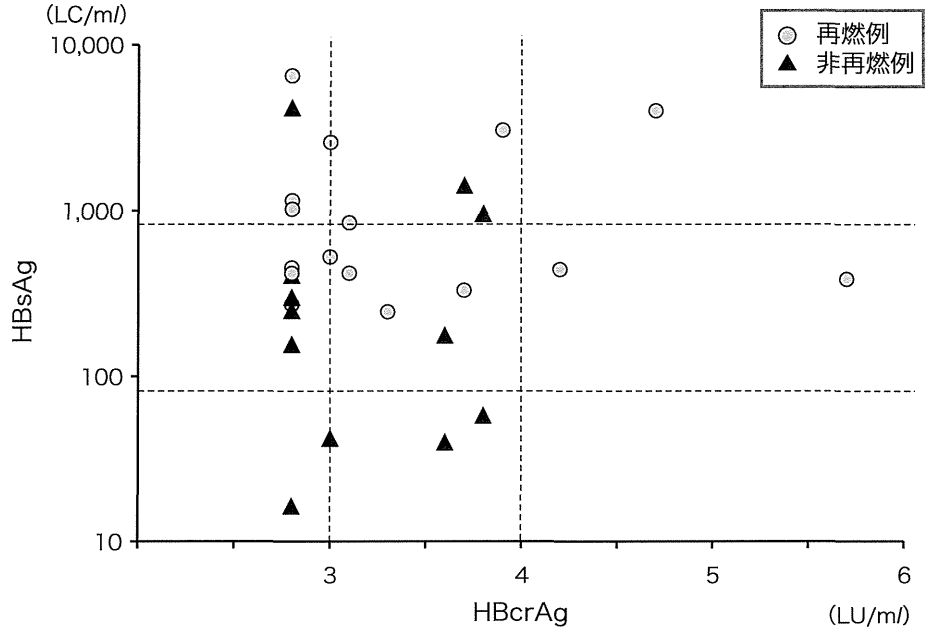


図6　HBe抗体陽性症例の核酸アナログ製剤投与中止時のHBs抗原，HBcr抗原と中止後の再燃

を24週後まで単独投与した12例について，治療開始後のHBVマーカーを経時的に測定した(図4)．

HBV DNA量は，両群ともに治療開始早期に減少したが，HBcr抗原量は，エンテカビル単独投与群での減少は緩徐であった．エンテカビル単独投与群と併用治療群の差分がIFNによる効果を反映する部分と考えられるが，HBcr抗原とHBV DNA量の減少量では，前者の減少量の方が2群間の差が大きく，IFNがウイルス抗原をより低下させていることを示唆している．

上記の症例について，治療開始を基準とした時の，治療開始後各時点までのHBcr抗原量とHBV DNA量の変化量を比較すると，IFNと核酸アナログ製剤併用症例の方が高値の傾向が示された(図5)．すなわち，併用例の方が，治療中のHBV DNAの減少度に比べてHBcr抗原の低下が大きいことを意味しており，同レベルのHBV DNA低下の場合には，併用例で，感染肝細胞排除，あるいは，細胞内HBV翻訳抑制が強いことを示唆している．

2. 核酸アナログ製剤投与中止時のHBVマーカーと再燃予測性

ラミブジン，あるいはエンテカビルを投与したHBe抗体陽性患者の中で，核酸アナログ投与中止後の再燃の有無と中止時のHBVレベルとの関連を検討した．

HBV DNA量は測定感度以下への低下を原則としており，該当する30例の中止時HBs抗原量，HBcr抗原量を測定した．いずれのマーカーも，中止後再燃群に比べて非再燃群でやや低い傾向を示したが，単一のマーカーによる2群比較では有意な差を認めなかった．

2012年に，厚生労働省「B型肝炎の核酸アナログ薬治療における治療中止基準の作成に関する研究班」(班長：信州大学，田中榮司教授)から，核酸アナログ投与中止に関わる指針が示された[12,13]．その指針では，核酸アナログ投与中止時のHBs抗原量とHBcr抗原量のレベルをスコア化し，両者の総スコアにより非再燃確率が示された．HBs抗原は，80

IU/m*l*（1.9 log IU/m*l*）と800 IU/m*l*（2.9 log IU/m*l*）で，HBcr抗原は，3.0 logU/m*l*，4.0 logU/m*l*で，それぞれ3群に分別してスコア0，1，2を配点し，総スコアを算定した．

われわれの症例についても，投与中止後にHBV DNA量が5.0（LC/m*l*）以上へ増加したものを再燃と判定し，治療中止時の検討を行った(図6)．上述のスコア判定を用いると，総スコア0の再燃低リスク群症例は2例で，ともに非再燃であった．また，総スコア1-2の中リスク群は20例中10例(50％)，総スコア3-4の再燃高リスク群では8例中2例(25％)が非再燃であった．

単独のHBVマーカーより，両者の組み合わせ判定の方が，中止後再燃の予測精度が上がると考えられる．

7 おわりに

HBVの構造や増殖機構の解析から，肝組織内のcccDNAが重要な位置にあることが判明したが，血液中のHBcr抗原は，日常臨床では測定が困難な肝内cccDNAレベルを反映した血液中のマーカーであり，抗HBV治療の評価にも有意義であることが明らかとなった．

