hum Mol Genet* 20: 3884–3892.
12. Li J, Yang D, He Y, Wang M, Wen Z, et al. (2011) Associations of HLA-DP variants with hepatitis B virus infection in southern and northern Han Chinese populations: a multicenter case-control study. *PLoS ONE* 6: e24221.
13. Guo X, Zhang Y, Li J, Ma J, Wei Z, et al. (2011) Strong influence of human leukocyte antigen (HLA)-DP gene variants on development of persistent chronic hepatitis B virus carriers in the Han Chinese population. *Hepatol* 53: 422–428.
14. Thursz MR, Kwiatkowski D, Allsopp CEM, Greenwood BM, Thomas HC, et al. (1995) Association between an MHC class II allele and clearance of hepatitis B virus in the Gambia. *N Engl J Med* 332: 1065–1069.
15. Godkin A, Davenport M, Hill AVS (2005) Molecular analysis of HLA class II associations with hepatitis B virus clearance and vaccine nonresponsiveness. *Hepatology* 41: 1383–1390.
16. An P, Winkler C, Guan L, O'Brien SJ, Zeng Z, et al. (2011) A common HLA-DPA1 variant is a major determinant of hepatitis B virus clearance in Han Chinese. *J Infect Dis* 203: 943–947.
17. Wang L, Wu X-P, Zhang W, Zhu D-H, Wang Y, et al. (2011) Evaluation of genetic susceptibility loci for chronic hepatitis B in Chinese: two independent case-control study.
18. Hu L, Zhai X, Liu J, Chu M, Pan S, et al. (2011) Genetic variants in HLA-DP/DQ influence both hepatitis B virus clearance and Hepatocellular carcinoma development. *Hepatology* (in press).
19. Nishida N, Koike A, Tajima A, Ogasawara Y, Ishibashi Y, et al. (2008) Evaluating the performance of Affymetrix SNP Array 6.0 platform. *BMC Genomics* 9: 431.
20. Miyagawa T, Nishida N, Ohashi J, Kimura R, Fujimoto A, et al. (2008) Appropriate data cleaning methods for genome-wide association study. *J Hum Genet* 53: 886–893.
21. Nishida N, Tanabe T, Takasu M, Suyama A, Tokunaga K (2007) Further development of multiplex single nucleotide polymorphism typing method, the DigiTag2 assay. *Anal Biochem* 364: 78–85.
22. Nishida N, Mawatari Y, Sageshima M, Tokunaga K (2012) Highly parallel and short-acting amplification with locus-specific primers to detect single nucleotide polymorphisms by the DigiTag2 assay. *PLoS ONE* 7: e29967.

Soluble MICA and a *MICA* Variation as Possible Prognostic Biomarkers for HBV-Induced Hepatocellular Carcinoma

Vinod Kumar^{1,2*}, Paulisally Hau Yi Lo¹, Hiromi Sawai³, Naoya Kato⁴, Atsushi Takahashi², Zhenzhong Deng¹, Yuji Urabe¹, Hamdi Mbarek¹, Katsushi Tokunaga^{3, 5}, Yasuhiro Tanaka⁵, Masaya Sugiyama⁶, Masashi Mizokami⁶, Ryosuke Muroyama⁴, Ryosuke Tateishi⁷, Masao Omata⁷, Kazuhiko Koike⁷, Chizu Tanikawa¹, Naoyuki Kamatani², Michiaki Kubo², Yusuke Nakamura¹, Koichi Matsuda¹

1 Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, Tokyo, Japan, **2** Center for Genomic Medicine, The Institute of Physical and Chemical Research (RIKEN), Kanagawa, Japan, **3** Department of Human Genetics, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan, **4** Unit of Disease Control Genome Medicine, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan, **5** Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, Aichi, Japan, **6** The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Chiba, Japan, **7** Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

Abstract

MHC class I polypeptide-related chain A (MICA) molecule is induced in response to viral infection and various types of stress. We recently reported that a single nucleotide polymorphism (SNP) rs2596542 located in the *MICA* promoter region was significantly associated with the risk for hepatitis C virus (HCV)-induced hepatocellular carcinoma (HCC) and also with serum levels of soluble MICA (sMICA). In this study, we focused on the possible involvement of MICA in liver carcinogenesis related to hepatitis B virus (HBV) infection and examined correlation between the *MICA* polymorphism and the serum sMICA levels in HBV-induced HCC patients. The genetic association analysis revealed a nominal association with an SNP rs2596542; a G allele was considered to increase the risk of HBV-induced HCC ($P=0.029$ with odds ratio of 1.19). We also found a significant elevation of sMICA in HBV-induced HCC cases. Moreover, a G allele of SNP rs2596542 was significantly associated with increased sMICA levels ($P=0.009$). Interestingly, HCC patients with the high serum level of sMICA (>5 pg/ml) exhibited poorer prognosis than those with the low serum level of sMICA (≤ 5 pg/ml) ($P=0.008$). Thus, our results highlight the importance of *MICA* genetic variations and the significance of sMICA as a predictive biomarker for HBV-induced HCC.

