postoperative interferon therapy. Br J Surg. 2002;89:418–22. doi: 10.1046/j.0007-1323.2001.02054.x.
29. Kudo M, Sakaguchi Y, Chung H, Hatanaka K, Hagiwara S, Ishikawa E, et al. Long-term interferon maintenance therapy improves survival in patients with HCV-related hepatocellular carcinoma after curative radiofrequency ablation. A matched case–control study. Oncology. 2007;72(Suppl 1):132–8. doi: 10.1159/000111719.
30. Singal AK, Freeman DH Jr, Anand BS. Meta-analysis: interferon improves outcomes following ablation or resection of hepatocellular carcinoma. Aliment Pharmacol Ther. 2010;32:851–8. doi:10.1111/j.1365-2036.2010.04414.x.
31. Miyake Y, Takaki A, Iwasaki Y, Yamamoto K. Meta-analysis: interferon-alpha prevents the recurrence after curative treatment of hepatitis C virus-related hepatocellular carcinoma. J Viral Hepat. 2010;17:287–92. doi:10.1111/j.1365-2893.2009.01181.x.
32. Arase Y, Ikeda K, Suzuki F, Suzuki Y, Kobayashi M, Akuta N, et al. Interferon-induced prolonged biochemical response reduces hepatocarcinogenesis in hepatitis C virus infection. J Med Virol. 2007;79:1485–90. doi:10.1002/jmv.20925.
33. Nomura H, Kashiwagi Y, Hirano R, Tanimoto H, Tsutsumi N, Higashi M, et al. Efficacy of low dose long-term interferon monotherapy in aged patients with chronic hepatitis C genotype 1 and its relation to alpha-fetoprotein: a pilot study. Hepatol Res. 2007;37:490–7. doi:10.1111/j.1872-034X.2007.00073.x.
34. Chen TM, Huang PT, Tsai MH, Lin LF, Liu CC, Ho KS, et al. Predictors of alpha-fetoprotein elevation in patients with chronic hepatitis C, but not hepatocellular carcinoma, and its normalization after pegylated interferon alfa 2a-ribavirin combination therapy. J Gastroenterol Hepatol. 2007;22:669–75. doi: 10.1111/j.1440-1746.2007.04898.x.
35. Osaki Y, Ueda Y, Marusawa H, Nakajima J, Kimura T, Kita R, et al. Decrease in alpha-fetoprotein levels predicts reduced incidence of hepatocellular carcinoma in patients with hepatitis C virus infection receiving interferon therapy: a single center study. J Gastroenterol. 2012;47:444–51.

Genome-wide Association Study Identifies *TNFSF15* and *POU2AF1* as Susceptibility Loci for Primary Biliary Cirrhosis in the Japanese Population

Minoru Nakamura,[1,2,3,30,*] Nao Nishida,[4,5,30] Minae Kawashima,[4] Yoshihiro Aiba,[1] Atsushi Tanaka,[6] Michio Yasunami,[7] Hitomi Nakamura,[1] Atsumasai Komori,[1] Makoto Nakamuta,[2] Mikio Zeniya,[8] Etsuko Hashimoto,[9] Hiromasa Ohira,[10] Kazuhide Yamamoto,[11] Morikazu Onji,[12] Shuichi Kaneko,[13] Masao Honda,[13] Satoshi Yamagiwa,[14] Kazuhiko Nakao,[15] Takafumi Ichida,[16] Hajime Takikawa,[6] Masataka Seike,[17] Takeji Umemura,[18] Yoshiyuki Ueno,[19] Shotaro Sakisaka,[20] Kentaro Kikuchi,[21] Hirotoshi Ebinuma,[22] Noriyo Yamashiki,[23] Sumito Tamura,[24] Yasuhiko Sugawara,[24] Akira Mori,[25] Shintaro Yagi,[25] Ken Shirabe,[26] Akinobu Taketomi,[26] Kuniaki Arai,[13] Kyoko Monoe,[10] Tatsuki Ichikawa,[15] Makiko Taniai,[9] Yasuhiro Miyake,[11] Teru Kumagi,[12] Masanori Abe,[12] Kaname Yoshizawa,[2,18] Satoru Joshita,[18] Shinji Shimoda,[27] Koichi Honda,[17] Hiroki Takahashi,[8] Katsuji Hirano,[16] Yasuaki Takeyama,[20] Kenichi Harada,[28] Kiyoshi Migita,[1] Masahiro Ito,[1] Hiroshi Yatsuhashi,[1] Nobuyoshi Fukushima,[2] Hajime Ota,[2] Tatsuji Komatsu,[2] Takeo Saoshiro,[2] Jinya Ishida,[2] Hirotsugu Kouno,[2] Hirotaka Kouno,[2] Michiyasu Yagura,[2] Masakazu Kobayashi,[2] Toyokichi Muro,[2] Naohiko Masaki,[2] Keiichi Hirata,[2] Yukio Watanabe,[2] Yoko Nakamura,[2] Masaaki Shimada,[2] Noboru Hirashima,[2] Toshiki Komeda,[2] Kazuhiro Sugi,[2] Michiaki Koga,[2] Keisuke Ario,[2] Eiichi Takesaki,[2] Yoshihiko Maehara,[26] Shinji Uemoto,[25] Norihiro Kokudo,[24] Hirohito Tsubouchi,[29] Masashi Mizokami,[5] Yasuni Nakanuma,[28] Katsushi Tokunaga,[4] and Hiromi Ishibashi[1]

For the identification of susceptibility loci for primary biliary cirrhosis (PBC), a genome-wide association study (GWAS) was performed in 963 Japanese individuals (487 PBC cases and 476 healthy controls) and in a subsequent replication study that included 1,402 other Japanese individuals (787 cases and 615 controls). In addition to the most significant susceptibility region, human leukocyte antigen (HLA), we identified two significant susceptibility loci, *TNFSF15* (rs4979462) and *POU2AF1* (rs4938534) (combined odds ratio [OR] = 1.56, $p = 2.84 \times 10^{-14}$ for rs4979462, and combined OR = 1.39, $p = 2.38 \times 10^{-8}$ for rs4938534). Among 21 non-HLA susceptibility loci for PBC identified in GWASs of individuals of European descent, three loci (*IL7R*, *IKZF3*, and *CD80*) showed significant associations (combined $p = 3.66 \times 10^{-8}$, 3.66×10^{-9}, and 3.04×10^{-9}, respectively) and *STAT4* and *NFKB1* loci showed suggestive association with PBC

[1]Clinical Research Center, National Hospital Organization (NHO) Nagasaki Medical Center, and Department of Hepatology, Nagasaki University Graduate School of Biomedical Sciences, Omura, Nagasaki 856-8562, Japan; [2]Headquaters of Primary Biliary Cirrhosis Research in the NHO Study Group for Liver Disease in Japan, Clinical Research Center, NHO Nagasaki Medical Center, Omura, Nagasaki 856-8562, Japan; [3]Headquaters of the gp210 Working Group in Intractable Liver Disease Research Project Team of the Ministry of Health and Welfare in Japan, Department of Hepatology, Nagasaki University Graduate School of Biomedical Sciences, Omura, Nagasaki 856-8562, Japan; [4]Department of Human Genetics, Graduate School of Medicine, The University of Tokyo, Tokyo 113-0033, Japan; [5]The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa 272-8516, Japan; [6]Department of Medicine, Teikyo University School of Medicine, Tokyo 173-8605, Japan; [7]Department of Clinical Medicine, Institute of Tropical Medicine, Nagasaki University, Nagasaki 852-8523, Japan; [8]Department of Gastroenterology and Hepatology, Tokyo Jikei University School of Medicine, Tokyo 105-8461, Japan; [9]Department of Medicine and Gastroenterology, Tokyo Women's Medical University, Tokyo 162-8666, Japan; [10]Department of Gastroenterology and Rheumatic Diseases, Fukushima Medical University, Fukushima 960-1295, Japan; [11]Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama 700-8558, Japan; [12]Department of Gastroenterology and Metabology, Ehime University Graduate School of Medicine, Matsuyama 791-0295, Japan; [13]Department of Gastroenterology, Kanazawa University Graduate School of Medicine, Kanazawa 920-0942, Japan; [14]Division of Gastroenterology and Hepatology, Niigata University Graduate School of Medical and Dental Sciences, Niigata 951-8510, Japan; [15]Department of Gastroenterology and Hepatology, Nagasaki University Graduate School of Biomedical Sciences, Nagaski 852-8501, Japan; [16]Department of Gastroenterology and Hepatology, Juntendo University Shizuoka Hospital, Shizuoka 410-2295, Japan; [17]First Department of Internal Medicine, Faculty of Medicine, Oita University, Oita 879-5593, Japan; [18]Division of Gastroenterology and Hepatology, Department of Medicine, Shinshu University School of Medicine, Matsumoto 390-8621, Japan; [19]Division of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai 980-8575, Japan; [20]Department of Gastroenterology and Medicine, Fukuoka University School of Medicine, Fukuoka 814-0180, Japan; [21]Deprtment of Internal Medicine, Teikyo University Mizonokuchi Hospital, Kawasaki 213-8507, Japan; [22]Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio Graduate School of Medicine, Tokyo 160-8582, Japan; [23]Organ Transplantation Service, The University of Tokyo, Tokyo 113-8655, Japan; [24]Hepatobiliary and Pancreatic Surgery Division and Artificial Organ and Transplantation Division, Department of Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo 113-8655, Japan; [25]Division of Hepato-pancreato-biliary Surgery and Transplantation, Department of Surgery, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan; [26]Department of Surgery and Science, Kyushu University Graduate School of Medical Sciences, Fukuoka 812-8582, Japan; [27]Department of Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences, Fukuoka 812-8582, Japan; [28]Department of Human Pathology, Kanazawa University Graduate School of Medicine, Kanazawa 920-0942, Japan; [29]Department of Digestive and Lifestyle-Related Disease, Kagoshima University Graduate School of Medical and Dental Science, Kagoshima 890-8520, Japan
[30]These authors contributed equally to this work
*Correspondence: nakamuram@nmc.hosp.go.jp
http://dx.doi.org/10.1016/j.ajhg.2012.08.010.

(combined $p = 1.11 \times 10^{-6}$ and 1.42×10^{-7}, respectively) in the Japanese population. These observations indicated the existence of ethnic differences in genetic susceptibility loci to PBC and the importance of TNF signaling and B cell differentiation for the development of PBC in individuals of European descent and Japanese individuals.

Primary biliary cirrhosis (PBC, MIM 109720) is a chronic and progressive cholestatic liver disease, presumably caused by autoimmune reactions against biliary epithelial cells, leading to liver cirrhosis and hepatic failure.[1] The incidence and prevalence of PBC range from 0.33 to 5.8 and from 2 to 40 per 100,000 inhabitants, respectively, in different geographical areas.[2] This may indicate the contribution of environmental or genetic factors in the development of PBC, whereas the clinical profiles of PBC are thought to be similar between different ethnicities and/or different geographical areas, including European-descent and eastern Asian populations. The high concordance rate in monozygotic twins compared to dizygotic twins[3] and familial clustering of individuals with PBC indicate the involvement of strong genetic factors in the development of PBC; however, the pathogenesis of PBC is still poorly understood. Previous genome-wide association studies (GWASs) and subsequent meta-analyses have identified *HLA* and 21 non-HLA susceptibility loci (*IL12A* [MIM 161560], *IL12RB2* [MIM 601642], *STAT4* [MIM 600558], *IRF5* [MIM 607218], *IKZF3* [MIM 606221], *MMEL1* [MIM 120520], *SPIB* [MIM 606802], *DENND1B* [MIM 613292], *CD80* [MIM 112203], *IL7R* [MIM 146661], *CXCR5* [MIM 601613], *TNFRSF1A* [MIM 191190], *CLEC16A* [MIM 611303], *NFKB* [MIM 164012], *RAD51L1* [MIM 602948], *MAP3K7IP1* [MIM 602615], *PLCL2* [MIM 614276], *RPS6KA4* [MIM 603606], *TNFAIP2* [MIM 603300], 7p14, and 16q24) to PBC in individuals of European descent,[4–7] indicating the important role of several autoimmune pathways (i.e., IL12A signaling, TNF/TLR-NF-κB signaling, and B cell differentiation) in the development of PBC. However, GWASs for PBC have never been reported for ethnicities other than European descent, limiting our knowledge of the genetic architecture of PBC. Here, we conducted a GWAS for PBC in the Japanese population to identify host genetic factors related to PBC, which would not only expand our knowledge of pathogenic pathways in PBC but also lead to the development of rationale for therapies in the future.