文　献

1) Ganem D, Prince AM : Hepatitis B virus infection - Natural history and clinical consequences. New Eng J Med 350 : 1118–1129, 2004
2) Ghany M, Liang TJ : Drug targets and molecular mechanisms of drug resistance in chronic hepatitis B. Gastroenterology 132 : 1574–1585, 2007
3) Addison WR, Wong WW, Fischer KP et al : A quantitative competitive PCR assay for the covalently closed circular form of the duck hepatitis B virus. Antivir Res 48 : 27–37, 2000
4) He ML, Wu J, Chen Y et al : A new and sensitive method for the quantification of HBV cccDNA by real-time PCR. Biochem Biophys Res Commun 295 : 1102–1107, 2002
5) Lee JM, Ahn SN : Quantification of HBsAg: basic virology for clinical practice. World J Gastroenterol 17 : 283–289, 2011
6) 髭　修平，寺下勝巳，小林智絵，他：HBs抗原測定 – 新たな臨床応用の話題．臨床消化器内科 27：125–129, 2012
7) Kimura T, Ohno N, Terada N et al : Hepatitis B virus DNA-negative Dane particles lack core protein but contain a 22-kDa precore protein without C-terminal arginine-rich domain. J Biol Chem 280 : 21713–21719, 2005
8) Kimura T, Rokuhara A, Sakamoto Y et al : Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. J Clin Microbiol 40 : 439–445, 2002
9) 髭　修平，堀本啓大，小原俊央，他：新しいB型肝炎ウイルスマーカー：HBコア関連抗原．臨床消化器内科 24：132–136, 2009
10) Suzuki F, Miyakoshi H, Kobayashi M et al : Correlation between serum hepatitis B virus core-related antigen and intrahepatic covalently closed circular DNA in chronic hepatitis B patients. J Med Virol 81 : 27–33, 2009
11) Randall RE, Goodbourn S : Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. J Gen Virol 89 : 1–47, 2008
12) 田中榮司，松本晶博，鈴木義之，他：核酸アナログ薬中止に伴うリスク回避のための指針2012－厚生労働省「B型肝炎の核酸アナログ薬治療における治療中止基準の作成と治療中止を目指したインターフェロン治療の有用性に関する研究」の報告－．肝臓 53：237–242, 2012
13) Matsumoto A, Tanaka E, Suzuki Y et al : Combination of hepatitis B viral antigens and DNA for prediction of relapse after discontinuation of nucleos(t)ide analogs in patients with chronic hepatitis B. Hepatol Res 42 : 139–149, 2012

*　　*　　*

Sawai et al. BMC Medical Genetics 2012, 13:47
http://www.biomedcentral.com/1471-2350/13/1/47

BMC Medical Genetics

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 Mbarek[3], Koichi Matsuda[3], Yoriko Mawatari[2], Megumi Yamaoka[1], Shuhei Hige[4], Jong-Hon Kang[5], Koichi Abe[6], Satoshi Mochida[7], Masaaki Watanabe[8], Masayuki Kurosaki[9], Yasuhiro Asahina[9], Namiki Izumi[9], Masao Honda[10], Shuichi Kaneko[10], Eiji Tanaka[11], Kentaro Matsuura[12], Yoshito Itoh[13], Eiji Mita[14], Masaaki Korenaga[15], Keisuke Hino[15], Yoshikazu Murawaki[16], Yoichi Hiasa[17], Tatsuya Ide[18], Kiyoaki Ito[2], Masaya Sugiyama[2], Sang Hoon Ahn[19], Kwang-Hyub Han[19], Jun Yong Park[19], Man-Fung Yuen[20], Yusuke Nakamura[3], Yasuhito Tanaka[12], Masashi Mizokami[2] and Katsushi Tokunaga[1]

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
[1]Department 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_{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 mete-analysis of all our study. The result did not show any association between rs17401966 and HBV-derived HCC (P_{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				OR		
	(cases/controls)	GG	AG	AA	GG	AG	AA	HWE p	(95 % CI)	P [a]	P_{het} [b]
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)		

[a]P value of fisher's exact test for allele model.
[b]Result of Breslow-Day test.
[c]Results 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 closed 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: $P_{het} = 0.0066$; Chan's study versus Zhang's study: $P_{het} = 0.035$). 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 hepatisis from The Japan Society of Hepatology (http://www.jsh.or.jp/medical/gudelines/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, Nationa 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-μl reaction mixture containing 1 μl of genomic DNA, 2.5 μl 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

[1]Department of Human Genetics, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. [2]The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa, Japan. [3]Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, Tokyo, Japan. [4]Department of Internal Medicine, Hokkaido University Graduate School of Medicine, Sapporo, Japan. [5]Department of Internal Medicine, Teine Keijinkai Hospital, Sapporo, Japan. [6]First Department of Internal Medicine, Iwate Medical University, Iwate, Japan. [7]Division of Gastroenterology and Hepatology, Internal Medicine, Saitama Medical University, Saitama, Japan. [8]Department of Gastroenterology, Kitasato University School of Medicine, Sagamihara, Kanagawa, Japan. [9]Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan. [10]Department of Gastroenterology, Kanazawa University Graduate School of Medicine, Kanazawa, Japan. [11]Department of Medicine, Shinshu University School of Medicine, Matsumoto, Japan. [12]Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan. [13]Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Kyoto, Japan. [14]National Hospital Organization Osaka National Hospital, Osaka, Japan. [15]Division of Hepatology and Pancreatology, Kawasaki Medical College, Kurashiki, Japan. [16]Second department of Internal Medicine, Faculty of Medicine, Tottori University, Yonago, Japan. [17]Department of Gastroenterology and Metabology, Ehime University Graduate School of Medicine, Ehime, Japan. [18]Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, Fukuoka, Japan. [19]Department of International Medicine, Yonsei University College of Medicine, Seoul, Korea. [20]Department 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 HI, 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, Yuan JM, Gao YT, Henderson BE, Wogan GN, 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 Sawai[2], Kentaro Matsuura[3], Masaya Sugiyama[1], Sang Hoon Ahn[4], Jun Yong Park[4], Shuhei Hige[5], Jong-Hon Kang[6], Kazuyuki Suzuki[7], Masayuki Kurosaki[8], Yasuhiro Asahina[8], Satoshi Mochida[9], Masaaki Watanabe[10], Eiji Tanaka[11], Masao Honda[12], Shuichi Kaneko[12], Etsuro Orito[13], Yoshito Itoh[14], Eiji Mita[15], Akihiro Tamori[16], Yoshikazu Murawaki[17], Yoichi Hiasa[18], Isao Sakaida[19], Masaaki Korenaga[20], Keisuke Hino[20], Tatsuya Ide[21], Minae Kawashima[2], Yoriko Mawatari[1,2], Megumi Sageshima[2], Yuko Ogasawara[2], Asako Koike[22], Namiki Izumi[8], Kwang-Hyub Han[4], Yasuhito Tanaka[3], Katsushi Tokunaga[2], Masashi Mizokami[1]