Citation: Kumar V, Yi Lo PH, Sawai H, Kato N, Takahashi A, et al. (2012) Soluble MICA and a *MICA* Variation as Possible Prognostic Biomarkers for HBV-Induced Hepatocellular Carcinoma. PLoS ONE 7(9): e44743. doi:10.1371/journal.pone.0044743

Editor: Erica Villa, University of Modena & Reggio Emilia, Italy

Received: May 3, 2012; **Accepted:** August 7, 2012; **Published:** September 14, 2012

Copyright: © 2012 Kumar et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was conducted as a part of the BioBank Japan Project that was supported by the Ministry of Education, Culture, Sports, Science and Technology of the Japanese government. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: koichima@ims.u-tokyo.ac.jp

Introduction

Hepatocellular carcinoma (HCC) reveals a very high mortality rate that is ranked the third among all cancers in the world [1]. HCC is known to develop in a multistep process which has been related to various risk factors such as genetic factors, environment toxins, alcohol and drug abuse, autoimmune disorders, elevated hepatic iron levels, obesity, and hepatotropic viral infections [2]. Among them, chronic infection with hepatitis B virus (HBV) is one of the major etiological factors for developing HCC with considerable regional variations ranging from 20% of HCC cases in Japan to 65% in China [3].

Interestingly, clinical outcome after the exposure to HBV considerably varies between individuals. The great majority of individuals infected with HBV spontaneously eliminate the viruses, but a subset of patients show the persistent chronic hepatitis B infection (CHB), and then progresses to liver cirrhosis and HCC through a complex interplay between multiple genetic and

environmental factors [4]. In this regard, genome wide association studies (GWAS) using single nucleotide polymorphisms (SNPs) have highlighted the importance of genetic factors in the pathogenesis of various diseases including CHB as well as HBV-induced HCC [5,6,7,8,9,10,11,12,13]. Recently, we identified a genetic variant located at 4.7 kb upstream of the *MHC class I polypeptide-related chain A (MICA)* gene to be strongly associated with hepatitis C virus (HCV) -induced HCC development [14].

MICA is highly expressed on viral-infected cells or cancer cells, and acts as ligand for NKG2D to activate antitumor effects of Natural killer (NK) cells and CD8⁺ T cells [15,16]. Our previous results indicated that a G allele of SNP rs2596542 was significantly associated with the lower cancer risk and the higher level of soluble MICA (sMICA) in the serum of HCV-induced HCC patients, demonstrating the possible role of MICA as a tumor suppressor. However, elevation of serum sMICA was shown to be associated with poor prognosis in various cancer patients [17,18,19,20].

Matrix metalloproteinases (MMPs) can cleave MICA at a transmembrane domain [21] and release sMICA proteins from cells. Since sMICA was shown to inhibit the antitumor effects of NK cells and CD8⁺ T cells by reduction of their affinity to binding to target cells [22,23], the effect of MICA in cancer cells would be modulated by the expression of MMPs. To elucidate the role of MICA in HBV-induced hepatocellular carcinogenesis, we here report analysis of the *MICA* polymorphism and serum sMICA level in HBV-induced HCC cases.

Materials and Methods

Study participants

The demographic details of study participants are summarized in Table 1. A total of 181 HCC cases, 597 CHB patients, and 4,549 non-HBV controls were obtained from BioBank Japan that was initiated in 2003 with the funding from the Ministry of Education, Culture, Sports, Science and Technology, Japan [24]. In the Biobank Japan Project, DNA and serum of patients with 47 diseases were collected through collaborating network of 66 hospitals throughout Japan. List of participating hospitals is shown in the following website (http://biobankjp.org/plan/member_hospital.html). A total of 226 HCC cases, 102 CHB patients, and 174 healthy controls were additionally obtained from the University of Tokyo. The diagnosis of chronic hepatitis B was conducted on the basis of HBsAg-seropositivity and elevated serum aminotransferase levels for more than six months according to the guideline for diagnosis and treatment of chronic hepatitis (The Japan Society of Hepatology, <http://www.jsh.or.jp/medical/guidelines/index.html>). Control Japanese DNA samples (n = 934) were obtained from Osaka-Midosuji Rotary Club, Osaka, Japan. All HCC patients were histopathologically diagnosed. Overall survival was defined as the time from blood sampling for sMICA test to the date of death due to HCC. Patients who were alive on the date of last follow-up were censored on that date. All participants provided written informed consent. This research project was approved by the ethics committee of the University of Tokyo and the ethics committee of RIKEN. All clinical assessments and specimen collections were conducted according to Declaration of Helsinki principles.

SNP genotyping

Genotyping platforms used in this study were shown in Table 1. We genotyped 181 HCC cases and 5,483 non-HBV control samples using either Illumina Human Hap610-Quad or Human Hap550v3. The other samples were genotyped at SNP rs2596542

by the Invader assay system (Third Wave Technologies, Madison, WI).

MICA variable number tandem repeat (VNTR) locus genotyping

Genotyping of the *MICA* VNTR locus in 176 HBV-induced HCC samples was performed using the primers reported previously by the method recommended by Applied Biosystems (Foster City, CA) [14]. Briefly, the 5' end of forward primer was labeled with 6-FAM, and reverse primer was modified with GTGTCTT non-random sequence at the 5' end to promote Plus A addition. The PCR products were mixed with Hi-Di Formamide and GeneScan-600 LIZ size standard, and separated by GeneScan system on a 3730x1 DNA analyzer (Applied Biosystems, Foster City, CA). GeneMapper software (Applied Biosystems, Foster City, CA) was employed to assign the repeat fragment size (Figure S1).