Samples from 2,395 individuals (1,295 cases with PBC and 1,100 healthy volunteers working at the National Hospital Organization (NHO) in Japan as a medical staff who declared having no apparent diseases, including chronic liver diseases and autoimmune diseases [healthy controls]) were collected by members of the Japan PBC-GWAS Consortium, which consists of 31 hospitals participating in the NHO Study Group for Liver Disease in Japan (NHOSLJ) and 24 university hospitals participating in the gp210 Working Group in Intractable Liver Disease Research Project Team of the Ministry of Health and Welfare in Japan. Most of the case and control samples were collected from the mainland and the neighboring islands of Japan (Honshu, Kyushu, and Shikoku). Previous studies have shown that there is little genetic heterogeneity in resident populations in these areas.[8] In fact, the genetic inflation factor was close to 1.00, and only a small portion of the samples were identified as outliers in the principal component analysis. The cases were diagnosed with PBC if they met at least two of the following internationally accepted criteria:[9] biochemical evidence of cholestasis based mainly on alkaline phosphatase elevation, presence of serum anti-mitochondrial antibodies, histological evidence of non-suppurative destructive cholangitis, and destruction of interlobular bile ducts. The demographic details of PBC cases are summarized in Table S1, available online. Of the 487 PBC cases in the GWAS, 57 were male and 430 were female, ages ranged from 33 to 90 years, the median age was 66 years, 320 cases had early-stage PBC (a stage without any signs indicating portal hypertension or liver cirrhosis), 110 had late-stage PBC without jaundice (a stage with signs of portal hypertension or liver cirrhosis but without persistent jaundice), and 57 were at the late stage with jaundice (persistent presence of jaundice [total bilirubin >2 mg/dl]). Of the 476 healthy controls in the GWAS, 170 were male and 306 were female, ages ranged from 25 to 87 years, and the median age was 40. Of the 808 PBC cases in the replication set, 120 were male and 688 were female, ages ranged from 24 to 85 years, the median age was 61 years, 646 had early-stage PBC, 121 had late-stage PBC without jaundice, and 39 were at the late stage with jaundice. Of the 624 healthy controls in the replication set, 271 were male and 353 were female, ages ranged from 24 to 74 years, and the median age was 33 years. Concomitant autoimmune diseases are also shown in Table S1. As for inflammatory bowel diseases such as Crohn disease (CD, MIM 266600) and ulcerative colitis (UC, MIM 266600), only one out of 1,274 PBC cases had UC, but none had CD. DNA was extracted from whole peripheral blood with the QIAamp DNA Blood Midi Kit (QIAGEN, Tokyo).

For the GWAS, we genotyped 1,015 samples (515 Japanese PBC cases and 500 Japanese healthy controls) using the Affymetrix Axiom Genome-Wide ASI 1 Array, according to the manufacturer's instructions. After excluding three PBC samples with a Dish QC of less than 0.82, we recalled the remaining 1,012 samples (512 cases and 500 controls) using the Genotyping Console v4.1 software. Here, Dish QC represents the recommended sample quality control (QC) metric for the Axiom arrays.[10] Of the 600,000 SNPs embedded in the array, samples with an overall call rate of less than 97% were also excluded. As a result, 508 cases and 484 controls were subjected to further analysis. All samples used for GWAS passed a heterozygosity check, and no duplicated and related samples were identified in identity by descent testing. Moreover, principal component analysis found 29 outliers to be excluded via the Smirnov-Grubbs test

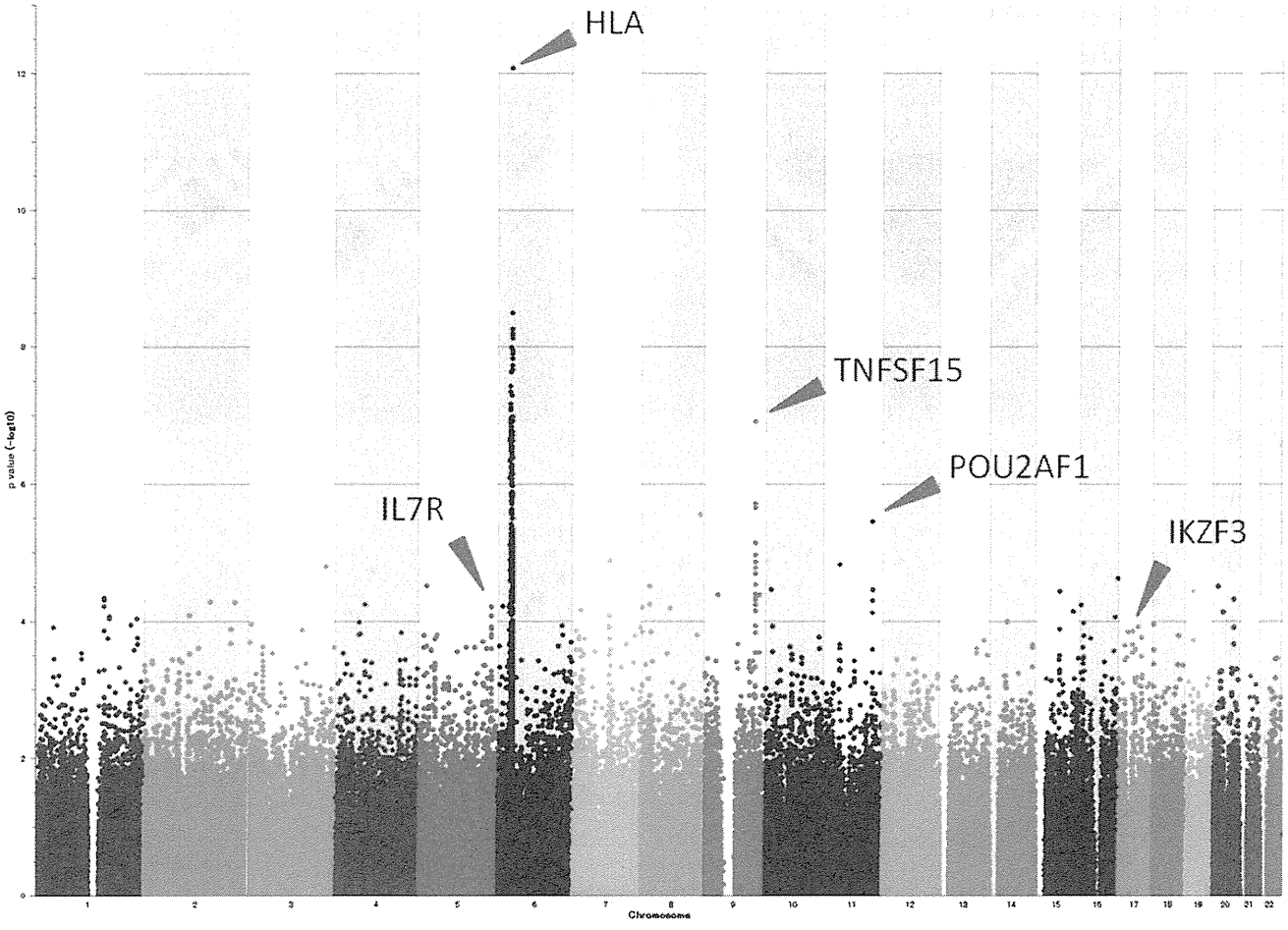

Figure 1. GWAS Results
From 963 samples (487 Japanese PBC cases and 476 Japanese healthy controls), p values were calculated with a chi-square test for allele frequencies among 420,928 SNPs.

and finally showed that all PBC cases (n = 487) and healthy controls (n = 476) formed a single cluster together with the HapMap JPT (Japanese in Tokyo from the CEPH collection), but not with CHB (Han Chinese in Beijing) samples (Figure S1, Table S2). These results indicate that the effect of population stratification was negligible. The average overall call rates of the remaining 487 PBC cases and 476 healthy controls were 99.38% (97.15–99.80) and 99.27% (97.01–99.81), respectively.[11] We then applied the following thresholds for SNP quality control during the data cleaning: SNP call rate ≥95%, minor allele frequency ≥5% in both PBC cases and healthy controls, and Hardy-Weinberg Equilibrium (HWE) p value ≥0.001 in healthy controls.[12] Of the SNPs on autosomal chromosomes and in the pseudoautosomal regions on the X chromosome, 420,928 and 317 passed the quality control filters and were used for the association analysis, respectively (Table S3). A quantile-quantile plot of the distribution of test statistics for the comparison of genotype frequencies in PBC cases and healthy controls showed that the inflation factor lambda was 1.039 for all the tested SNPs, including those in the HLA region, and was 1.026 when SNPs in the HLA region were excluded (Figures S2A and S2B). Table S4 shows the 298 SNPs with $p < 0.0001$ in the GWAS. All cluster plots for the SNPs with a $p < 0.0001$ from a chi-square test of the allele frequency model were checked by visual inspection, and SNPs with ambiguous genotype calls were excluded. For the GWAS and replication study, a chi-square test was applied to a two-by-two contingency table in an allele frequency model.

Figure 1 shows a genome-wide view of the single-point association data, which are based on allele frequencies. We found that the *HLA-DQB1* locus (MIM 604305) had the strongest association with susceptibility to PBC (rs9275175, odds ratio [OR] = 1.94; 95% confidence interval [CI] = 1.62–2.33, $p = 8.30 \times 10^{-13}$) (Figure 1 and Table S4); this finding was consistent with findings from previous studies.[4–7] In addition to the HLA class II region, loci *TNFSF15* and *POU2AF1* showed evidence indicative of association with PBC (rs4979462, OR = 1.63; 95% CI = 1.36–1.95, $p = 1.21 \times 10^{-7}$ for *TNFSF15*; rs4938534, OR = 1.53; 95% CI = 1.28–1.83, $p = 3.51 \times 10^{-6}$ for *POU2AF1*).

In a subsequent replication analysis, 27 SNPs with $p < 0.0001$ in the initial GWAS were also studied, in addition to SNPs at the *TNFSF15* and *POU2AF1* loci. Tagging SNPs were selected from the regions surrounding *TNFSF15* and