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, Sagamihara, 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 Metabology, 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

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

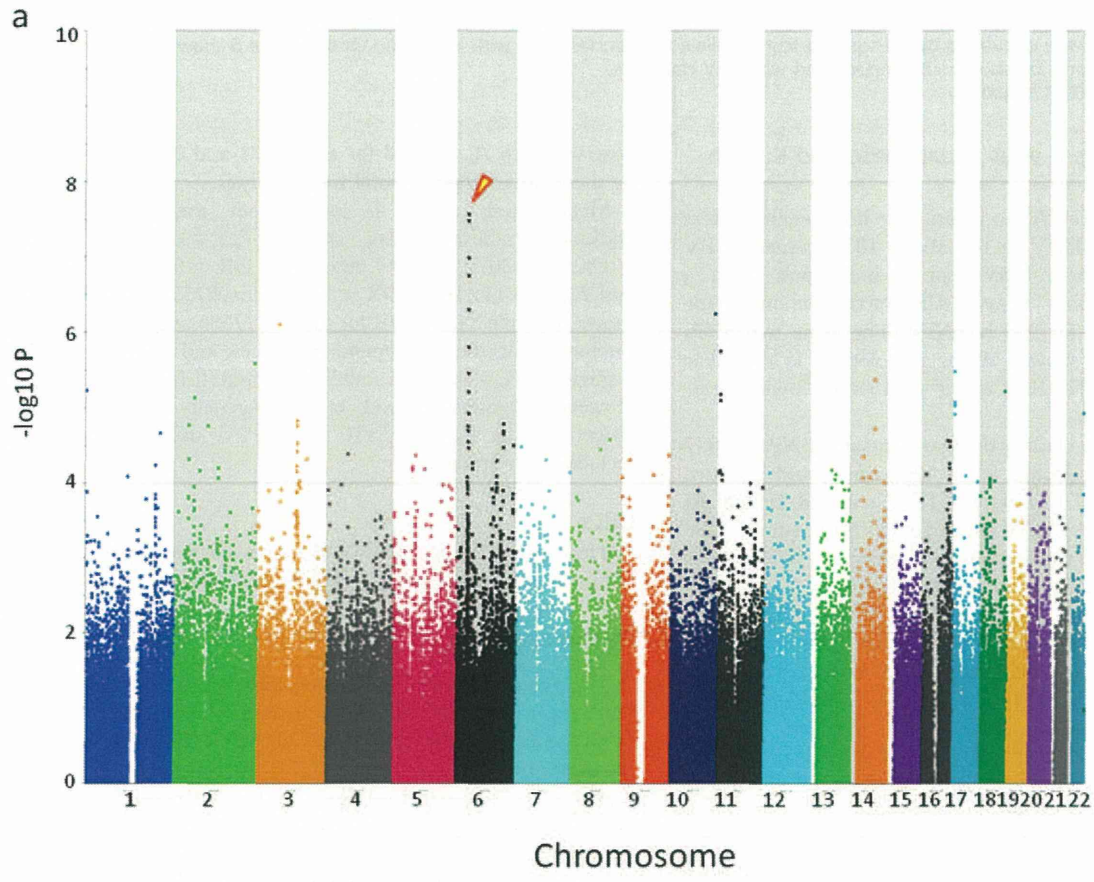
Protective Effects Against Chronic Hepatitis B in Japanese and Korean Individuals

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 ≥95%; (ii) minor allele frequency (MAF) ≥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 ≥ 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).


a
10
8
6
4
2
0
-log10 P
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Chromosome

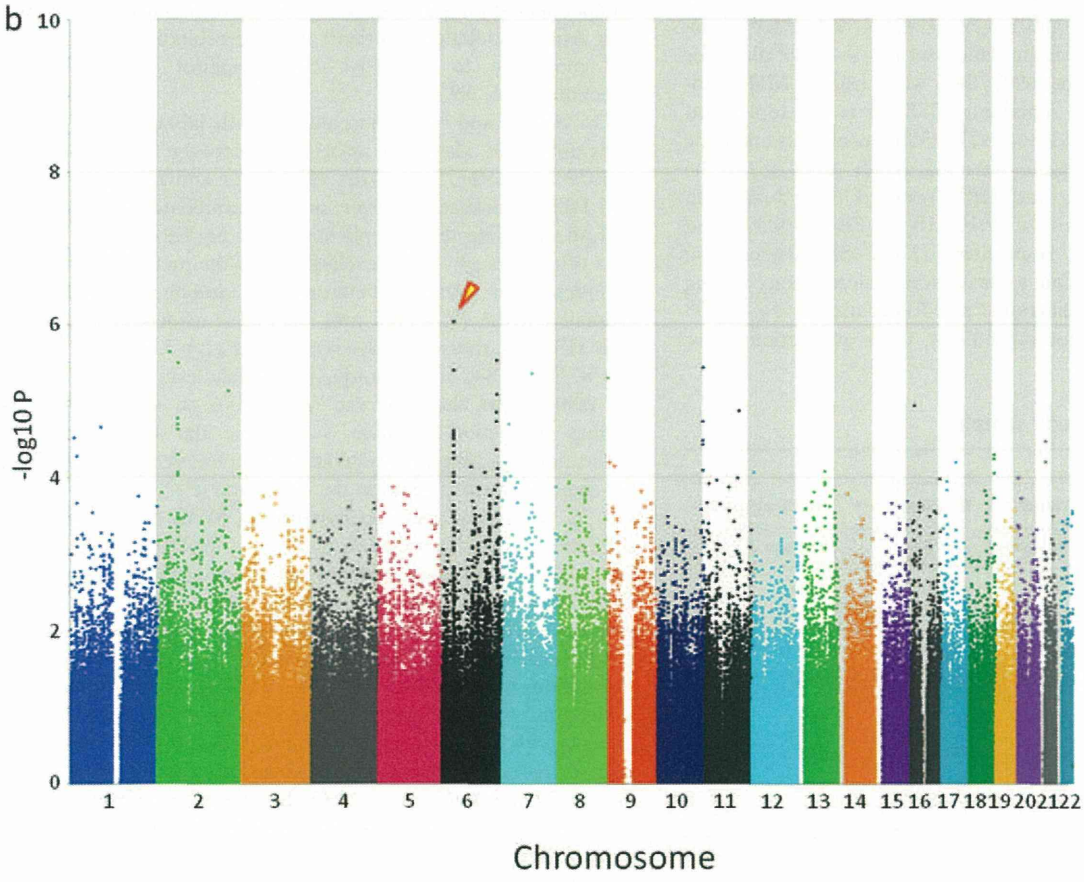

b
10
8
6
4
2
0
-log10 P
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Chromosome