Quantification of soluble MICA

We obtained serum samples of 111 HBV-positive HCC samples, 129 HCV-positive HCC samples, and 60 non-HBV controls from Biobank Japan. Soluble MICA levels were measured by sandwich enzyme-linked immunosorbent assay, as described in the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Statistical analysis

The association between an SNP rs2596542 and HBV-induced HCC was tested by Cochran-Armitage trend test. The Odds ratios were calculated by considering a major allele as a reference. Statistical comparisons between genotypes and sMICA levels were performed by Kruskal-Wallis test (if more than two classes for comparison) or Wilcoxon rank test using R. Overall survival rate of the patients was analyzed by Kaplan-Meier method in combination with log-rank test with SPSS 20 software. The period for the survival analysis was calculated from the date of blood sampling to the recorded date of death or the last follow-up date. Differences with a P value of <0.05 were considered statistically significant.

Results

Association of SNP rs2596542 with HBV-induced HCC

In order to examine the effect of rs2596542 genotypes on the susceptibility to HBV-induced HCC, a total of 407 HCC cases and 5,657 healthy controls were genotyped. The Cochran Armitage trend test of the data revealed a nominal association

Table 1. Demographic details of subjects analyzed.

Subjects	Source	Genotyping platform	Number of Sample	Female (%)	Age (mean+/-sd)
Liver Cancer	BioBank Japan	Illumina Human Hap610-Quad	181	17.9	62.94±9.42
	University of Tokyo	Invader assay	226		
Control	BioBank Japan	Illumina Human Hap550v3	4549	47.95	55.19±12.5
	Osaka**	Illumina Human Hap550v3	934		
Chronic hepatitis B*	BioBank Japan	Invader assay	597	45.66	61.31±12.6
	University of Tokyo	Invader assay	102		

*Chronic hepatitis B patients without liver cirrhosis and liver cancer during enrollment.

**Healthy volunteers from Osaka Midosuji Rotary Club, Osaka, Japan.

doi:10.1371/journal.pone.0044743.t001

between HBV-induced HCC and rs2596542 in which a risk allele G was more frequent among HBV-induced HCC cases than an A allele ($P=0.029$, OR = 1.19, 95% CI: 1.02–1.4; Table 2). To further investigate the effect of rs2596542 on the progression from CHB to HBV-induced HCC, we genotyped a total of 699 CHB cases without HCC. Although the progression risk from CHB to HBV-induced HCC was not statistically significant with rs2596542 ($P=0.197$ by the Cochran Armitage trend test with an allelic OR = 1.3 (0.94–1.36); Table 2), we found a similar trend of association in which the frequency of a risk-allele G was higher among HBV-induced HCC patients than that of CHB subjects. Since we previously revealed that an A allele was associated with a higher risk of HCV-induced HCC with OR of 1.36 [14], the rs2596542 alleles that increased the risk of HCC were opposite in HBV-induced HCC and HCV-induced HCC.

Soluble MICA levels are associated with SNP rs2596542

We subsequently performed measurement of soluble MICA (sMICA) in serum samples using the ELISA method in 176 HBV-positive HCC cases and 60 non-HBV controls. Nearly 30% of the HBV-induced HCC cases revealed the serum sMICA level of >5 pg/ml (defined as high) while the all control individuals except one showed that of ≤ 5 pg/ml (defined as low) ($P=4.5 \times 10^{-6}$; Figure 1A). Then, we examined correlation between SNP rs2596542 genotypes and serum sMICA levels in HBV-positive HCC cases. Interestingly, rs2596542 genotypes were significantly associated with serum sMICA levels ($P=0.009$; Figure 1B); 39% of individuals with the GG genotype and 20% of those with the AG genotype were classified as high for serum sMICA, but only 11% of those with the AA genotype were classified as high (AA+AG vs GG; $P=0.003$) (Figure 1B). These findings were similar with our previous reports in which a G allele was associated with higher serum sMICA levels in HCV-induced HCC patients [14].

Negative association of variable number of tandem repeat (VNTR) with sMICA level

The *MICA* gene harbors a VNTR locus in exon 5 that consists of 4, 5, 6, or 9 repeats of GCT as well as a G nucleotide insertion into a five-repeat allele (referred as A4, A5, A6, A9, and A5.1, respectively). The insertion of G (A5.1) causes a premature translation termination and results in loss of a transmembrane domain, which may produce the shorter form of the MICA protein that is likely be secreted into serum [25]. However, the association of this VNTR locus with serum sMICA level was controversial among studies [14,26,27,28]. Therefore, we examined the association between the VNTR locus and sMICA level in HBV-induced HCC patients, and found no significant association (Figure S1 and S2), concordant with our previous report for HCV-induced HCC patients [14].