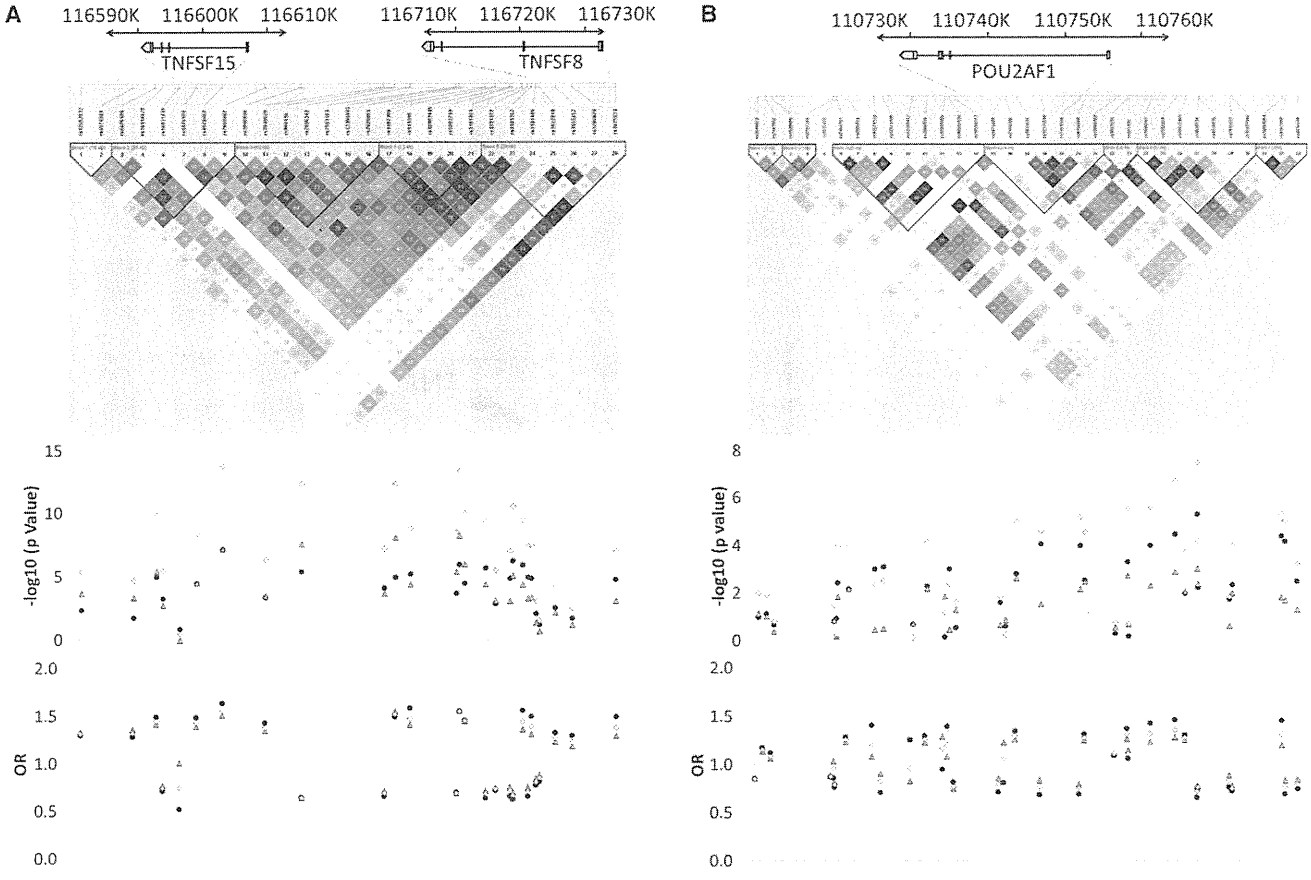

Figure 2. LD Structure, p Values, and OR Plots in the Association Analysis
LD maps (A) around *TNFSF15* (chr9: nucleotide position: 116561403–116733452; build 36.3) and (B) around *POU2AF1* (chr11: nucleotide position: 110684600–110802128; build 36.3). The middle panels show estimates of pairwise r^2 for (A) 28 SNPs and (B) 33 SNPs in the high-density mapping with a total of 2,365 samples used. The bottom panels show p values and OR-based chi-square tests for the allelic model for the left panels of 963 samples in the GWAS (●), the right panels of 1,402 samples in the replication study (▲), and the combined analysis (◇).

POU2AF1 (28 and 33, respectively) for high-density association mapping (Table S5, Figures 2A and 2B). For this follow-up replication analysis, an independent set of 1,402 samples (787 Japanese PBC cases and 615 Japanese healthy controls) and the original set of 963 samples (487 PBC cases and 476 healthy controls) were genotyped with the DigiTag2[13] and custom TaqMan SNP genotyping assays (Applied Biosystems, Foster City, CA, USA) on the LightCycler 480 Real-Time PCR System (Roche, Mannheim, Germany). The strongest associations identified in the initial GWAS were replicated in the independent set of 1,402 samples (OR = 1.52, $p = 5.79 \times 10^{-8}$ for rs4979462; OR = 1.29, $p = 9.32 \times 10^{-4}$ for rs4938534, Table 1). The combined p values were 2.84×10^{-14} (OR = 1.56; 95% CI = 1.39–1.76) for rs4979462 and 2.38×10^{-8} (OR = 1.39; 95% CI = 1.24–1.56) for rs4938534 (Table 1), both of which reached the genome-wide significance level of $p < 5 \times 10^{-8}$. In contrast, the other 27 weakly associated SNPs identified in the initial GWAS (p values <0.0001) were not found to have significant associations with PBC (Table S5). Moreover, no strongly associated SNPs were observed when comparing PBC cases between the early and late stages (Table S5).

A haplotype analysis of the *TNFSF15* and *POU2AF1* regions was conducted with the use of the genotype data from all 2,365 samples (1,274 PBC cases and 1,091 healthy controls). Linkage disequilibrium (LD) blocks were analyzed with Gabriel's algorithm,[14] and five blocks were observed in the *TNFSF15* region and seven blocks in the *POU2AF1* region (Figures 2A and 2B). There were no differences in the LD blocks between PBC cases and healthy controls. The risk haplotypes in each region showed a lower level of association than did the individual SNPs ($p = 8.26 \times 10^{-14}$ for *TNFSF15* and $p = 1.00 \times 10^{-4}$ for *POU2AF1*) (Tables S6 and S7).

Next, we focused on data from our initial GWAS in 21 loci that are reportedly associated with susceptibility to PBC in populations of European descent.[4–7] We found that three such loci (*IL7R*, *IKZF3*, and *STAT4*) had p values of less than 0.001 and eight other such loci (*RAD51L1*, *CXCR5*, *PLCL2*, *IL12RB2*, *NFKB1*, *CD80*, *DENND1B*, and 7p14) showed evidence of marginal associations ($p < 0.05$) in the initial GWAS in 487 Japanese PBC cases and 476 Japanese healthy controls (data not shown). We genotyped three SNPs (rs6890503 for *IL7R*, rs9303277 for *IKZF3*, and rs7574865 for *STAT4*) in an independent set

Table 1. *TNFSF15* SNP rs4979462 and *POU2AF1* SNP rs4938534 Associated with Susceptibility to PBC

dbSNP rsID	Nearest Gene	Risk Allele	Allele (1/2)	Stage	PBC Cases				Healthy Controls				OR[a]	
					11	12	22	RAF	11	12	22	RAF	95% CI	p Value[b]
rs4979462	*TNFSF15*	T	T/C	GWAS	154 (31.8)	244 (50.4)	86 (17.8)	0.57	98 (20.7)	230 (48.5)	146 (30.8)	0.45	1.63 (1.36–1.95)	1.21×10^{-7}
				Replication	253 (32.3)	390 (49.7)	141 (18.0)	0.57	131 (21.6)	305 (50.3)	170 (28.1)	0.47	1.52 (1.30–1.76)	5.79×10^{-8}
				Combined	407 (32.1)	634 (50.0)	227 (17.9)	0.57	229 (21.2)	535 (49.5)	316 (29.3)	0.46	1.56 (1.39–1.76)	2.84×10^{-14}
rs4938534	*POU2AF1*	A	G/A	GWAS	114 (23.6)	229 (47.3)	141 (29.1)	0.53	151 (31.8)	247 (52.0)	77 (16.2)	0.42	1.53 (1.28–1.83)	3.51×10^{-6}
				Replication	179 (22.8)	391 (49.8)	215 (27.4)	0.52	179 (29.4)	299 (49.2)	130 (21.4)	0.46	1.29 (1.11–1.50)	9.32×10^{-4}
				Combined	293 (23.1)	620 (48.9)	356 (28.1)	0.52	330 (30.5)	546 (50.4)	207 (19.1)	0.44	1.39 (1.24–1.56)	2.38×10^{-8}

Parenthetical numbers indicate the percentage of allele 11, 12, or 22 among total alleles in PBC cases or healthy controls. The following abbreviations are used: PBC, primary biliary cirrhosis; RAF, risk allele frequency; and GWAS, genome-wide association study.
[a]Odds ratio (OR) of minor allele from the two-by-two allele frequency table.
[b]p value of Pearson's chi-square test for the allelic model.

of 1,402 samples (787 Japanese PBC cases and 615 Japanese healthy controls) and the original set of 963 samples (487 PBC cases and 476 healthy controls) using the DigiTag2[13] and custom TaqMan SNP genotyping assays. Two SNPs, rs6890853 and rs9303277 located in loci *IL7R* and *IKZF3*, respectively, showed significant associations and the *STAT4* locus (rs7574865) showed suggestive association with PBC in 2,365 Japanese samples (1,274 PBC cases and 1,091 healthy controls) (rs6890853, combined p value = 3.66×10^{-8}, OR = 1.47 for *IL7R*; rs9303277, combined p value = 3.66×10^{-9}, OR = 1.44 for *IKZF3*; rs7574865, combined p value = 1.11×10^{-6}, OR = 1.35 for *STAT4*) (Tables S5 and S8).

Moreover, we genotyped 16 additional associated SNPs, all of which were the same SNPs as identified in previous studies,[4–7] and revealed that six out of 16 SNPs (located on *CXCR5*, *NFKB1*, *CD80*, *DENND1B*, *MAP3K7IP1*, and *TNFAIP2*) were replicated ($p < 0.05$) in 2,365 Japanese samples (Table S8). The SNP rs2293370, located in the *CD80* locus, showed a significant association and the *NFKB1* locus (rs7665090) showed a suggestive association with PBC in the Japanese population (rs2293370, combined p value = 3.04×10^{-9}, OR = 1.48 for *CD80*; rs7665090, combined p value = 1.42×10^{-7}, OR = 1.35 for *NFKB1*). Although further study for determining the primary SNP at each locus is necessary, the remaining ten loci (*RAD51L1*, *PLCL2*, *IL12RB2*, *IRF5*, *SPIB*, *RPS6KA4*, *CLEC16A*, *TNFRSF1A*, *IL12A*, and *MMEL1*) did not show significant association ($p < 0.05$) with PBC in the Japanese population (Table S8).

In the current GWAS in the Japanese population, we identified two significant susceptibility loci for PBC, *TNFSF15* (rs4979462) and *POU2AF1* (rs4938534), which had not been identified in the previous GWAS in populations of European descent. In addition, of the 21 PBC susceptibility loci that have been identified in populations of European descent, three loci (*IL7R*, *IKZF3*, and *CD80*) showed significant associations and two loci (*STAT4* and *NFKB1*) showed suggestive associations with PBC in the Japanese population. Eight other loci (*RAD51L1*, *CXCR5*, *PLCL2*, *IL12RB2*, *DENND1B*, *MAP3K7IP1*, *TNFAIP2*, and 7p14) also showed marginal associations with PBC in the Japanese population. These results indicate the presence of additional important disease pathways (via TNFSF15 and POU2AF1)—differentiation to T helper 1 (Th1) cells (via IL7R and STAT4), B cell differentiation (via IL7R and IKZF3), T cell activation (via CD80), and NF-κB signaling—in addition to the previously reported disease pathways in the development of PBC in Japanese populations.

TNFSF15 is a newly described member of the TNF superfamily that interacts with death receptor 3 (*DR3* [MIM 603366], also known as *TNFRSF25*) not only to promote effector T cell expansion (i.e., Th1 and Th17 cells) and cytokine production (i.e., interferon-γ [IFN-γ, MIM 147570]) at the site of inflammation, but also to induce apoptosis in cells that overexpress DR3.[15] Interestingly, genetic polymorphisms in *TNFSF15* are associated with susceptibility to CD, UC, ankylosing spondylitis (AS, MIM 106300), and leprosy (MIM 609888)[16–20] (Table S8). Strong association of five SNPs (rs3810936, rs6478108, rs6478109, rs7848647, and rs7869487) in the *TNFSF15* region with CD was first reported for a Japanese population,[16] and the finding was replicated in an independent Japanese population and in European-descent and Korean populations.[21–25] Another SNP within *TNFSF15* (rs4263839) is also associated with susceptibility to CD in populations of European descent.[17,20,26] In addition, the risk alleles of the SNPs were significantly associated with *TNFSF15* mRNA expression in peripheral blood.[27,28] Given that there exists strong LD among SNPs in *TNFSF15*, including those in the promoter region (rs6478109 and

rs7848647) and introns (rs4263839 and rs4979462), it is very probable that the PBC susceptibility haplotype containing rs4979462 also influences *TNFSF15* mRNA expression. Additionally, TNFSF15 signaling via DR3 synergizes with interleukin-12 (IL-12) and IL-18 to promote IFN-γ production.[15] The IL-12 signaling pathway includes *IL12A* and *IL12RB* (MIM 601604), variants of which have been identified as PBC susceptibility loci in previous GWASs of peoples of European ancestry, and has been implicated as a key player in the pathogenesis of PBC.[4–7] STAT4 is essential for IL-12 signal transduction via the IL-12 receptor (IL12R) for IFN-γ production and Th1 polarization.[29] Thus, the evidence that *TNFSF15* and *STAT4* were identified and confirmed as PBC susceptibility loci in the present study might indicate that the IL-12 signaling pathway via IL12R is also operative in PBC pathogenesis in Japanese populations, as it is in populations of European descent.