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 ($P=9.24\times10^{-7}$ and $P=3.15\times10^{-5}$) 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, $P=3.32\times10^{-2}$, OR $=0.72$ and rs9277542, $P=1.25\times10^{-2}$, OR $=0.68$; replication-2 (Korean): rs3077, $P=2.35\times10^{-7}$, OR $=0.41$ and rs9277542, $P=4.97\times10^{-6}$, OR $=0.46$; Table 3]. Meta-analysis using random effects model showed $P_{meta}=1.56\times10^{-4}$ 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 $P_{meta}=3.28\times10^{-12}$ 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 $P_{meta}=1.48\times10^{-9}$, OR $=0.60$ for rs3077, $P_{meta}=1.08\times10^{-17}$, OR $=0.66$ for rs9277535 and $P_{meta}=5.14\times10^{-5}$, 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 $P_{het}=9.57\times10^{-6}$ across 5 independent studies, including our study.

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

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. Mata-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 $P_{meta}=5.88\times10^{-24}$, 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 ($P=3.32\times10^{-2}$, OR $=0.72$). Moreover, meta-analysis using GWAS and replication-2 showed significant association of $P_{meta}=1.89\times10^{-12}$, 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 2. Results of replication study for protective effects against CHB.

	Position		MAF[a]	Allele	Stage	HBV carriers			Healthy controls				OR[b]		
dbSNP rsID	Chr	Buld 36.3	Nearest Gene	(allele)	(1/2)	(population)	11	12	22	11	12	22	HWEp	95% CI	*P*-value[c]	P_{het}[d]
rs3077	6	33141000	HLA-DPA1	0.44	T/C	GWAS	13	51	117	28	88	67	0.919	0.42	1.14×10^{-7}	
				(T)		(Japanese)	(7.2)	(28.2)	(64.6)	(15.3)	(48.1)	(36.6)		(0.30–0.58)		
						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/C	GWAS	18	53	110	29	102	52	0.073	0.42	5.32×10^{-8}	
				(T)		(Japanese)	(9.9)	(29.3)	(60.8)	(15.8)	(55.7)	(28.4)		(0.31–0.58)		
						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)		

[a]Minor allele frequency and minor allele in 198 healthy Japanese (ref#19).
[b]Odds ratio of minor allele from two-by-two allele frequency table.
[c]P value of Pearson's chi-square test for allelic model.
[d]Heterogeneity was tested using general variance-based method.
[e]Meta-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 rs92775542) 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-DP*A1/−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.

	Position		MAF[a]	Allele	Stage	HBV carriers			Resolved individuals			OR[b]		
dbSNP rsID	Chr	Buld 36.3 Nearest Gene	(allele)	(1/2)	(population)	11	12	22	11	12	22	95% CI	*P*-value[c]	P_{het}[d]
rs3077	6	33141000 HLA-DPA1	0.44	T/C	GWAS	13	51	117	29	82	74	0.44	9.24×10^{-7}	
			(T)		(Japanese)	(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}	
					(Japanese)	(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}	
					(Korean)	(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)		
					Meta-analysis[e]							0.43	1.89×10^{-12}	0.75
					(GWAS+replication-2)							(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}	
			(T)		(Japanese)	(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}	
					(Japanese)	(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}	
					(Korean)	(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)		
					Meta-analysis[e]							0.49	9.69×10^{-10}	0.65
					(GWAS+replication-2)							(0.39–0.61)		

[a]Minor allele frequency and minor allele in 198 healthy Japanese (ref#19).
[b]Odds ratio of minor allele from two-by-two allele frequency table.
[c]P value of Pearson's chi-square test for allelic model.
[d]Heterogeneity was tested using general variance-based method.
[e]Meta-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/cm^3, 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 agreement (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 $P=8.36\times10^{-8}$ (0.05/597,789) and $P=8.47\times10^{-8}$ (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 Cochrane-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. [b]Odds ratio of minor allele from two-by-two allele frequency table. [c]Meta-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. [a]Minor allele frequency and minor allele in 198 healthy Japanese (ref#19). [b]Odds ratio of minor allele from two-by-two allele frequency table. [c]*P* value of Pearson's chi-squared test for allele model. [d]Heterogeneity was tested using general variance-based method. [e]Meta-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. [b]Odds ratio of minor allele from two-by-two allele frequency table. [c]Meta-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. [a]Minor allele frequency and minor allele in 198 healthy Japanese (ref#19). [b]Odds ratio of minor allele from two-by-two allele frequency table. [c]*P* value of Pearson's chi-squared test for allele model. [d]Heterogeneity was tested using general variance-based method. [e]Meta-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, Magnius 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. Szmuness 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, Yatsuhashi H, Komori A, Abiru S, et al. (2010) Dynamic epidemiology of acute viral hepatitis in Japan. Intervirology 53: 70–75.
10. 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.
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.