Soluble MICA levels are associated with survival of HCC patients

In order to evaluate the prognostic significance of serum sMICA levels in HCC patients, we performed survival analysis of HCC patients. A total of 111 HBV-infected HCC patients and 129 HCV-infected HCC patients were included in this analysis. The mean survival period for HBV- and HCV-infected patients with less than 5 pg/ml of serum sMICA were 67.1 months (95% CI: 61.1–73.1, $n=83$), and 58.2 months (95% CI: 51.4–65.0, $n=85$), respectively. On the other hand, for patients with more than 5 pg/ml of serum sMICA, the mean survival periods were 47.8 months (95% CI: 34.8–30.9, $n=28$) for HBV-induced HCC patients and 59.5 months (95% CI: 51.9–67.1, $n=44$) for HCV-induced HCC patients. The Kaplan-Maier analysis and log-rank test indicated that among HBV-induced HCC subjects, the patients in the high serum sMICA group showed a significantly shorter survival than those in the low serum sMICA ($P=0.008$; Figure 2). In addition, we performed multi-variate analysis to test whether sMICA is an independent prognostic factor by including age and gender as covariates. The results revealed significant association of sMICA levels with overall survival ($P=0.017$) but not with age and gender (Table S1). However, we found no association between the serum sMICA level and the overall survival in the HCV-induced HCC subjects ($P=0.414$; Figure S3). Taken together, our findings imply the distinct roles of the *MICA* variation and sMICA between HBV- and HCV-induced hepatocellular carcinogenesis.

Vascular invasion in HBV-related HCC patients is associated with soluble MICA levels

Since sMICA levels were associated with the overall survival of HBV-related HCC patients, we tested whether sMICA levels affect survival through modulating invasive properties of tumors or size of the tumors. We tested the association between sMICA levels and vascular invasion in 35 HBV-related HCC cases, among whom 7 cases were positive and 21 cases were negative for vascular invasion. We found significant association between sMICA levels and vascular invasion (Figure 3; $P=0.014$) in which 7 cases with positive vascular invasion showed high levels of sMICA (mean = 54 pg/ml) than 21 cases without vascular invasion (mean = 7.51 pg/ml). However, we found no association between tumor size and sMICA levels ($P=0.56$; data not shown). These results suggest that sMICA may reduce the survival of HBV-related HCC patients by affecting the invasive properties of tumors.

Discussion

Several mechanisms such as HBV-genome integration into host chromosomal DNA [29] and effects of viral proteins including HBx [30] are shown to contribute to development and progression of HCC, while the immune cells such as NK and T cells function as key antiviral and antitumor effectors. MICA protein has been

Table 2. Association between HCC and rs2596542.

SNP	Comparison	Chr	Locus	Case MAF	Control MAF	P^*	OR*	95% CI
rs2596542	HCC vs. Healthy control	6	<i>MICA</i>	0.294	0.332	0.029	1.19	1.02–1.4
rs2596542	HCC vs. CHB	6	<i>MICA</i>	0.294	0.320	0.197	1.13	0.94–1.36

Note: 407 HCC cases, 699 CHB subjects and 5,657 non-HBV controls were used in the analysis.

Chr., chromosome; MAF, minor allele frequency; OR, odds ratio for minor allele; CI, confidence interval.

*Obtained by Armitage trend test.

doi:10.1371/journal.pone.0044743.t002

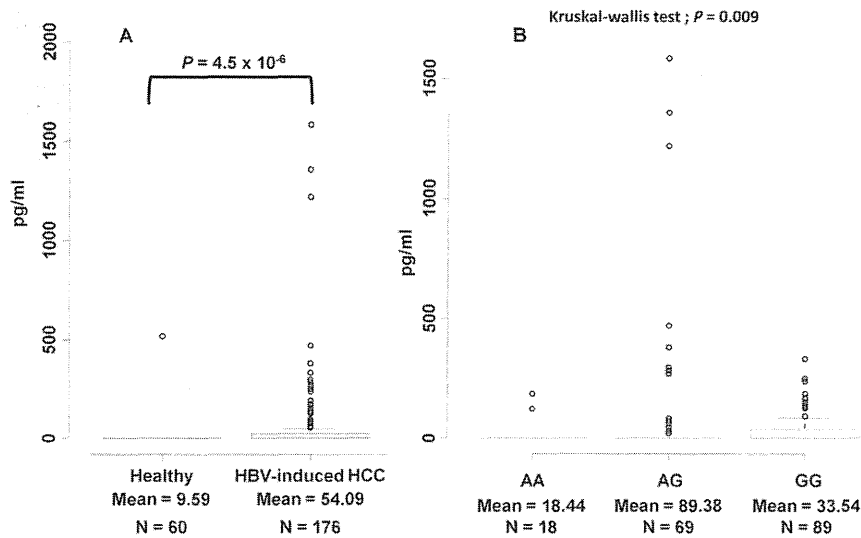


Figure 1. Soluble MICA levels are associated with HBV-related HCC. (A) Correlation between soluble MICA levels and HBV-induced HCC subjects. The y-axis displays the concentration of soluble MICA in pg/ml. The number of independent samples tested in each group is shown in the x-axis. Each group is shown as a box plot and the mean values are shown in the x-axis. The difference between two groups is tested by Wilcoxon rank test. The box plots are plotted using default settings in R. (B) Correlation between soluble MICA levels and rs2596542 genotype in HBV-positive HCC subjects. The x-axis shows the genotypes at rs2596542 and y-axis display the concentration of soluble MICA in pg/ml. Each group is shown as a box plot. $P=0.027$ and 0.013 for AA vs. GG and AA vs. AG, respectively. The association between genotypes and sMICA levels was tested by Kruskal-wallis test, whereas the difference in the sMICA levels between AA and GG is tested by Wilcoxon rank test. The box plots are plotted using default settings in R.