POU2AF1 is a B cell-specific transcriptional factor that coactivates octamer-binding transcriptional factors *POU2F1* (MIM 164175) and *POU2F2* (MIM 164176) on B cell-specific promoters; thus, *POU2AF1* is essential for B cell maturation and germinal center formation.[30] The E-twenty six transcription factor *Spi-B* was recently identified as a direct target of the coactivator *POU2AF1*.[31] *Spi-B* is an important mediator of both B cell receptor signaling and early T cell lineage decisions.[32,33] *Spi-B* also induces IL7R-induced CD40 (MIM 109535, MIM 300386) expression.[34] Given that *Spi-B* has been identified as a PBC susceptibility gene in previous GWASs of peoples of European ancestry,[6,7,35] variation of *POU2AF1* might function along with *Spi-B* in this pathway of B cell signaling and differentiation. The lack of *POU2AF1* reportedly prevents the development of autoimmunity in *Aiolos* (also known as *IKZF3*) mutant mice, which have a systemic lupus erythematosus (MIM 152700)-like phenotype, and in MRL-lpr mice.[36,37] *IKZF3* and *IL7R* were both replicated and confirmed as PBC susceptibility loci in this study; *IKZF3* functions as a transcription factor that participates in the generation of high-affinity bone marrow plasma cells responsible for long-term immunity, and *IL7R* participates in pre-B cell expansion.[38,39] Collectively, these results strengthen the notion that the B cell signaling pathway is involved in the development of PBC.

In conclusion, *TNFSF15* and *POU2AF1* were identified as significant susceptibility loci for PBC in a Japanese population. Our results provide further evidence for the presence of (1) ethnic differences in genetic susceptibility loci (i.e., *TNFSF15*, *IL12A*, and *IL12RB2*), (2) a new autoimmune pathway (i.e., TNFSF15 signaling) shared with other autoimmune diseases (CD, UC, and AS), and (3) common pathogenic pathways such as B cell differentiation (i.e., *POU2AF1*, *IKZF3*, and *SPIB*), IL-12 signaling (i.e., *IL12A*, *IL12RB2*, and *STAT4*), and T cell activation (i.e., *CD80*) for the development of PBC in individuals of European descent and Japanese individuals (Table S8). Functional analysis of these genetic loci, as well as the identification of additional susceptibility loci associated with PBC in eastern Asian populations, should facilitate the analysis of the pathogenesis of PBC worldwide and aid the development of rationale for therapies in the future.

Supplemental Data

Supplemental Data include two figures, eight tables, and Supplemental Acknowledgments and can be found with this article online at http://www.cell.com/AJHG/.

Acknowledgments

The study was approved by the ethics committees of Nagasaki Medical Center and all institutes and hospitals throughout Japan that participated in this collaborative study. All participants provided written informed consent for participation in this study. This study was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (#20590800, #23591006) to M.N., by a Grant-in-Aid for Clinical Research from the NHO to M. Nakamura, by a grant from the Research Program of Intractable Disease provided by the Ministry of Health, Labor, and Welfare of Japan to H.I., and by a grant for Scientific Research on Innovative Areas (Genome Science) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT) to K.T. We thank Yoriko Mawatari, Megumi Sageshima, Yuko Ogasawara, Natsumi Baba, and Rieko Hayashi (University of Tokyo) for technical assistance. We also thank Shinya Nagaoka and Seigo Abiru (NHO Nagasaki Medical Center, Omura, Japan) and Shigeki Hayashi, Hiroshi Mano, Yukio Ohara, Haruhiro Yamashita, Kouki Matsushita, Takeaki Sato, Tsutomu Yamashita, Masahiko Takahashi, Tetsuo Yamamoto, Hironori Sakai, Michio Kato, Fujio Makita, Hitoshi Takaki, and Hideo Nishimura (members of NHOSLJ) for collecting clinical data and blood samples and for obtaining informed consent from PBC cases.

Received: April 15, 2012
Revised: June 3, 2012
Accepted: August 8, 2012
Published online: September 20, 2012

Web Resources

The URLs for data presented herein are as follows:

MEXT Integrated GWAS Database, https://gwas.biosciencedbc.jp/cgi-bin/gwasdb/gwas_top.cgi
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org

References

1. Kaplan, M.M., and Gershwin, M.E. (2005). Primary biliary cirrhosis. N. Engl. J. Med. *353*, 1261–1273.
2. Boonstra, K., Beuers, U., and Ponsioen, C.Y. (2012). Epidemiology of primary sclerosing cholangitis and primary biliary cirrhosis: a systematic review. J. Hepatol. *56*, 1181–1188.
3. Selmi, C., Mayo, M.J., Bach, N., Ishibashi, H., Invernizzi, P., Gish, R.G., Gordon, S.C., Wright, H.I., Zweiban, B., Podda, M., and Gershwin, M.E. (2004). Primary biliary cirrhosis in monozygotic and dizygotic twins: genetics, epigenetics, and environment. Gastroenterology *127*, 485–492.

4. Hirschfield, G.M., Liu, X., Xu, C., Lu, Y., Xie, G., Lu, Y., Gu, X., Walker, E.J., Jing, K., Juran, B.D., et al. (2009). Primary biliary cirrhosis associated with HLA, IL12A, and IL12RB2 variants. N. Engl. J. Med. *360*, 2544–2555.
5. Hirschfield, G.M., Liu, X., Han, Y., Gorlov, I.P., Lu, Y., Xu, C., Lu, Y., Chen, W., Juran, B.D., Coltescu, C., et al. (2010). Variants at IRF5-TNPO3, 17q12-21 and MMEL1 are associated with primary biliary cirrhosis. Nat. Genet. *42*, 655–657.
6. Liu, X., Invernizzi, P., Lu, Y., Kosoy, R., Lu, Y., Bianchi, I., Podda, M., Xu, C., Xie, G., Macciardi, F., et al. (2010). Genome-wide meta-analyses identify three loci associated with primary biliary cirrhosis. Nat. Genet. *42*, 658–660.
7. Mells, G.F., Floyd, J.A., Morley, K.I., Cordell, H.J., Franklin, C.S., Shin, S.Y., Heneghan, M.A., Neuberger, J.M., Donaldson, P.T., Day, D.B., et al.; UK PBC Consortium; Wellcome Trust Case Control Consortium 3. (2011). Genome-wide association study identifies 12 new susceptibility loci for primary biliary cirrhosis. Nat. Genet. *43*, 329–332.
8. Yamaguchi-Kabata, Y., Nakazono, K., Takahashi, A., Saito, S., Hosono, N., Kubo, M., Nakamura, Y., and Kamatani, N. (2008). Japanese population structure, based on SNP genotypes from 7003 individuals compared to other ethnic groups: effects on population-based association studies. Am. J. Hum. Genet. *83*, 445–456.
9. Lindor, K.D., Gershwin, M.E., Poupon, R., Kaplan, M., Bergasa, N.V., and Heathcote, E.J.; American Association for Study of Liver Diseases. (2009). Primary biliary cirrhosis. Hepatology *50*, 291–308.
10. Hoffmann, T.J., Kvale, M.N., Hesselson, S.E., Zhan, Y., Aquino, C., Cao, Y., Cawley, S., Chung, E., Connell, S., Eshragh, J., et al. (2011). Next generation genome-wide association tool: design and coverage of a high-throughput European-optimized SNP array. Genomics *98*, 79–89.
11. Nishida, N., Koike, A., Tajima, A., Ogasawara, Y., Ishibashi, Y., Uehara, Y., Inoue, I., and Tokunaga, K. (2008). Evaluating the performance of Affymetrix SNP Array 6.0 platform with 400 Japanese individuals. BMC Genomics *9*, 431.
12. Miyagawa, T., Nishida, N., Ohashi, J., Kimura, R., Fujimoto, A., Kawashima, M., Koike, A., Sasaki, T., Tanii, H., Otowa, T., et al. (2008). Appropriate data cleaning methods for genome-wide association study. J. Hum. Genet. *53*, 886–893.
13. Nishida, N., Mawatari, Y., Sageshima, M., and 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.
14. Gabriel, S.B., Schaffner, S.F., Nguyen, H., Moore, J.M., Roy, J., Blumenstiel, B., Higgins, J., DeFelice, M., Lochner, A., Faggart, M., et al. (2002). The structure of haplotype blocks in the human genome. Science *296*, 2225–2229.
15. Meylan, F., Richard, A.C., and Siegel, R.M. (2011). TL1A and DR3, a TNF family ligand-receptor pair that promotes lymphocyte costimulation, mucosal hyperplasia, and autoimmune inflammation. Immunol. Rev. *244*, 188–196.
16. Yamazaki, K., McGovern, D., Ragoussis, J., Paolucci, M., Butler, H., Jewell, D., Cardon, L., Takazoe, M., Tanaka, T., Ichimori, T., et al. (2005). Single nucleotide polymorphisms in TNFSF15 confer susceptibility to Crohn's disease. Hum. Mol. Genet. *14*, 3499–3506.
17. Barrett, J.C., Hansoul, S., Nicolae, D.L., Cho, J.H., Duerr, R.H., Rioux, J.D., Brant, S.R., Silverberg, M.S., Taylor, K.D., Barmada, M.M., et al.; NIDDK IBD Genetics Consortium; Belgian-French IBD Consortium; Wellcome Trust Case Control Consortium. (2008). Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. Nat. Genet. *40*, 955–962.
18. Zhang, F.R., Huang, W., Chen, S.M., Sun, L.D., Liu, H., Li, Y., Cui, Y., Yan, X.X., Yang, H.T., Yang, R.D., et al. (2009). Genomewide association study of leprosy. N. Engl. J. Med. *361*, 2609–2618.
19. Zinovieva, E., Bourgain, C., Kadi, A., Letourneur, F., Izac, B., Said-Nahal, R., Lebrun, N., Cagnard, N., Vigier, A., Jacques, S., et al. (2009). Comprehensive linkage and association analyses identify haplotype, near to the TNFSF15 gene, significantly associated with spondyloarthritis. PLoS Genet. *5*, e1000528.
20. Latiano, A., Palmieri, O., Latiano, T., Corritore, G., Bossa, F., Martino, G., Biscaglia, G., Scimeca, D., Valvano, M.R., Pastore, M., et al. (2011). Investigation of multiple susceptibility loci for inflammatory bowel disease in an Italian cohort of patients. PLoS ONE *6*, e22688.
21. Kakuta, Y., Kinouchi, Y., Negoro, K., Takahashi, S., and Shimosegawa, T. (2006). Association study of TNFSF15 polymorphisms in Japanese patients with inflammatory bowel disease. Gut *55*, 1527–1528.
22. Tremelling, M., Berzuini, C., Massey, D., Bredin, F., Price, C., Dawson, C., Bingham, S.A., and Parkes, M. (2008). Contribution of TNFSF15 gene variants to Crohn's disease susceptibility confirmed in UK population. Inflamm. Bowel Dis. *14*, 733–737.
23. Yang, S.K., Lim, J., Chang, H.S., Lee, I., Li, Y., Liu, J., and Song, K. (2008). Association of TNFSF15 with Crohn's disease in Koreans. Am. J. Gastroenterol. *103*, 1437–1442.
24. Michelsen, K.S., Thomas, L.S., Taylor, K.D., Yu, Q.T., Mei, L., Landers, C.J., Derkowski, C., McGovern, D.P., Rotter, J.I., and Targan, S.R. (2009). IBD-associated TL1A gene (TNFSF15) haplotypes determine increased expression of TL1A protein. PLoS ONE *4*, e4719.
25. Thiébaut, R., Kotti, S., Jung, C., Merlin, F., Colombel, J.F., Lemann, M., Almer, S., Tysk, C., O'Morain, M., Gassull, M., et al. (2009). TNFSF15 polymorphisms are associated with susceptibility to inflammatory bowel disease in a new European cohort. Am. J. Gastroenterol. *104*, 384–391.
26. Wang, K., Baldassano, R., Zhang, H., Qu, H.Q., Imielinski, M., Kugathasan, S., Annese, V., Dubinsky, M., Rotter, J.I., Russell, R.K., et al. (2010). Comparative genetic analysis of inflammatory bowel disease and type 1 diabetes implicates multiple loci with opposite effects. Hum. Mol. Genet. *19*, 2059–2067.
27. Kakuta, Y., Ueki, N., Kinouchi, Y., Negoro, K., Endo, K., Nomura, E., Takagi, S., Takahashi, S., and Shimosegawa, T. (2009). TNFSF15 transcripts from risk haplotype for Crohn's disease are overexpressed in stimulated T cells. Hum. Mol. Genet. *18*, 1089–1098.
28. Zucchelli, M., Camilleri, M., Andreasson, A.N., Bresso, F., Dlugosz, A., Halfvarson, J., Törkvist, L., Schmidt, P.T., Karling, P., Ohlsson, B., et al. (2011). Association of TNFSF15 polymorphism with irritable bowel syndrome. Gut *60*, 1671–1677.
29. Lleo, A., Gershwin, M.E., Mantovani, A., and Invernizzi, P. (2012). Towards common denominators in primary biliary cirrhosis: the role of IL-12. J. Hepatol. *56*, 731–733.
30. Strubin, M., Newell, J.W., and Matthias, P. (1995). OBF-1, a novel B cell-specific coactivator that stimulates immunoglobulin promoter activity through association with octamer-binding proteins. Cell *80*, 497–506.