J Infect Chemother (2012) 18:883–890
DOI 10.1007/s10156-012-0433-4

ORIGINAL ARTICLE

Chronic hepatitis B in patients coinfected with human immunodeficiency virus in Japan: a retrospective multicenter analysis

Shintaro Yanagimoto · Hiroshi Yotsuyanagi · Yoshimi Kikuchi · Kunihisa Tsukada · Michio Kato · Junki Takamatsu · Shuhei Hige · Kazuaki Chayama · Kyoji Moriya · Kazuhiko Koike

Received: 14 January 2012 / Accepted: 10 May 2012 / Published online: 4 July 2012

Abstract A nationwide survey in Japan revealed that about 6 % of human immunodeficiency virus (HIV)-positive patients are coinfected with hepatitis B virus (HBV). To further analyze the features of liver disease in HIV/HBV-coinfected patients, we analyzed 252 patients from six hospitals in the HIV/AIDS (acquired immunodeficiency syndrome) Network of Japan. The mean age was 39.5 years, and the proportion of male patients was very high (243 of 252; 96 %). The main transmission route was male homosexual contact (186 of 252; 74 %), followed by heterosexual contact. The HBV genotype was determined in 77 patients. Among them, genotype A HBV was the most frequent (58 of 77; 75 %) and was detected almost exclusively in homosexual patients. Acute hepatitis B was documented in 21 patients (8 %). Three of the 252 HIV/HBV-coinfected patients developed advanced liver disease with the complication of ascites, hepatic encephalopathy, or hepatocellular carcinoma. A comparison between patients not treated and those treated with antiretroviral drugs including anti-HBV drugs revealed that the baseline liver function was worse in treated patients. However, the serum albumin levels and platelet counts in both groups increased after treatment and were similar. Liver disease-associated death was not observed. Here, we characterize the clinical features of liver disease in HIV/HBV-coinfected patients in Japan for the first time. The findings suggest that antiretroviral therapy with anti-HBV drugs may retard the progression of a liver disease and prevent liver disease-associated death in such patients.

Keywords Acquired immunodeficiency syndrome · Chronic liver disease · HBV DNA · Genotype

S. Yanagimoto · H. Yotsuyanagi (✉) · K. Moriya · K. Koike
Department of Internal Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
e-mail: hyotsu-tky@umin.ac.jp

Y. Kikuchi · K. Tsukada
AIDS Clinical Center, International Medical Center of Japan, Tokyo, Japan

M. Kato
Department of Gastroenterology, Osaka National Hospital, Osaka, Japan

J. Takamatsu
Division of Transfusion Medicine, Nagoya University Hospital, Nagoya, Japan

S. Hige
Department of Gastroenterology and Hematology, Hokkaido University Graduate School of Medicine, Sapporo, Japan

K. Chayama
Department of Medicine and Molecular Science, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

Introduction

The number of human immunodeficiency virus (HIV)-positive patients is growing in Japan [1]. Although combination therapy with antiretroviral agents has made HIV infection itself somewhat controllable in many cases since its introduction in 1996, and mortality from opportunistic infection has decreased, existing comorbidities are the focus of current patient care. In fact, more than 50 % of deaths in HIV-1-infected patients are not related to acquired immunodeficiency syndrome (AIDS); the mortality from liver disease is second only to AIDS-related mortality [2]. Risk factors related to significant liver

diseases among HIV-positive patients include a diagnosis of viral hepatitis [3], nonalcoholic fatty liver disease [4], and excessive alcohol consumption [5]. Among these factors, hepatitis B and hepatitis C are of particular importance because they can often lead to life-threatening diseases such as cirrhosis and hepatocellular carcinoma by themselves.

The estimated prevalence of chronic hepatitis B virus (HBV) infection in Japan is less than 1 %, or 0.9 million carriers [6]. However, about 6 % of HIV-positive patients are coinfected with HBV [7]; this coinfection rate is more than six times higher than that in the non-HIV population. In the United States, the HIV/HBV coinfection rate is reported to be in the range of 6–14 % [8–10].

Several issues make the management of HIV/HBV coinfection complicated. HBV infection tends to be persistent in HIV-positive patients [9, 11, 12]. Chronic HBV infection may lead to hepatitis, cirrhosis, or hepatocellular carcinoma. The progression of a liver disease associated with chronic HBV infection is more rapid in HIV/HBV-coinfected patients than in HBV-monoinfected patients [13].

Combination regimens of antiretroviral therapy (ART) for coinfected patients should be carefully determined. Initial combination regimens of ART for HIV/hepatitis C virus (HCV)-coinfected patients are basically the same as those for HIV patients without HCV infection. However, because some nucleoside reverse transcriptase inhibitors (NRTIs) used in HIV treatment have activity against HBV, and some NRTIs mainly used in HBV treatment have partial activity against HIV [14], careful choice of treatment agents is necessary in HIV/HBV coinfection. Abrupt discontinuation of NRTIs that are active against HBV may aggravate viral hepatitis. Administration of entecavir, which has a weak activity against HIV, to HIV/HBV-coinfected patients without simultaneous effective HIV treatment may cause the accumulation of drug-resistant HIV strains [15–17]. In such cases, drug resistance of HBV may occur as well [18].

Drug-induced liver injury following ART is another concern. HIV/HBV-coinfected patients show an increase in transaminase level at a higher rate [19, 20]. However, it is often unclear whether this increase is caused by drug hepatotoxicity because the treatment of HIV infection causes immune reconstruction in patients, which alone could contribute to the transaminase level increase in viral hepatitis.

The objective of this study is to clarify the clinical features of HIV/HBV coinfection in Japan and to clarify the impact of ART on liver function among HIV/HBV-coinfected patients. The estimated prevalence of chronic HBV infection among the general population in Japan is decreasing yearly, but it remains much higher than that in the United States [21], where universal hepatitis B vaccination is introduced. Thus, the detailed analysis of HIV/HBV coinfection in Japan is of particular importance.

Patients and methods

We have conducted a multicenter retrospective study based on the data from a nationwide survey in 2006 conducted by sending questionnaires to 372 member hospitals of the HIV/AIDS network of Japan as of January 2006, and part of the results was reported earlier [7]. Following the survey, 6 of the 207 hospitals that responded to the survey—Hokkaido University Hospital (Hokkaido, Japan), University of Tokyo Hospital (Tokyo, Japan), Nagoya University Hospital (Aichi, Japan), International Medical Center of Japan (currently, National Center for Global Health and Medicine, Tokyo, Japan), Osaka National Hospital (Osaka, Japan), and Hiroshima University Hospital (Hiroshima, Japan)—were chosen for further studies because more than two-thirds of the HIV/HBV-coinfected patients identified in the survey went to these hospitals, and because both HIV experts and hepatologists were following up those patients there.