doi:10.1371/journal.pone.0044743.g001

considered as a stress marker of gastrointestinal epithelial cells because of its induced expression by several external stimuli such as heat, DNA damage, and viral infections [31,32,33,34]. Here,

we examined the association of rs2596542 and serum sMICA levels with HBV-induced HCC. Like in HCV-induced HCC [14], our results from ELISA revealed a significantly higher proportion

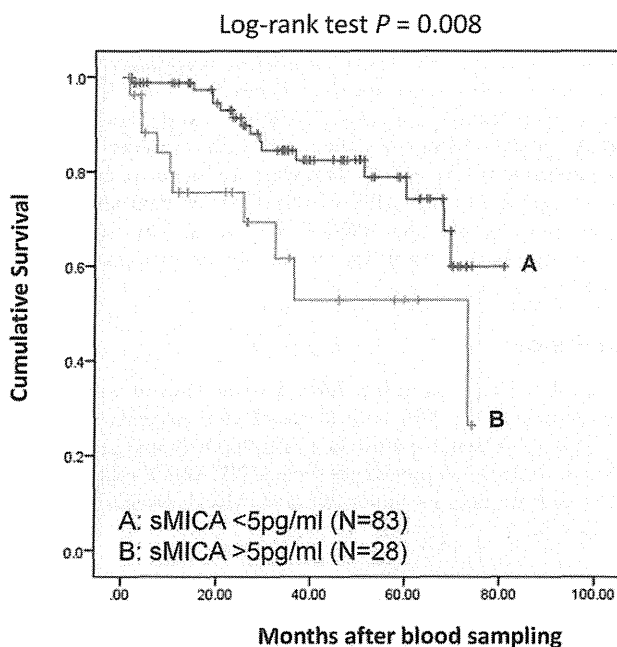


Figure 2. Kaplan-Meier curves of the patients with HBV-induced HCC. The patients were divided into two groups according to their sMICA concentration (high: >5 pg/ml and low: ≤ 5 pg/ml). Statistical difference was analyzed by log-rank test. The y-axis shows the cumulative survival probability and x-axis display the months of the patient's survival after blood sampling. doi:10.1371/journal.pone.0044743.g002

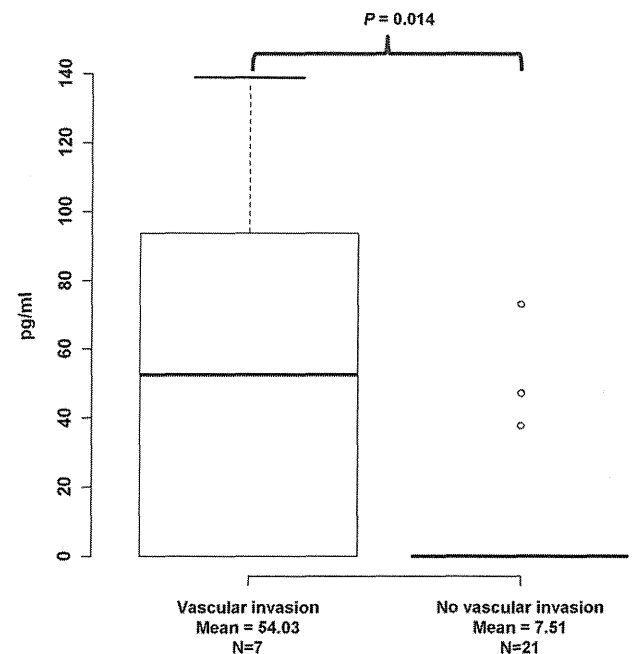


Figure 3. Correlation between soluble MICA levels and vascular invasion in HBV-induced HCC subjects. The y-axis displays the concentration of soluble MICA in pg/ml. The number of independent samples tested in each group is shown in the x-axis. Each group is shown as a box plot and the mean values are shown in the x-axis. The difference between two groups is tested by Wilcoxon rank test. The box plots are plotted using default settings in R. doi:10.1371/journal.pone.0044743.g003

of high serum sMICA cases (nearly 30%) in the HBV-induced HCC group, compared to non-HBV individuals (1.7%). Moreover, the serum sMICA level was significantly associated with rs2596542, but not with the copy number differences of the VNTR locus, as concordant with our previous report [14].

Several studies have already indicated the roles of sMICA as prognostic markers for different types of malignant diseases [17,18,19,20]. Therefore, it is of medical importance to test whether serum sMICA levels can be used as a prognostic marker for patients with HCC. To our best knowledge, this is the first study to demonstrate the prognostic potential of sMICA for HBV-positive HCC patients; we found 19.3 months of improvement in survival among patients carrying less than 5 pg/ml of serum sMICA, compared to those having more than 5 pg/ml.