31. Bartholdy, B., Du Roure, C., Bordon, A., Emslie, D., Corcoran, L.M., and Matthias, P. (2006). The Ets factor Spi-B is a direct critical target of the coactivator OBF-1. Proc. Natl. Acad. Sci. USA *103*, 11665–11670.
32. Garrett-Sinha, L.A., Su, G.H., Rao, S., Kabak, S., Hao, Z., Clark, M.R., and Simon, M.C. (1999). PU.1 and Spi-B are required for normal B cell receptor-mediated signal transduction. Immunity *10*, 399–408.
33. David-Fung, E.S., Yui, M.A., Morales, M., Wang, H., Taghon, T., Diamond, R.A., and Rothenberg, E.V. (2006). Progression of regulatory gene expression states in fetal and adult pro-T-cell development. Immunol. Rev. *209*, 212–236.
34. Nguyen, V.T., and Benveniste, E.N. (2000). Involvement of STAT-1 and ets family members in interferon-gamma induction of CD40 transcription in microglia/macrophages. J. Biol. Chem. *275*, 23674–23684.
35. Hirschfield, G.M., Xie, G., Lu, E., Sun, Y., Juran, B.D., Chellappa, V., Coltescu, C., Mason, A.L., Milkiewicz, P., Myers, R.P., et al. (2012). Association of primary biliary cirrhosis with variants in the CLEC16A, SOCS1, SPIB and SIAE immunomodulatory genes. Genes Immun. *13*, 328–335.
36. Sun, J., Matthias, G., Mihatsch, M.J., Georgopoulos, K., and Matthias, P. (2003). Lack of the transcriptional coactivator OBF-1 prevents the development of systemic lupus erythematosus-like phenotypes in Aiolos mutant mice. J. Immunol. *170*, 1699–1706.
37. Zuo, J., Ge, H., Zhu, G., Matthias, P., and Sun, J. (2007). OBF-1 is essential for the generation of antibody-secreting cells and the development of autoimmunity in MRL-lpr mice. J. Autoimmun. *29*, 87–96.
38. Cortés, M., and Georgopoulos, K. (2004). Aiolos is required for the generation of high affinity bone marrow plasma cells responsible for long-term immunity. J. Exp. Med. *199*, 209–219.
39. Mackall, C.L., Fry, T.J., and Gress, R.E. (2011). Harnessing the biology of IL-7 for therapeutic application. Nat. Rev. Immunol. *11*, 330–342.

Digestive Diseases

Dig Dis 2012;30:554–560
DOI: 10.1159/000343064

Retreatment with Peginterferon α-2a + Ribavirin in Patients Who Failed Previous Peginterferon α-2b + Ribavirin Combination Therapy

Taisuke Ueda[a] Kaoru Tsuchiya[b] Satoru Hashimoto[c] Taisuke Inoue[d] Nobuyuki Enomoto[d] Mie Inao[e] Atsushi Tanaka[f] Masahiko Kaito[g] Fumio Imazeki[h] Shuhei Nishiguchi[i] Satoshi Mochida[e] Osamu Yokosuka[h] Hiroshi Yatsuhashi[c] Namiki Izumi[b] Masatoshi Kudo[a] for the RETRY Study Group

[a]Department of Gastroenterology and Hepatology, Kinki University School of Medicine, Osaka, [b]Department of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, [c]Clinical Research Center, National Hospital Organization, Nagasaki Medical Center, Nagasaki, [d]First Department of Internal Medicine, Faculty of Medicine, University of Yamanashi, Yamanashi, [e]Department of Gastroenterology and Hepatology, Faculty of Medicine, Saitama Medical University, Saitama, [f]Department of Medicine, Teikyo University School of Medicine, Tokyo, [g]Mie Gastroenterological Clinic, Mie, [h]Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, Chiba, and [i]Division of Hepatobiliary and Pancreatic Disease, Department of Internal Medicine, Hyogo College of Medicine, Hyogo, Japan

Key Words
Hepatitis C · Interleukin-28B · Peginterferon · Retreatment · Sustained viral response

Abstract
Background/Aims: Peginterferon (PEG-IFN) + ribavirin (RBV) combination therapy is the current standard of care for chronic hepatitis C. However, more than half of the patients cannot achieve sustained viral response (SVR). In Japan, the clinical benefit of retreatment with PEG-IFN + RBV combination retreatment is still unknown. ***Methods:*** We collected clinical data in 106 chronic hepatitis C patients who failed to achieve SVR with PEG-IFNα-2b + RBV combination therapy and were retreated with PEG-IFNα-2a + RBV. This retrospective study examined the efficacy of retreatment with PEG-IFNα-2a + RBV by evaluating the time to eradication of hepatitis C virus RNA, early virological response (EVR), and SVR. We compared the results of the previous therapy and retreatment in terms of efficacy and analyzed the factors influencing SVR. ***Results:*** The SVR rates in the non-responders and relapsers were 11 and 53%, respectively. EVR and prolonged treatment duration were associated with SVR. We also found that a prior response to PEG-IFN + RBV therapy was more important than the Interleukin-28B genotype for predicting the response to retreatment. ***Conclusions:*** Retreatment with PEG-IFNα-2a + RBV should be considered for relapsers and partial responders. Our results suggest that prolonged administration is also favorable for EVR cases to attain a higher SVR.

Introduction

The development of a combination therapy consisting of peginterferon (PEG-IFN) and ribavirin (RBV) has increased the hepatitis C virus (HCV) RNA response rate to 65–69% at the end of therapy with a sustained HCV RNA response (sustained viral response, SVR) in 54–56% of chronic hepatitis C (CHC) patients. Conversely, this

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0257–2753/12/0306–0554$38.00/0

Accessible online at:
www.karger.com/ddi

Masatoshi Kudo
Department of Gastroenterology and Hepatology
Kinki University Faculty of Medicine, 377-2, Ohno-Higashi
Osaka-Sayama, Osaka 589-8511 (Japan)
E-Mail m-kudo@med.kindai.ac.jp

indicates that PEG-IFN + RBV therapy does not induce a response in 31–35% of such patients either because HCV RNA is not eliminated during therapy or the therapy is not completed. Furthermore, HCV RNA reappears in 11–13% of patients after the end of therapy [1, 2].

Retreatment with PEG-IFN + RBV for non-responding or relapse patients has been studied in Western countries [3, 4], but no large-scale studies have been performed in Japan. The AASLD guidelines [5] do not recommend retreatment with PEG-IFN + RBV. However, the Japanese guidelines [6] state that 'after examining cases with no effect in previous treatment, a treatment for SVR or for maintenance should be selected'; thus, retreatment with PEG-IFN + RBV is not completely excluded according to the Japanese guidelines.

A high SVR rate is observed after the addition of telaprevir to PEG-IFN + RBV [7–10]. However, some patients in Japan have not benefited from the launch of telaprevir, because CHC patients in Japan are older than those in Western countries [11, 12] and are often anemic. Thus, we retrospectively analyzed the results of retreatment with PEG-IFN + RBV in Japanese relapse and non-responding patients who previously received therapy with PEG-IFN + RBV. Core amino acid substitutions at position 70 [13] and host genome single-nucleotide polymorphism (SNP) genotyping of rs8099917, an interleukin-28B (IL28B) SNP [14, 15], are related to the efficacy of PEG-IFN + RBV therapy. Therefore, these factors were also examined in the study.

Materials and Methods

A total of 106 patients received combination therapy with PEG-IFNα-2a + RBV at 12 medical facilities in Japan from 2007 to 2009. We retrospectively evaluated the data of CHC patients who failed to achieve SVR (i.e. non-responders) or became HCV RNA negative on PEG-IFNα-2b + RBV therapy but relapsed again after the end of treatment (i.e. relapsers). The non-responders were divided into two groups according to the maximum decrease in HCV RNA titer during the initial treatment. Retreatment with PEG-IFNα-2a + RBV was performed to examine the relationships between SVR and patient background factors, timing of the HCV RNA response, and treatment duration.

For the previous treatment, PEG-IFNα-2b (PegIntron; MSD, Tokyo, Japan) at a dose of 1.5 μg kg^{-1} per week subcutaneously and RBV (Rebetol; MSD) were used. PEG-IFNα-2a (Pegasys; Chugai Pharmaceutical Co., Ltd, Tokyo, Japan) + RBV (Copegus; Chugai Pharmaceutical Co., Ltd) was started between 2007 and 2009. In principle, as a starting dose, PEG-IFN was given once weekly at 180 μg PEG-IFNα-2a while RBV was given at 600–1,000 mg/day based on body weight (body weight <60 kg, 600 mg; 60–80 kg, 800 mg; >80 kg, 1,000 mg), according to the standard treatment protocol in Japan.

Serum HCV RNA after PEG-IFNα-2b + RBV treatment was assessed by quantification using a Cobas Amplicor HCV monitor test (high range method: detection range, 5–5,000 KIU ml^{-1}, or version 2.0: limit of quantitation, 500 IU ml^{-1}; Roche Diagnostics Co. Ltd, Tokyo, Japan).

HCV RNA in retreatment was measured using a Cobas TaqMan HCV test (Roche Diagnostics Co. Ltd) at 4-week intervals; the linear dynamic range was 1.2–7.8 log IU ml^{-1}. Samples with undetectable HCV RNA levels were reported as <1.2 log IU ml^{-1} (i.e. no detectable HCV RNA). Patients were judged to have attained SVR status if HCV RNA was not detected for 24 weeks after the end of treatment. Rapid viral response (RVR) was defined when HCV RNA was not detected at week 4; early virological response (EVR) was defined when HCV RNA was not detected at week 12 of treatment.

Univariate analysis was performed using a χ^2 test and Fisher's exact test. Multivariate analysis was performed using logistic regression using the stepwise method.

As a rule, dose modification followed the manufacturer's drug information on the intensity of potential adverse hematologic effects. The PEG-IFN doses were reduced to 50% of the original dose if the neutrophil count fell below 750/mm^3 and discontinued if the neutrophil count fell below 500/mm^3 or the platelet count (PLT) fell below 50,000/mm^3. RBV was also reduced from 1,000 to 600, 800 to 600, or 600 to 400 mg when hemoglobin (Hb) was below 10 g dl^{-1} and was discontinued when the Hb was below 8.5 g dl^{-1}. Both PEG-IFN and RBV were discontinued if there was a need to discontinue one of the drugs.

The baseline data of the patients are expressed as median values and ranges. In order to analyze the differences between baseline data and the factors associated with SVR, univariate analysis using the Mann-Whitney U test or a χ^2 test was performed; multivariate analysis was performed using stepwise and multiple logistic-regression models. p values <0.05 were considered significant.

This study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki amended in 2008, and informed consent was obtained from each patient.