The questionnaire sent to the hospitals included items regarding the number of patients who visited the hospitals at least once between January and December in 2006 as follows: (1) the number of HIV-positive patients; (2) the number of hepatitis B surface antigen (HBsAg)-positive patients among (1); (3) the number of patients among (2) who were determined at least once to have a serum alanine aminotransferase (ALT) level higher than 100 IU/l; (4) the number of HIV-positive patients who contracted HIV from blood products; (5) the number of HBsAg-positive patients among (4); (6) the number of patients among (5) who were determined at least once to have a serum ALT level higher than 100 IU/l; (7) the number of HIV-positive patients whose presumed transmission route is through homosexual contact; (8) the number of HBsAg-positive patients among (7); (9) the number of patients among (8) who were determined at least once to have a serum ALT level higher than 100 IU/l; (10) the number of HIV-positive patients who presumably contracted HIV through injection drug use; (11) the number of HBsAg-positive patients among (10); (12) the number of patients among (11) who were determined at least once to have a serum ALT level higher than 100 IU/l; (13) the number of HIV-positive patients whose transmission routes were classified as "others"; (14) the number of HBsAg-positive patients among (13); and (15) the number of patients among (15) who were determined at least once to have a serum ALT level higher than 100 IU/l.

We defined confirmed HIV infection with positivity for serum HBsAg as the criterion for HIV/HBV coinfection.

After identifying HIV/HBV-coinfected patients, medical records including laboratory data of these patients were reviewed between the date of the oldest available record for these patients and the final date of the record acquired by the end of the study. The laboratory data at the diagnosis or first recognition of HBV infection and the latest data in the study period were compared for analysis unless otherwise noted. HBV genotypes (A through D) were determined serologically by enzyme immunoassay (EIA) using commercial kits (HBV GENOTYPE EIA; Institute of Immunology, Tokyo, Japan) on the basis of the pattern of detection using monoclonal antibodies of a combination of epitopes on preS2-region products, each of which was specific for each genotype [22, 23].

Ethical issues

The respective ethics committees of the six hospitals approved the study. Informed consent was obtained from each study participant.

Statistical analyses

For the comparison of means of collected data, Student's *t* test (paired *t* test) was performed unless otherwise specified. The chi-square test was performed to determine the independence of clinical parameters.

Results

Two hundred and fifty-two patients were identified to have HIV/HBV coinfection. The mean age was 39.5 years, and the proportion of male patients was very high (243 of 252; 96.4 %). The main presumed transmission route of HIV was male homosexual contact (186 of 252; 73.8 %), followed by heterosexual contact. Among those HIV/HBV-coinfected patients, 21 of the 252 (8.3 %) acquired acute hepatitis during the study period (Table 1).

Table 1 Clinical background of HIV/HBV-coinfected patients

Number (male:female)	243:9
Age (year)	39.5 ± 9.6[a]
Presumed Transmission Route	
Transfusion	14
Homosexual contact	186
Heterosexual contact	24
Injection drug use	2
Others	4
Onset as acute hepatitis	21

[a] Mean ± standard deviation

The HBV genotype was determined in 77 patients. Among them, genotype A HBV was the most frequent (58 of 77; 75.3 %), followed far behind by genotype C (7 of 77; 9.1 %), which is the predominant genotype in the entire chronic hepatitis B population in Japan. Genotype B, which is also common in Japan, was found only in three patients (3.9 %). Genotype A was detected almost exclusively in homosexual patients (57 of 58; 98.3 %) (Fig. 1).

At the end of the study period, 113 patients (44.8 %) received some type of anti-HBV drug such as interferon, lamivudine, adefovir, or entecavir, not as part of anti-HIV treatment. Ninety-seven (38.5 %) patients were still taking anti-HBV drugs by the end of the study period. The median ALT level was 30.0 IU/l (5th percentile, 11.1; 95th percentile, 128.9), suggesting the existence of some liver injury. Liver function was normal in most HIV/HBV-coinfected patients. The mean serum albumin level was 4.1 ± 0.6 g/dl, and the median serum total bilirubin level was 0.8 mg/dl (5th percentile, 0.3; 95th percentile, 3.8). The mean platelet count was 21.0 ± 6.1 × 10^4/ml. The hepatitis B e antigen (HBeAg) was detected in 84 patients, and the HBV DNA level was high (higher than 100,000 IU/l) in 55 patients (Table 2). Three of the 252 (1.1 %) HIV/HBV-coinfected patients developed advanced chronic liver diseases, such as cirrhosis with the complication of ascites and/or hepatic encephalopathy, or hepatocellular carcinoma. Although we tried to retrieve information on alcohol consumption of the patients, it was available for only a limited number of patients (26 of 252); among the 26, only 2 patients had a habit of taking more than 60 g alcohol per day. The remaining 24 patients took alcohol only on social occasions. The antiretroviral agents used for these study patients are listed in detail in Table 3. Among those who had a known history of ART, 158 of 252 (62.7 %) received regimens that include anti-HBV drugs at least once previously, whereas 42 (16.7 %) did not, and no information is available for the remaining 52. The most common drug combination for HIV/HBV-coinfected patients was ATV/r + FTC/TDF (22 of 172; 12.8 %) (Table 4). FTC/TDF, composed of two drugs active against HBV, is recommended for HIV/HBV-coinfected patients

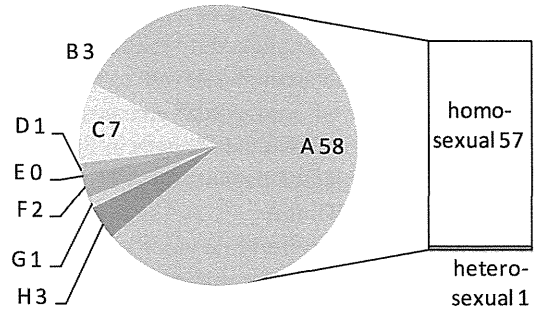


Fig. 1 Hepatitis B virus (HBV) genotype

Table 2 Liver function and related parameters of HIV/HBV-coinfected patients

Albumin (g/dl)	4.1 ± 0.6
Bilirubin[a] (mg/dl)	0.8 (5th percentile, 0.3; 95th percentile, 3.8)
ALT[a] (IU/l)	30.0 (5th percentile, 11.1; 95th percentile, 128.9)
WBC ($\times 10^3/\mu l$)	5.2 ± 1.6
Platelet ($\times 10^4/\mu l$)	21.0 ± 6.1
HBeAg (positive:negative)	84:68
HBV DNA (high:low)[b]	55:127

[a] Median and percentiles are provided instead of mean and standard deviation because of the nonnormality of the distribution

[b] HBV DNA level of 100,000 IU/l or higher is categorized as "high"

as one of the preferred NRTI backbones of the ART regimen [24].