On the contrary, we found no significant correlation between sMICA levels and the prognosis of HCV-induced HCC cases. These opposite effects of *MICA* variation could be explained by the following mechanism. The individuals who carry the G allele would express high levels of membrane-bound MICA upon HCV infection and thus lead to the activation of immune cells against virus infected cells. On one hand, HBV infection results in increased expression of membrane-bound MICA as well as MMPs through viral protein HBx [35], which would result in the elevation of sMICA and the reduction of membrane-bound MICA. Since sMICA could block CD8+T cells, NK-CTL, and NK cells, higher sMICA would cause the inactivation of immune surveillance system against HBV infected cells. In other words, HBV may use this strategy to evade immune response and hence, higher levels of sMICA could be associated with lower survival rate among HBV-associated HCC. On the other hand, since HCV is not known to induce the cleavage of membrane bound MICA, individuals with low level membrane bound MICA expression (carriers of rs2596542-allele A) could be inherently susceptible for HCV-induced HCC. Thus, HBx-mediated induction of MMPs could partially explain the intriguing contradictory effect of MICA between HBV-induced HCC and HCV-induced HCC. Since we observed significant correlation of sMICA levels with vascular invasion, it may be the case that high levels of sMICA cause poor prognosis of HBV-related HCC cases by making tumors more aggressive and invasive. However it is important in future to determine the ratio of membrane-bound MICA to sMICA in case of HCV- and HBV-related HCC.

Interestingly, the immune therapy against melanoma patients induced the production of auto-antibodies against MICA [36]. Anti-MICA antibodies would exert antitumor effects through antibody-dependent cellular cytotoxicity against cells expressing membrane-bound MICA and/or activation of NK cells by inhibiting the sMICA-NKG2D interaction. However, further studies are necessary, using well-defined HBV-related HCC

References

- Kew MC (2010) Epidemiology of chronic hepatitis B virus infection, hepatocellular carcinoma, and hepatitis B virus-induced hepatocellular carcinoma. *Pathol Biol (Paris)* 58: 273–277.
- Sherman M (2010) Hepatocellular carcinoma: epidemiology, surveillance, and diagnosis. *Semin Liver Dis* 30: 3–16.
- Perz J, Armstrong G, Farrington L, Hutin Y, Bell B (2006) The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. *J Hepatol* 45: 529–538.
- Chen CJ, Chen DS (2002) Interaction of hepatitis B virus, chemical carcinogen, and genetic susceptibility: multistage hepatocarcinogenesis with multifactorial etiology. *Hepatology* 36: 1046–1049.
- Cui R, Okada Y, Jang SG, Ku JL, Park JG, et al. (2011) Common variant in 6q26–q27 is associated with distal colon cancer in an Asian population. *Gut* 60: 799–805.
- Kumar V, Matsuo K, Takahashi A, Hosono N, Tsunoda T, et al. (2011) Common variants on 14q32 and 13q12 are associated with DLBCL susceptibility. *J Hum Genet* England. pp. 436–439.
- Cui R, Kamatani Y, Takahashi A, Usami M, Hosono N, et al. (2009) Functional variants in ADH1B and ALDH2 coupled with alcohol and smoking synergistically enhance esophageal cancer risk. *Gastroenterology* 137: 1768–1775.
- Urabe Y, Tanikawa C, Takahashi A, Okada Y, Morizono T, et al. (2012) A genome-wide association study of nephrolithiasis in the Japanese population identifies novel susceptible loci at 5q35.3, 7p14.3 and 13q14.1. *PLOS Genet* 8(3): e1002541.
- Tanikawa C, Urabe Y, Matsuo K, Kubo M, Takahashi A, et al. (2012) A genome-wide association study identifies two susceptibility loci for duodenal ulcer in the Japanese population. *Nat Genet* 44(4): 430–434.

cohort, to investigate whether sMICA levels could be included as an additional factor to predict the survival rate among HBV-related HCC subjects. Taken together, our results indicate the potential of *MICA* variant and sMICA as prognostic biomarkers. Thus, MICA could be a useful therapeutic target for HBV-induced HCC.

Supporting Information

Figure S1 MICA repeat genotyping using capillary-based method. The alleles are annotated using GeneMapper software based on the size of the PCR product (185 bp = A4 allele, 188 bp = A5, 189 bp = A5.1, 191 bp = A6 and 200 bp = A9). The inset at the base of each peak shows the size of the PCR product with corresponding allele call by the software. The figure display all observed heterozygotes at A5.1 allele. (TIF)

Figure S2 MICA VNTR alleles are not associated with soluble MICA levels. Each group is shown as a box plot. The difference in the sMICA values among each group is tested by Wilcoxon rank test. The box plots are plotted using default settings in R. (TIF)

Figure S3 Kaplan-Meier curves of the patients with HCV-induced HCC. The patients were divided into two groups according to their sMICA concentration (<5 pg/ml or >5 pg/ml). Statistical difference was analyzed by log-rank test. The y-axis shows the cumulative survival probability and x-axis display the months of the patients' survival after blood sampling. (TIF)

Table S1 Clinical parameters of HBV-related HCC patients available for prognostic analyses. (XLS)

Acknowledgments

We would like to thank all the patients and the members of the Rotary Club of Osaka-Midosuji District 2660 Rotary International in Japan, who donated their DNA for this work. We also thank Ayako Matsui and Hiroe Tagaya (the University of Tokyo), and the technical staff of the Laboratory for Genotyping Development, Center for Genomic Medicine, RIKEN for their technical support.