Results

Characteristics

The median age of the 106 subjects was 60 years. There were 63 non-responders and 43 relapsers who received previous treatment. Non-responders were divided into two groups according to their virological response to the previous treatment: partial responders, maximum HCV RNA decrease of >2 log; null responders, maximum HCV RNA decrease <2 log. HCV RNA genotypes 1 and 2 were detected in 101 and 5 subjects, respectively. The baseline characteristics of the study patients are shown in table 1.

Efficacy

Retreatment of the 106 subjects with PEG-IFNα-2a + RBV therapy resulted in an SVR rate of 28% (30/106). The

Table 1. Baseline patients' characteristics

	Relapser (n = 43)	Partial responder (n = 39)	Null responder (n = 24)
Age, years	64 (44–75)	58 (33–77)	60 (30–69)
Male/female	18/25	19/20	12/12
Body mass index	22.2 (16.6–31.0)	23.4 (18.2–32.3)	22.2 (17.8–29.8)
Genotype 1/2	42/1	36/3	23/1
Viral load, log IU/ml	6.3 (3.7–7.2)	5.7 (1.2–7.2)	6.4 (3.7–7.5)
ALT, IU/ml	39 (15–189)	41 (12–379)	43 (18–275)
γ-GTP, IU/ml	24 (9–175)	51 (17–326)	39 (19–366)
WBC count, /mm^3	4,500 (1,900–8,300)	4,100 (2,000–9,400)	3,495 (1,700–7,100)
Platelet count, $\times 10^4$/mm^3	13.8 (7.6–24.9)	14.1 (4.5–28.6)	11.2 (4.5–29.3)
Hemoglobin, g/dl	13.8 (11.9–16.5)	13.3 (9.3–16.5)	12.4 (9.6–15.9)
LDL cholesterol, mg/dl	100 (42–167)	86 (32–128)	74 (15–122)
α-Fetoprotein, ng/ml	4 (1–48)	5.9 (1–30)	7.8 (2–108)
Core aa70, wild/non-wild	13/9	8/7	3/3
IL28B rs8099917 (TT/TG, GG)	21/5	12/7	3/7

Values are medians with ranges in parentheses or numbers.

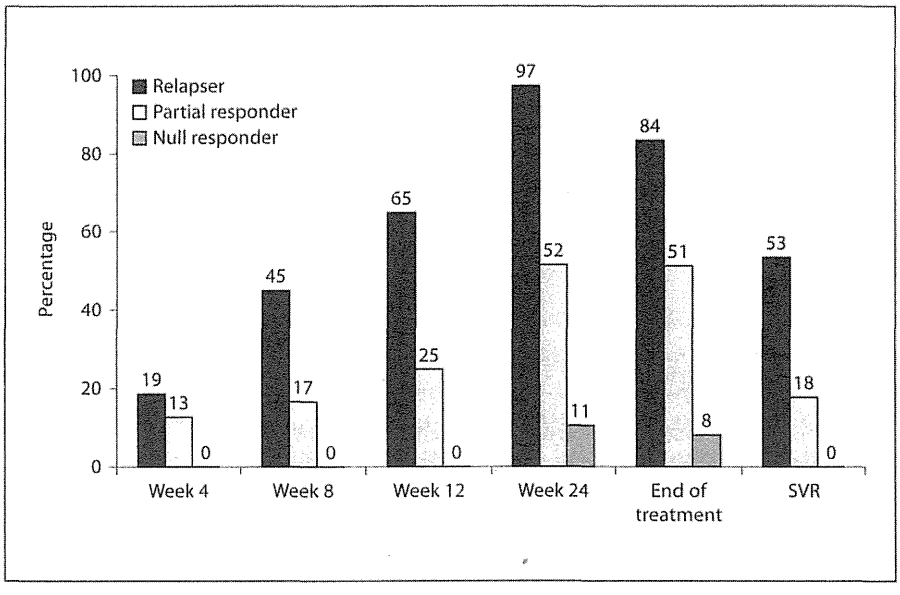

Fig. 1. Virological responses of PEG-IFNα-2a + RBV.

SVR rates in the relapsers, partial responders, and null responders were 53% (23/43), 18% (7/39), and 0% (0/24), respectively. The HCV RNA response rate in the relapsers was 65% at week 12 of the PEG-IFNα-2a + RBV therapy and 84% at the end of treatment (fig. 1). In contrast, the HCV RNA response rates in the non-responders were 15, 37, and 37% at week 12, week 24, and the end of retreatment, respectively. These rates were lower than those of the relapsers.

Examination of Previous Relapsers

The durations before an HCV RNA response was observed in the relapsers were ≤12 weeks in 65% (28/43) and 13–24 weeks in 26% (11/43) of the patients. In the 28 patients who achieved EVR, the SVR rates were 56% (9/16) and 92% (11/12) in the patients for whom retreatment commenced within 48 weeks and who received prolonged therapy, respectively; this difference was significant (p = 0.04).

Table 2. Factors associated to SVR

	Univariate analysis		Multivariate analysis	
	OR (95% CI)	p value	OR (95% CI)	p value
Response of previous treatment, relapsers/non-responders	9.20 (6.3–54)	<0.0001	–	n.s.
γ-GTP, <35/≥35 IU/l	2.92 (1.2–7.3)	0.0189	–	n.s.
Platelet counts, $\geq 12 \times 10^4 / < 12 \times 10^4$/mm^3	2.96 (1.1–7.7)	0.0232	–	n.s.
Hemoglobin, ≥13/<13 g/dl	3.24 (1.2–8.8)	0.0179	–	n.s.
LDL cholesterol, ≥100/<100 mg/dl	4.61 (1.5–14)	0.0046	–	n.s.
α-Fetoprotein, <6/≥6 ng/ml	4.16 (1.5–11)	0.0112	–	n.s.
Treatment duration, prolonged/standard	3.32 (1.4–8:0)	0.0063	5.16 (1.13–23.6)	0.0343
HCV RNA at week 12, negative/positive	18.5 (6.3–54)	<0.0001	10.3 (1.65–64.7)	0.0022
PEG-IFN adherence, ≥80/<80%	4.80 (1.6–14)	0.0025	–	n.s.
Ribavirin adherence, ≥60/<60%	3.69 (1.2–12)	0.0214	–	n.s.

We also compared the RVR and EVR rates between initial treatment and retreatment among the 41 patients whose timing of HCV negativity was available (fig. 2). The rates of RVR and EVR were higher in the retreatment patients (2 and 44% in initial treatment, and 20 and 66% in retreatment, respectively).

Examination of Previous Non-Responders

Among the non-responders, an HCV RNA response after retreatment was achieved only in partial responders. Of these patients, 23% (9/39) achieved EVR, 23% (9/39) within 13–24 weeks, and 8% (3/37) after 25 weeks. In the 9 subjects who achieved EVR among partial responders, the SVR rate was 25% (1/4) during the retreatment within 48 weeks but was 60% (3/5) when the retreatment period was prolonged. No SVR was achieved in retreatment subjects with an HCV RNA response that required ≥25 weeks.

Factors Associated with SVR

Analysis revealed that the SVR rate was significantly high in the following factors: relapsers; prolonged duration of retreatment; Hb level ≥13 g dl^{-1}; PLT ≥12 × 10^4/mm^3; α-fetoprotein (AFP) <6 ng ml^{-1}; low-density lipoprotein cholesterol (LDL-Chol) ≥100 mg dl^{-1}; γ-glutamyl transpeptidase (γ-GTP) ≥35 IU l^{-1}; adherence of PEG-IFN ≥80%; adherence of RBV ≥60%, and EVR. A multivariate analysis of SVR including the effect of initial treatment, duration of retreatment, Hb, PLT, AFP, LDL-Chol, γ-GTP, PEG-IFN adherence, RBV adherence, and EVR as variables showed that EVR and the period of retreatment were major factors contributing to SVR (table 2).

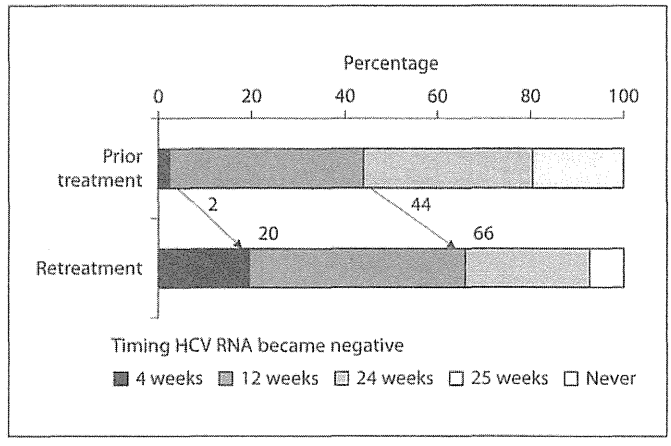

Fig. 2. Comparison of time to eradicate HCV RNA. Prior treatment: PEG-IFNα-2b + RBV combination treatment. Retreatment: PEG-IFNα-2a + RBV combination treatment.

IL28B SNP Genotype

55 patients agreed to undergo a test for IL28B SNP genotyping. Among them, 36, 18, and 1 had the TT, TG, and GG genotypes, respectively. The patients with TT had significantly lower γ-GTP and AFP levels; moreover, relapse patients were more frequently found to have TT. Among the patients with TT, 40% (14/35) showed an HCV RNA response at week 12, 70% (21/29) at week 24, and 56% (20/36) at the end of treatment. Among the non-TT patients, 16% (3/19) showed an HCV RNA response at week 12, 44% (6/16) at week 24, and 42% (7/19) at the end of treatment (fig. 3).

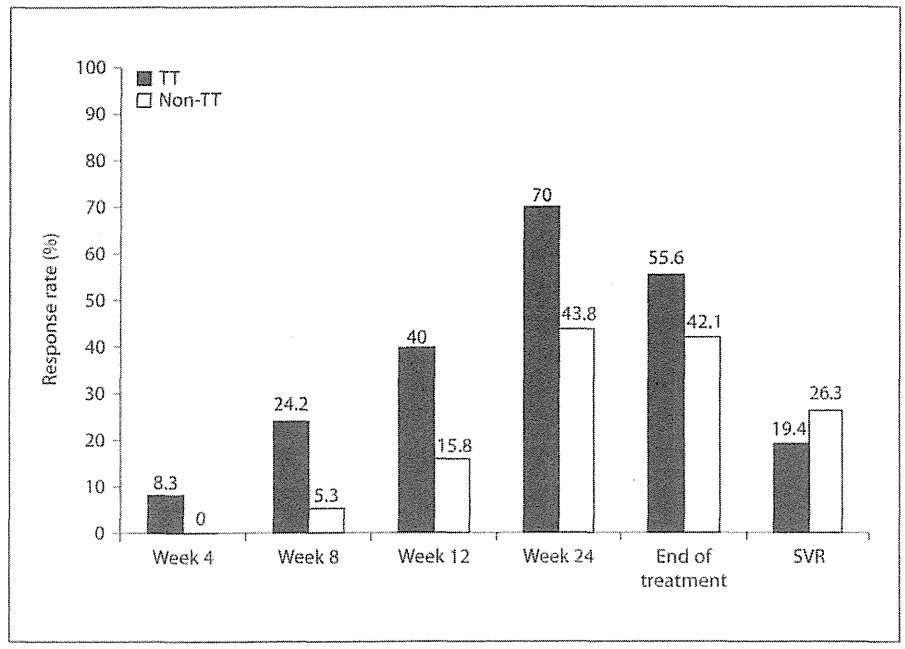

Fig. 3. Virological responses of PEG-IFNα-2a + RBV by IL28B genotype.