We compared the clinical characteristics between patients who received the full ART and those who did not. Regarding the baseline statistical data, the observation period was longer for patients on ART, and there were more patients with AIDS in the ART group (10 of 64 vs. 52 of 162) (Table 5a). No significant difference was observed between the non-ART and ART groups in male/female ratio, age, transmission route, HBV markers, or advanced liver disease. Liver-related death was not observed, but hepatic failure with ascites and/or hepatic encephalopathy developed in 2 patients on ART and hepatocellular carcinoma developed in another patient.

Comparison between the ART group and the non-ART group revealed that the baseline liver function was worse in the ART group. At the beginning of the study period, the ART group showed a significantly lower CD4+ T-cell count than the non-ART group. The total white blood cell count and platelet count were also lower in the ART group. Although it is not statistically significant, the serum albumin level and prothrombin time (PT) index were lower in the ART group. However, at the end of the observation period, these parameters improved significantly in the ART group. The difference in CD4+ T-cell count between the ART and non-ART groups became marginal and became statistically insignificant (Table 5b).

Changes in the liver function of HIV/HBV-coinfected patients may not be fully explained by the changes in HBV activity because some parameters relevant to the estimation of liver function showed paradoxical changes. To clarify this observation, we compared the changes in liver function among HIV/HBV-coinfected patients on ART with respect to protease inhibitor (PI) use.

The mean serum total bilirubin level in patients on ART with PI use (PI group) at the beginning of the observation period was 1.1 mg/dl, whereas that in patients without PI use (non-PI group) was 0.8 mg/dl. The means at the end of the study period were 1.6 mg/dl in the PI group and 0.7 mg/dl in the non-PI group. Because the sample distribution of serum total bilirubin level did not follow the normal distribution by logarithmic transformation, we compared the means statistically. At the beginning, the difference in the mean between the PI group and the non-PI group was not significant ($p = 0.257$). At the end of the observation period, a statistically significant difference ($p = 0.001$) was observed. We then calculated the

Table 3 Antiretroviral treatment of HIV/HBV-coinfected patients

Antiretroviral drugs	Number of patients
NRTIs	
Zidovudine (AZT)	34
Didanosine (ddI)	9
Ddl / enteric coated	7
Zalcitabine (ddC)	1
Stavudine (d4T)	4
Lamivudine[a] (3TC)	84
Abacavir[3] (ABC)	38
Tenofovir[3] (TDF)	27
Emtricitabine (FTC) / TDF[a]	57
NNRTIs	
Nevirapine (NVP)	10
Efavirenz (EFV)	34
Delavirdine (DLV)	1
PIs	
Indinavir (IDV)	4
Ritonavir (RTV)	50
Nelfinavir (NFV)	8
Lopinavir (LPV)	3
Ritonavir-boosted LPV (LPV/r)	40
Atazanavir (ATV)	39
ATV/r	6
Fosamprenavir (FPV)	13

NRTI nucleoside reverse transcriptase inhibitor, *NNRTI* non-nucleoside reverse transcriptase inhibitor, *PI* protease inhibitor

[a] Agents with anti-HBV activity

Table 4 Antiretroviral regimens used for HIV/HBV-coinfected patients

Antiretroviral regimen	Number of patients
ATV/r + FTC/TDF	22
LPV/r + 3TC + TDF	8
LPV/r + FTC/TDF	7
EFV + FTC/TDF	6
ATV/r + 3TC + TDF	5

Table 5 Comparison of changes in clinical parameters of HIV/HBV-coinfected patients with or without antiretroviral therapy (ART)

a. Baseline statistical data	Natural course[a] (without ART)	With ART	*p* value (with vs. without ART)
Number (male:female)	84:6	159:3	0.105†
Age (year)	37.0 ± 10.3	39.0 ± 9.1	0.362
Observation period (month)	34.5 ± 55.5	50.9 ± 43.9	0.022*
Presumed transmission route	Blood products:homosexual contact:heterosexual contact:injection drug use:other		
	5:60:12:2:3	9:126:12:0:1	0.052†
Recognized acute hepatitis	10	11	0.243†
HBeAg (positive:negative)	42:18	100:40	0.394†
HBV DNA (high:low)	29:18	83:37	0.356†
HBV genotype	A:B:C:D:F:G:H		
	17:0:1:1:1:0:1	31:3:6:0:1:1:2	0.372†
Ascites	1/56	2/144	1.000†
Hepatocellular carcinoma	0/62	1/159	1.000†
Acquired immunodeficiency syndrome (AIDS)	10/64	52/162	0.012*,†