Author Contributions

Conceived and designed the experiments: VK KM YN. Performed the experiments: VK PHL YU HM ZD. Analyzed the data: VK PHL CT RM. Contributed reagents/materials/analysis tools: YN NK AT MK HS KT YT MS MM RT MO KK NK. Wrote the paper: VK PHL KM YN.

10. Hata J, Matsuda K, Ninomiya T, Yonemoto K, Matsushita T, et al. (2007) Functional SNP in an Sp1-binding site of AGTRL1 gene is associated with susceptibility to brain infarction. *Hum Mol Genet* 16: 630–639.
11. Kamatani Y, Wattanapokayakit S, Ochi H, Kawaguchi T, Takahashi A, et al. (2009) A genome-wide association study identifies variants in the HLA-DP locus associated with chronic hepatitis B in Asians. *Nat Genet* 41: 591–595.
12. Mbarek H, Ochi H, Urabe Y, Kumar V, Kubo M, et al. (2011) A genome-wide association study of chronic hepatitis B identified novel risk locus in a Japanese population. *Human Molecular Genetics* 20: 3884–3892.
13. Zhang H, Zhai Y, Hu Z, Wu C, Qian J, et al. (2010) Genome-wide association study identifies 1p36.22 as a new susceptibility locus for hepatocellular carcinoma in chronic hepatitis B virus carriers. *Nat Genet* 42: 755–758.
14. Kumar V, Kato N, Urabe Y, Takahashi A, Muroyama R, et al. (2011) Genome-wide association study identifies a susceptibility locus for HCV-induced hepatocellular carcinoma. *Nature genetics* 43: 455–458.
15. Jinushi M, Takehara T, Tatsumi T, Kanto T, Groh V, et al. (2003) Expression and role of MICA and MICB in human hepatocellular carcinomas and their regulation by retinoic acid. *Int J Cancer* 104: 354–361.
16. Bauer S, Groh V, Wu J, Steinle A, Phillips JH, et al. (1999) Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* 285: 727–729.
17. Holdenrieder S, Stieber P, Peterfi A, Nagel D, Steinle A, et al. (2006) Soluble MICA in malignant diseases. *Int J Cancer* 118: 684–687.
18. Nüchel H, Switala M, Sellmann L, Horn PA, Dürig J, et al. (2010) The prognostic significance of soluble NKG2D ligands in B-cell chronic lymphocytic leukemia. *Leukemia* 24: 1152–1159.
19. Tamaki S, Sanefuzi N, Kawakami M, Aoki K, Imai Y, et al. (2008) Association between soluble MICA levels and disease stage IV oral squamous cell carcinoma in Japanese patients. *Hum Immunol* 69: 88–93.
20. Li K, Mandai M, Hamanishi J, Matsumura N, Suzuki A, et al. (2009) Clinical significance of the NKG2D ligands, MICA/B and ULBP2 in ovarian cancer: high expression of ULBP2 is an indicator of poor prognosis. *Cancer Immunol Immunother* 58: 641–652.
21. Salih H, Rammensee H, Steinle A (2002) Cutting edge: down-regulation of MICA on human tumors by proteolytic shedding. *J Immunol* 169: 4098–4102.
22. Groh V, Wu J, Yee C, Spies T (2002) Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. *Nature* 419: 734–738.
23. Jinushi M, Takehara T, Tatsumi T, Hiramatsu N, Sakamori R, et al. (2005) Impairment of natural killer cell and dendritic cell functions by the soluble form of MHC class I-related chain A in advanced human hepatocellular carcinomas. *J Hepatol* 43: 1013–1020.
24. Nakamura Y (2007) The BioBank Japan Project. *Clin Adv Hematol Oncol* 5: 696–697.
25. Ota M, Katsuyama Y, Mizuki N, Ando H, Furihata K, et al. (1997) Trinucleotide repeat polymorphism within exon 5 of the MICA gene (MHC class I chain-related gene A): allele frequency data in the nine population groups Japanese, Northern Han, Hui, Uyghur, Kazakhstan, Iranian, Saudi Arabian, Greek and Italian. *Tissue Antigens* 49: 448–454.
26. Tamaki S, Sanefuzi N, Ohgi K, Imai Y, Kawakami M, et al. (2007) An association between the MICA-A5.1 allele and an increased susceptibility to oral squamous cell carcinoma in Japanese patients. *J Oral Pathol Med* 36: 351–356.
27. Tamaki S, Kawakami M, Yamanaka Y, Shimomura H, Imai Y, et al. (2009) Relationship between soluble MICA and the MICA A5.1 homozygous genotype in patients with oral squamous cell carcinoma. *Clin Immunol* 130: 331–337.
28. Lü M, Xia B, Ge L, Li Y, Zhao J, et al. (2009) Role of major histocompatibility complex class I-related molecules A*5.1 allele in ulcerative colitis in Chinese patients. *Immunology* 128: e230–236.
29. Bonilla Guerrero R, Roberts LR (2005) The role of hepatitis B virus integrations in the pathogenesis of human hepatocellular carcinoma. *J Hepatol* 42: 760–777.
30. Bouchard MJ, Schneider RJ (2004) The enigmatic X gene of hepatitis B virus. *J Virol* 78: 12725–12734.
31. Groh V, Bahram S, Bauer S, Herman A, Beauchamp M, et al. (1996) Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. *Proc Natl Acad Sci U S A* 93: 12445–12450.
32. Groh V, Steinle A, Bauer S, Spies T (1998) Recognition of stress-induced MHC molecules by intestinal epithelial gamma delta T cells. *Science* 279: 1737–1740.
33. Groh V, Rhinehart R, Randolph-Habecker J, Topp M, Riddell S, et al. (2001) Costimulation of CD8alpha beta T cells by NKG2D via engagement by MIC induced on virus-infected cells. *Nat Immunol* 2: 255–260.
34. Gasser S, Orsulic S, Brown EJ, Raulat DH (2005) The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. *Nature* 436: 1186–1190.
35. Lara-Pezzi E, Gomez-Gavero MV, Galvez BG, Mira E, Iniguez MA, et al. (2002) The hepatitis B virus X protein promotes tumor cell invasion by inducing membrane-type matrix metalloproteinase-1 and cyclooxygenase-2 expression. *J Clin Invest* 110: 1831–1838.
36. Jinushi M, Hodi F, Dranoff G (2006) Therapy-induced antibodies to MHC class I chain-related protein A antagonize immune suppression and stimulate antitumor cytotoxicity. *Proc Natl Acad Sci U S A* 103: 9190–9195.