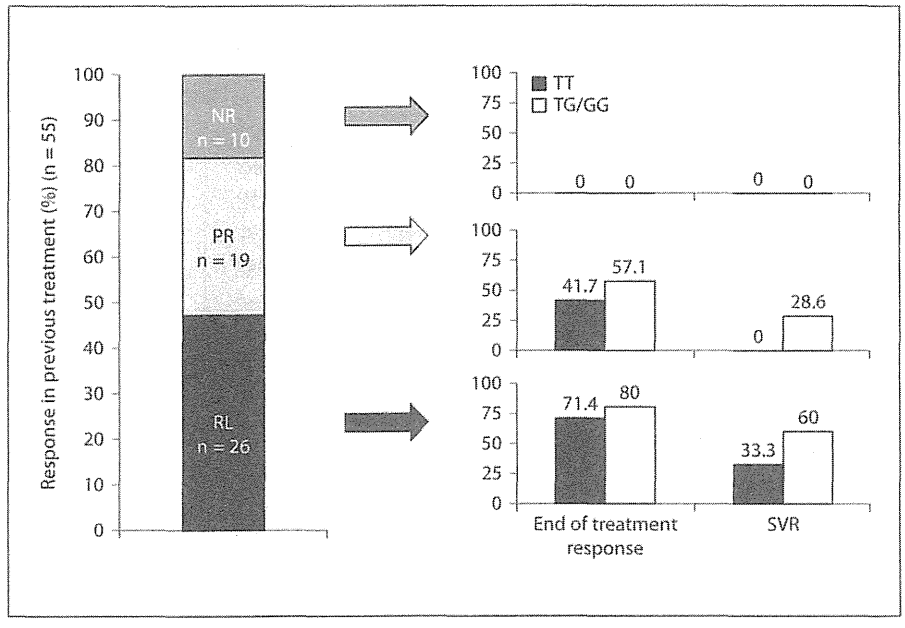

Fig. 4. Virological responses of PEG-IFNα-2a + RBV by IL28B genotype and response of prior treatment. NR = Null responders; PR = partial responders; RL = relapsers.

When we limited the analysis to relapsers, the EVR rates were 57% (12/21) and 40% (2/5) in the TT and non-TT patients, respectively. At week 24, the HCV RNA response rates were 90% (19/21) and 80% (4/5) in the TT and non-TT patients, respectively (fig. 3, 4).

Treatment Discontinuation

33 patients (31.1%) discontinued treatment during the study. 17 patients discontinued treatment due to lack of virological response, 5 due to personal reasons, 3 due to the incidence of hepatocellular carcinoma, and 8 (7.5%) due to adverse events. The reasons for the discontinuation of the 8 patients due to adverse events were anemia

(n = 2), fatigue (n = 2), depressed mental state (n = 1), unidentified fever (n = 1), heart failure (n = 1), and interstitial pneumonia (n = 1).

Discussion

We retrospectively examined the therapeutic efficacy and effective predictors after retreatment with PEG-IFNα-2a + RBV combination therapy in 106 CHC patients. These patients showed non-response or relapse after previous PEG-IFNα-2b + RBV combination therapy. SVR was achieved in 53% of the relapsers and 18% of the partial responders. In the relapsers, SVR was more likely to be achieved when HCV RNA became negative within 12 weeks after the commencement of therapy and the therapeutic effect was enhanced with prolonged administration. Among the non-responders, SVR was not achieved in cases without a ≥2 log decrease in HCV RNA during the previous therapy or in cases with an HCV RNA response after ≥25 weeks during the retreatment period. Similar to that observed in the relapsers, SVR in the non-responders was achieved after prolonged retreatment in patients who showed an HCV RNA response within 12 weeks.

Patients with genotype 1 who receive PEG-IFN + RBV therapy for the first time undergo response-guided therapy (RGT) [16], in which the therapeutic period is determined according to the timing of the HCV RNA response after the commencement of therapy. In RGT, PEG-IFN + RBV therapy is recommended for 48 weeks for patients who exhibit an HCV RNA response within 12 weeks after the commencement of therapy (i.e. complete early viral response, cEVR). However, the results of the present study suggest that prolonged administration is favorable for cEVR cases. Moreover, a different RGT is required in patients who receive retreatment and who have previously received PEG-IFN + RBV therapy.

We also examined the status of the IL28B genotype and found that the IL28B non-TT genotype is associated with factors that are unlikely to be related to the response including AFP levels, γ-GTP levels, and mutations in the core regions. However, when limiting the analysis to relapse patients, the rate of HCV RNA negativity did not differ among IL28B genotypes. This suggests that a prior response to PEG-IFN + RBV therapy is more important for predicting the response to retreatment than the IL28B genotype.

As stated earlier, some patients in Japan have not benefited from the launch of telaprevir, because CHC patients in Japan are older than those in Western countries [11, 12] and are often anemic. We believe that retreatment for elderly patients who cannot receive telaprevir-based therapy, especially relapsers and non-responders with a maximum virus decrease of ≥2 log during the previous therapy, should be considered after PEG-IFN + RBV therapy.

Disclosure Statement

The authors have no conflicts of interest to disclose.

References

1 Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr, Haussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J: Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 2002;347:975–982.

2 Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK: Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet 2001;358:958–965.

3 Jensen DM, Marcellin P, Freilich B, Andreone P, Di Bisceglie A, Brandao-Mello CE, Reddy KR, Craxi A, Martin AO, Teuber G, Messinger D, Thommes JA, Tietz A: Retreatment of patients with chronic hepatitis C who do not respond to peginterferon-α2b: a randomized trial. Ann Intern Med 2009;150:528–540.

4 Poynard T, Colombo M, Bruix J, Schiff E, Terg R, Flamm S, Moreno-Otero R, Carrilho F, Schmidt W, Berg T, McGarrity T, Heathcote EJ, Goncales F, Diago M, Craxi A, Silva M, Bedossa P, Mukhopadhyay P, Griffel L, Burroughs M, Brass C, Albrecht J: Peginterferon alfa-2b and ribavirin: effective in patients with hepatitis C who failed interferon alfa/ribavirin therapy. Gastroenterology 2009;136:1618–1628 e1612.

5 Ghany MG, Strader DB, Thomas DL, Seeff LB: Diagnosis, management, and treatment of hepatitis C: an update. Hepatology 2009;49:1335–1374.

6 Kumada H, Okanoue T, Onji M, Moriwaki H, Izumi N, Tanaka E, Chayama K, Sakisaka S, Takehara T, Oketani M, Suzuki F, Toyota J, Nomura H, Yoshioka K, Seike M, Yotsuyanagi H, Ueno Y: Guidelines for the treatment of chronic hepatitis and cirrhosis due to hepatitis C virus infection for the fiscal year 2008 in Japan. Hepatol Res 2010;40:8–13.

7 McHutchison JG, Manns MP, Muir AJ, Terrault NA, Jacobson IM, Afdhal NH, Heathcote EJ, Zeuzem S, Reesink HW, Garg J, Bsharat M, George S, Kauffman RS, Adda N, Di Bisceglie AM: Telaprevir for previously treated chronic HCV infection. N Engl J Med;362:1292–1303.

8 Zeuzem S, Andreone P, Pol S, Lawitz E, Diago M, Roberts S, Focaccia R, Younossi Z, Foster GR, Horban A, Ferenci P, Nevens F, Mullhaupt B, Pockros P, Terg R, Shouval D, van Hoek B, Weiland O, Van Heeswijk R, De Meyer S, Luo D, Boogaerts G, Polo R, Picchio G, Beumont M: Telaprevir for retreatment of HCV infection. N Engl J Med 2011;364:2417–2428.

9 Hayashi N, Okanoue T, Tsubouchi H, Toyota J, Chayama K, Kumada H: Efficacy and safety of telaprevir, a new protease inhibitor, for difficult-to-treat patients with genotype 1 chronic hepatitis C. J Viral Hepat 2012; 19:e134–e142.
10 Kumada H, Toyota J, Okanoue T, Chayama K, Tsubouchi H, Hayashi N: Telaprevir with peginterferon and ribavirin for treatment-naive patients chronically infected with HCV of genotype 1 in japan. J Hepatol 2012; 56:78–84.
11 Chung H, Ueda T, Kudo M: Changing trends in hepatitis c infection over the past 50 years in Japan. Intervirology 2010;53:39–43.
12 Tanaka Y, Kurbanov F, Mano S, Orito E, Vargas V, Esteban JI, Yuen MF, Lai CL, Kramvis A, Kew MC, Smuts HE, Netesov SV, Alter HJ, Mizokami M: Molecular tracing of the global hepatitis C virus epidemic predicts regional patterns of hepatocellular carcinoma mortality. Gastroenterology 2006;130:703–714.
13 Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, Kobayashi M, Saitoh S, Watahiki S, Sato J, Matsuda M, Arase Y, Ikeda K, Kumada H: Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. Intervirology 2005;48:372–380.
14 Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, Heinzen EL, Qiu P, Bertelsen AH, Muir AJ, Sulkowski M, McHutchison JG, Goldstein DB: Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 2009;461:399–401.
15 Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, Nakagawa M, Korenaga M, Hino K, Hige S, Ito Y, Mita E, Tanaka E, Mochida S, Murawaki Y, Honda M, Sakai A, Hiasa Y, Nishiguchi S, Koike A, Sakaida I, Imamura M, Ito K, Yano K, Masaki N, Sugauchi F, Izumi N, Tokunaga K, Mizokami M: Genome-wide association of IL28B with response to pegylated interferon-α and ribavirin therapy for chronic hepatitis C. Nat Genet 2009;41:1105–1109.
16 Lee SS, Ferenci P: Optimizing outcomes in patients with hepatitis C virus genotype 1 or 4. Antivir Ther 2008;13(suppl 1):9–16.

GENETIC POLYMORPHISM-DISEASE ASSOCIATION

HLA-DP gene polymorphisms and hepatitis B infection in the Japanese population

KIYOSHI MIGITA, SEIGO ABIRU, MASASHI OHTANI, YUKA JIUCHI, YUMI MAEDA, SUNG KWAN BAE, SHIGEMUNE BEKKI, SATORU HASHIMOTO, KAKHARMAN YESMEMBETOV, SHINYA NAGAOKA, MINORU NAKAMURA, ATSUMASA KOMORI, TATSUKI ICHIKAWA, KAZUHIKO NAKAO, HIROSHI YATSUHASHI, HIROMI ISHIBASHI, and MICHIO YASUNAMI

OMURA AND NAGASAKI, JAPAN

The mechanisms underlying the different outcomes of hepatitis B virus (HBV) infection are not fully understood.[1] Kamatani et al[2] identified an association of the single nucleotide polymorphisms (SNPs) human leukocyte antigen (*HLA*)-*DPA1* (rs3077) and *HLA-DPB1* (rs9277535) with chronic HBV infection in a genome-wide association study (GWAS). Additional studies confirmed that rs3077 and rs9277535 were associated with chronic HBV infection in the Han-Chinese population and strengthened the findings from previous GWAS.[3-6] Furthermore, Hu et al[7] reported that SNPs in *HLA-DP* (rs3077 and rs9277535) were associated with both HBV clearance and hepatocellular carcinoma (HCC) development. To investigate the association of these *HLA-DP* variants with the disease progression of HBV infection, we genotyped the 2 SNPs (rs3077 and rs9277535) in different clinical stages of liver disease in Japanese HBV carriers.