b. Comparison of clinical parameters between pre- and post-ART among patients with and without ART	Natural course (without ART)	With ART	*p* value (with vs. without ART)
CD4 count (per μl)			
Start[b]	402.9 ± 180.1	242.5 ± 187.6	0.000*
End[c]	406.4 ± 212.4	398.1 ± 195.9	0.883
p value (start vs. end)	0.893	0.000*	
Albumin (g/dl)			
Start	4.1 ± 0.4	3.8 ± 0.8	0.292
End	3.9 ± 0.8	4.2 ± 0.4	0.025*
p value	0.473	0.001*	
Bilirubin[d] (mg/dl)			
Start	0.7 (0.30, 4.26)	0.5 (0.30, 2.62)	0.138
End	0.5 (0.25, 1.30)	0.9 (0.36, 4.32)	0.000*
p value	0.046*	0.000*	
ALT[d] (IU/l)			
Start	46.0 (15.0, 1418.2)	34.0 (12.8, 1,068.8)	0.120
End	27.0 (9.9, 229.9)	31.5 (12.73, 89.3)	0.713
p value	0.003*	0.000*	
Prothrombin time index (%)			
Start	89.4 ± 13.1	78.8 ± 23.0	0.650
End	78.8 ± 27.3	84.2 ± 16.3	0.531
p value	0.377	0.218	
WBC ($\times 10^3$/μl)			
Start	6.1 ± 2.4	4.8 ± 2.1	0.000*
End	5.4 ± 1.4	5.1 ± 1.6	0.404
p value	0.044*	0.247	
Platelet ($\times 10^4$ /μl)			
Start	22.2 ± 6.5	19.3 ± 6.3	0.010*
End	21.2 ± 6.5	20.8 ± 6.1	0.649
p value	0.204	0.001*	

* $p < 0.05$

† Chi-square test was performed

[a] Two patients with habitual alcohol intake were included in this group

[b] Start of observation period

[c] End of observation period

[d] Means were compared by log transformation because of the nonnormality of the distribution; median and percentiles (5th percentile, 95th percentile) are provided

difference in serum total bilirubin level between the beginning and the end of the observation period [Dbilirubin level = (bilirubin level at the end) − (bilirubin level at the beginning)] in individual patients and compared it between the PI group and the non-PI group. The mean Dbilirubin level in the PI group was 0.5 ± 3.4 mg/dl and that in the non-PI group was −0.2 ± 1.6 mg/dl ($p = 0.250$). The Dbilirubin level in a patient in the PI group who was coinfected with HCV besides HIV/HBV as well was −27.4 mg/dl. Excluding this single outlier, the mean Dbilirubin level was significantly different between the PI and non-PI groups (mean Dbilirubin level 0.8 vs. −0.2; $p = 0.01$).

Discussion

We have summarized here the data from our comprehensive survey of HIV/HBV coinfection in Japan, focusing particularly on the clinical features of the patients and the effect of ART on liver function. As we reported earlier, HIV/HBV coinfection was observed in 6.3 % of Japanese HIV-positive patients [7]. Certain considerations for HBV coinfection are important in HIV patient care.

The major transmission route of HIV was male homosexual contact, which accounted for the infection in about 80 % of the patients; thus, male patients were the majority in the present cohort. The most frequently found genotype of HBV was genotype A, which is infrequent in HIV-negative patients in Japan. Genotype A is often found in the United States, Europe, India, and the west coast of Sub-Saharan Africa [25]. Although the data on HBV subgenotypes were not available in our study, some reports showed that most genotype A strains detected in HIV/HBV-coinfected individuals are of genotype Ae [26]. These findings suggest that HBV infection among Japanese HIV carriers is not caused by the spread of indigenous HBV, such as transmission in the perinatal period, but rather specific strains are circulating among the homosexual population in Japan. Genotypes B and C accounted for more than 96 % of the entire Japanese chronic HBV infection [27, 28]. These findings are compatible with the report that the presumed transmission route of HBV in HIV/HBV-coinfected patients is not from Japanese female partners but from male partners, as shown by Koibuchi et al. [29].

Seventy-five percent of HIV/HBV-coinfected patients received ART with two agents against HBV, and its efficacy against HBV as well as HIV is considered to be high. As recommended by the United States Department of Health and Human Services (DHHS) and the Japanese guidelines on HIV treatment, the initiation of ART with NRTIs with anti-HBV activity as the backbone is indicated for HIV/HBV-coinfected patients regardless of HIV viral load or CD4+ T lymphocyte count [30]. Nucleoside analogues can improve liver function in HBV-monoinfected patients [31]. Our study shows that ART decreased the levels of ALT and albumin in HIV/HBV-coinfected patients. It is noteworthy that the regimen used in ART includes multiple drugs with anti-HBV activity such as lamivudine plus abacavir, which is unusual for HBV-monoinfected patients.

When we compared the characteristics of patients on ART with those not on ART, there were some notable differences in their immune status and liver function. At the beginning of the observation period, patients on ART showed a lower CD4+ T-cell count and poorer liver function. Our study is a retrospective observation, and patients were not grouped randomly. These observations are rather understandable because those who had a low CD4+ T cell count were more likely candidates for ART. Additionally, patients on ART had a longer observation period and were more likely to develop AIDS. These findings are also understandable because the longer the duration of HIV infection, the more likely is the immune system of the patient to deteriorate. Moreover, once ART is started, patients need to visit clinics or hospitals regularly for a long period; in reality, for the rest of their life. Following current recommendations for the initiation of ART for HIV infection, patients with worse immune status are more likely to receive the treatment. These findings can explain our observation.

Our data show that the serum albumin level and platelet count improved in the patients who were on ART. As the regimen of ART usually contains two drugs against HBV, ART suppresses HBV replication, which may lead to an improved liver function, as observed in HBV-monoinfected patients treated with nucleoside analogues [31]. Long-term treatment with lamivudine was shown to regress the fibrosis of the liver [32, 33] and decrease the proportion of patients with hepatocellular carcinoma complication [34]. In view of these findings, ART for HIV/HBV-coinfected patients may markedly improve the prognosis of patients. In our study, only a small number of patients with advanced liver diseases associated with HBV infection such as cirrhosis or hepatocellular carcinoma were observed, which could be attributable in part to the short observation period and the short duration of HBV infection. If we had a longer observational period, we would be able to clarify the difference in clinical course between the ART and non-ART groups, and the actual significance of ART for HIV/HBV-coinfected patients should become clearer.

We found that some parameters related to liver function changed paradoxically, particularly in the ART group. Although the mean serum albumin level, ALT level, and platelet count improved, the mean serum bilirubin level worsened, from 0.5 to 0.9 mg/dl. On the other hand, the serum bilirubin level in the non-ART group decreased. Both changes are statistically significant, which suggests