ORIGINAL ARTICLE

Specific mutations of basal core promoter are associated with chronic liver disease in hepatitis B virus subgenotype D1 prevalent in Turkey

Mustafa Sunbul¹, Masaya Sugiyama², Fuat Kurbanov³, Hakan Leblebicioglu¹, Anis Khan³, Abeer Elkady³, Yasuhito Tanaka³, and Masashi Mizokami²

¹Department of Infectious Diseases, School of Medicine, Ondokuz Mayıs University, Kurupelit, Samsun, Turkey, ²Department of Hepatic Diseases, The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa, and ³Department of Virology, Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

ABSTRACT

The role of hepatitis B virus (HBV) genetics in the clinical manifestations of infection is being increasingly recognized. Genotype D is one of eight currently recognized major HBV genotypes. The virus is ubiquitous worldwide, but shows different features in different regions. One hundred and ninety-eight patients with chronic HBV infection were enrolled in this study, 38 of whom had been diagnosed with cirrhosis of the liver and/or hepatocellular carcinoma. HBV DNA was isolated from the patients' blood samples and the entire genome and/or the basal core promoter/core promoter region sequenced. Phylogenetic analysis of the complete genomes revealed that subgenotype D1 is the most prevalent subgenotype in Turkey, but there was no definite phylogenetic grouping according to geography for isolates from different regions within Turkey, or for isolates in Turkey relative to other parts of the world. Turkish isolates tended to be genetically similar to European and central Asian isolates. Overall, HBV-infection in Turkey appears to be characterized by early HBeAg seroconversion, a high incidence of the A1896 core promoter mutation and a small viral load. Genotype D characteristic mutations A1757 and T1764/G1766 were found in the BCP region. T1773 was associated with T1764/G1766 and a larger viral load. In conclusion, infection with HBV genotype D in Turkey has a similar clinical outcome to that of Europe and central Asia. Genotypic mutations in genotype D may be linked with disease prognosis in Turkey, but further studies with higher sample numbers and balanced clinical groups are needed to confirm this.

Key words basal core promoter, genotype D, hepatitis B virus, Turkey.

Hepatitis B virus infection is a global public health problem, affecting more than 350 million people worldwide. The clinical manifestations of this infection vary greatly and include acute self-limiting disease, an inactive carrier state and CH with progression to LC and

HCC (1, 2). An accumulating body of evidence indicates that the viral genotype (3, 4) and specific mutations in the viral genome (5, 6) are important viral factors contributing to the development of HCC. The main eight genotypes of HBV (A–H) have been identified based

Correspondence

Masaya Sugiyama, Department of Hepatic Diseases, The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa 272-8516, Japan.

Tel: +81 47 372 3501; fax: +81 47 375 4766; email: m.sugiyama@hospk.ncgm.go.jp

Received 3 October 2012; revised 12 November 2012; accepted 23 November 2012.

List of Abbreviations: γ -GTP, γ -glutamyl transpeptidase; AFP, alpha-fetoprotein; Alb, albumin; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate aminotransferase; BCP/CP, basal core promoter/core promoter; CH, chronic hepatitis; DB, direct bilirubin; EIA, enzyme-linked immunoassay; Glob, globulin; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinomas; HCV, hepatitis C virus; Kozak, Kozak sequence used in translation initiation site of eukaryotic mRNA; LC, liver cirrhosis; PLT, platelet; PT-INR, Prothrombin time-international normalized ratio; TB, total bilirubin; TP, total protein.