CLINICAL SUMMARY

A total of 241 HBV carriers (positive for hepatitis B surface antigen) who visited the clinics for liver diseases at the Nagasaki University Hospital or Nagasaki Medical Center between 1999 and 2007 were enrolled. As controls, 143 healthy Japanese volunteers (56 men and 87 women aged 16–63 years, with a mean age of 31.3 ± 8.9 years) without any history of liver disease were enrolled. All patients did not have any other types of liver diseases, such as chronic hepatitis C, alcoholic liver disease, autoimmune liver disease, or metabolic liver disease. The study protocol was approved by the Ethics Committees of National Nagasaki Medical Center, and informed consent was obtained from each individual. Of the 241 HBV carriers, 69 were considered to be asymptomatic carriers on the basis of sustained normalization of the serum alanine aminotransferase (ALT) levels together with seropositivity for anti-hepatitis Be antigen throughout the study. On the other hand, 172 of the 241 HBV carriers were considered to have chronic liver disease, such as chronic hepatitis (57), cirrhosis (65), or HCC (50) manifested by elevated ALT levels and by clinical or histologic findings on examination of liver tissue during the follow-up period. Of the 50 patients with HCC, 6 (12%) were found to have chronic hepatitis and 44 (88%) had cirrhosis. All patients were regularly followed with measurements of serum ALT and HBV markers, such as hepatitis B surface antigen, hepatitis Be antigen, anti-hepatitis Be antibody, and HBV-DNA. A total of 79 patients had undergone liver biopsy during the study to assess the degree of liver fibrosis. However, liver biopsy was not performed in patients who had apparent biochemical, endoscopic, and ultrasound features of liver cancer. Tumor markers such as alpha-fetoprotein and des-γ-carboxy-prothrombin were measured with ultrasonography of the liver every 6 months to detect HCC in an early stage. The diagnosis of HCC was made by several imaging modalities in all patients and confirmed histologically by sonography-guided fine-needle tumor biopsy specimens. The genotype of rs3077 (*HLA-DPA1*) and rs9277535 (*HLA-DPB1*) was determined by direct sequencing. The apolipoprotein B mRNA-editing enzyme catalytic peptide 3G (*APOBEC3G* H186R) genotyping was performed on the basis of the report by An et al.[8]

The frequencies of the 2 SNPs of *HLA-DPA1* (rs3077) and *HLA-DPB1* (rs9277535) are listed in Table I. There was a significant difference in the frequencies between these 2 SNPs between Japanese HBV carriers and healthy subjects, as described previously.[3] We divided HBV carriers into 2 groups: a nonadvanced group (asymptomatic carriers or chronic hepatitis, n = 115) and an advanced group (liver cirrhosis or HCC, n = 126). The frequencies of CC (rs3077) or GG (rs9277535) genotypes were higher in the advanced group compared with those in the nonadvanced group; however, the difference was not significant (Table I). Next, we stratified the HBV carriers for the presence or absence of the *APOBEC3G* H186R variant and examined the effects of *HLA-DP* polymorphisms on the progression of HBV-related liver disease. Both C and G alleles of rs3077 and rs9277535 significantly increased the risk for advanced liver disease in HBV carriers lacking the H186R variant (Table II).

A 2-stage GWAS identified SNPs including rs3077 and rs9277535 located in *HLA-DPA1* and *HLA-DPB1*, which were associated with a susceptibility to chronic HBV infection.[2] After the first Japanese GWAS, 5 studies replicated the association of these 2 *HLA-DP* SNPs (rs3077 and rs9277535) and chronic HBV infection in the Han-Chinese population.[3-7] Among these studies, an association between HBV-related HCC and rs9277535 or rs3077 was demonstrated.[7] In this study, we examined whether these 2 SNPs (rs3077 and rs9277535) in *HLA-DP* genes were associated with the disease progression and susceptibility to HBV infection in a Japanese population. As demonstrated previously, we reconfirmed that rs3077 and rs9277535 in the *HLA-DPA1* and *HLA-DPB1* genes were significantly associated with HBV infection. Although some differences in the frequencies of rs3077 and rs9277535 genotypes between HBV carriers with advanced liver disease (liver cirrhosis and HCC) and those without advanced liver disease were observed, these differences were not statistically significant.

Recent evidence suggests that APOBEC3G inhibits HBV production by interfering with HBV replication through hypermutation of the majority of the HBV genome.[8] Because of the *APOBEC3G* gene's ability to regulate HBV replication, mutations of the gene may cause a deleterious variation that may affect the outcome of HBV infection. Among the SNPs identified in the *APOBEC3G* gene, H186R variant was strongly associated with a decline in $CD4^+$ T-cell numbers and accelerated progression to acquired immune deficiency syndrome–defining conditions in human immunodeficiency virus–infected individuals.[9,10] Viral disease outcome is influenced by host variability in immune response genes and genes that control viral replication or mutation rate.[11] *APOBEC3G* coding region variant might influence the progression of HBV infection by inducing the replication of HBV.[12] Therefore, genetic diversity of immune response genes, such as *HLA*, and genes that control viral replication, such as *APOBEC3G*, could contribute to the variability in outcome of HBV infection. To minimize the effects

From the Clinical Research Center, NHO Nagasaki Medical Center, Omura, Japan; Department of Gastroenterology, Nagasaki University Hospital, Nagasaki, Japan; Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan.

Conflict of interests: None

Funding: This study was supported by a grant from the National Hospital Organization (Liver-net work study).

Submitted for publication May 25, 2012; revision submitted June 8, 2012; accepted for publication June 9, 2012.

Reprint requests: Kiyoshi Migita, MD, Clinical Research Center, NHO Nagasaki Medical Center, Kubara 2-1001-1, Omura 856-8652, Japan; e-mail: migita@nmc.hosp.go.jp.

1931-5244/$ - see front matter

http://dx.doi.org/10.1016/j.trsl.2012.06.003

Table I. Association between *HLA-DP* polymorphisms (rs3077, rs9277535) and HBV infection

SNP ID	HBV carrier n = 241 (%)	Healthy subjects n = 143 (%)	*P* value*	OR (95% CI)	Advanced HBV carrier n = 115 (%)	Nonadvanced HBV carrier n = 126 (%)	*P* value*	OR (95% CI)
rs3077								
C/C	148 (61.4)	47 (32.9)			77 (67.0)	71 (56.3)		
C/T	79 (32.8)	72 (50.3)			33 (28.7)	46 (36.5)		
T/T	14 (5.8)	24 (16.8)			5 (4.3)	9 (7.1)		
C allele (allele frequencies)	375 (77.8)	166 (58.0)	<0.0001	2.533 (1.843–3.483)	187 (81.3)	188 (74.6)	0.077	1.480 (0.957–2.290)
rs9277535								
G/G	143 (59.3)	45 (31.5)			73 (63.5)	70 (55.6)		
A/G	82 (34.0)	72 (50.3)			36 (31.3)	46 (36.5)		
A/A	16 (6.6)	26 (18.2)			6 (5.2)	10 (7.9)		
G allele (allele frequencies)	368 (76.3)	162 (56.6)	<0.0001	2.471 (1.804–3384)	182 (79.1)	186 (73.8)	0.170	1.345 (0.880–2.056)

Abbreviations: CI, confidence interval; *HBV,* hepatitis B virus; *OR,* odds ratio; *SNP,* single-nucleotide polymorphism.
**P* values were calculated using the chi-square test.

Table II. Association between *HLA-DP* polymorphisms (rs3077, rs9277535) and the outcome of HBV infection in HBV carrier without H186R variant

SNP ID	Advanced HBV carrier n = 90 (%)	Nonadvanced HBV carrier n = 108 (%)	*P* value*	OR (95% CI)
rs3077				
C/C	64 (71.1)	60 (55.6)		
C/T	22 (24.4)	40 (37.0)		
T/T	4 (4.4)	8 (7.4)		
C allele (allele frequencies)	150 (83.3)	160 (74.1)	0.026	1.750 (1.065–2.874)
rs9277535				
G/G	5 (5.6)	10 (9.3)		
A/G	24 (26.7)	39 (36.1)		
A/A	61 (67.8)	59 (54.6)		
G allele (allele frequencies)	146 (81.1)	157 (72.7)	0.049	1.614 (1.000–2.604)

Abbreviations: CI, confidence interval; *HBV,* hepatitis B virus; *OR,* odds ratio; *SNP,* single-nucleotide polymorphism.
**P* values were calculated using the chi-square test.

of viral factors, such as APOBEC3G-mediated HBV editing, and evaluate the effect of *HLA-DP* more precisely, we focused on the subjects without the H186R variant. Because the *APOBEC3G* coding region variant might influence the progression of HBV infection,[11] we investigated the effect of *HLA-DP* polymorphisms on the outcome of HBV infection in HBV carriers lacking the H186R variant.

Our results showed that *HLA-DP* polymorphisms were associated with the progression of HBV infection and that this association was significant in Japanese HBV carriers lacking H186R variants. Our data demonstrated that *HLA-DP* polymorphisms are important in determining the susceptibility and the progression of HBV infection in the Japanese population.

One limitation of our study is the lack of information of HBV genotypes in the patients studied. Another limitation is that the number of HBV carriers (n = 241) is relatively small. Larger studies are needed to confirm the results of our study.

CONCLUSIONS

We confirmed that rs3077 and rs9277535 SNPs in the *HLA-DP* locus are associated with the susceptibility and progression of HBV infection in the Japanese population. Further functional analyses are warranted to validate the biological plausibility of these SNPs in chronic HBV infection.

REFERENCES

1. Ganem D, Prince AM. Hepatitis B virus infection–natural history and clinical consequences. N Engl J Med 2004;350:1118–29.
2. Kamatani Y, Wattanapokayakit S, Ochi H, et al. A genome-wide association study identifies variants in the HLA-DP locus associated with chronic hepatitis B in Asians. Nat Genet 2009;41:591–5.
3. An P, Winkler C, Guan L, O'Brien SJ, Zeng Z. HBV Study Consortium. A common HLA-DPA1 variant is a major determinant of hepatitis B virus clearance in Han Chinese. J Infect Dis 2011;203:943–7.
4. Guo X, Zhang Y, Li J, et al. Strong influence of human leukocyte antigen (HLA)-DP gene variants on development of persistent chronic hepatitis B virus carriers in the Han Chinese population. Hepatology 2011;53:422–8.
5. Wang L, Wu XP, Zhang W, et al. Evaluation of genetic susceptibility loci for chronic hepatitis B in Chinese: two independent case-control studies. PLoS One 2011;6:e17608.
6. Li J, Yang D, He Y, et al. Associations of HLA-DP variants with hepatitis B virus infection in southern and northern Han Chinese populations: a multicenter case-control study. PLoS One 2011;6:e24221.
7. Hu L, Zhai X, Liu J, et al. Genetic variants in HLA-DP/DQ influence both hepatitis B virus clearance and hepatocellular carcinoma development. Hepatology 2012;55:1426–31.
8. An P, Bleiber G, Duggal P, et al. APOBEC3G genetic variants and their influence on the progression to AIDS. J Virol 2004;78:11070–6.
9. Suspène R, Guétard D, Henry M, Sommer P, Wain-Hobson S, Vartanian JP. Extensive editing of both hepatitis B virus DNA strands by APOBEC3 cytidine deaminases in vitro and in vivo. Proc Natl Acad Sci U S A 2005;102:8321–6.
10. Do H, Vasilescu A, Diop G, et al. Exhaustive genotyping of the CEM15 (APOBEC3G) gene and absence of association with AIDS progression in a French cohort. J Infect Dis 2005;191:159–63.
11. Martin MP, Carrington M. Immunogenetics of viral infections. Curr Opin Immunol 2005;17:510–6.
12. Bonvin M, Greeve J, Hepatitis B. modern concepts in pathogenesis–APOBEC3 cytidine deaminases as effectors in innate immunity against the hepatitis B virus. Curr Opin Infect Dis 2008;21:298–303.

Microbiol Immunol 2012; **56**: 318–323
doi:10.1111/j.1348-0421.2012.00439.x

ORIGINAL ARTICLE

Development of new *IL28B* genotyping method using Invader Plus assay

Satomi Kani[1,2], Yasuhito Tanaka[1,2], Kentaro Matsuura[1], Tsunamasa Watanabe[1], Hiroshi Yatsuhashi[3], Etsuro Orito[4], Ken Inose[5], Nao Motojuku[5], Yukio Wakimoto[2] and Masashi Mizokami[6]

[1]Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, [2]Department of Clinical Laboratory, Nagoya City University Hospital, [4]Nagoya Daini Red Cross Hospital, Nagoya, [3]Clinical Research Center, National Nagasaki Medical Center, Oomura, [5]Third Wave Japan Incorporated, Tokyo, [6]Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa, Japan

ABSTRACT

***IL28B* polymorphism is associated with the response to pegylated interferon-α with ribavirin (PEG-IFN-α/RBV) treatment in chronic hepatitis C patients. As a genotyping assay for *IL28B* single nucleotide polymorphisms (SNPs) in clinical practice, the Invader Plus assay was developed. The accuracy, intra-assay, inter-assay precision, and the limit of detection of the Invader Plus assay were evaluated. Two SNPs (rs8099917 and rs12979860) associated with *IL28B* were genotyped by the Invader Plus and TaqMan assay in 512 Japanese patients. In comparison with direct sequencing, the Invader Plus assay showed 99% accuracy in rs8099917 and 100% accuracy in rs12979860. Intra-assay and inter-assay precision were sufficient to use in clinical practice and the detection limit was 1ngDNA/assay. Genotyping by rs8099917 showed that 361 (71%), 144 (28%) and seven (1%) of the patients were major homozygous, heterozygous and minor homozygous types, respectively. Five of the 512 cases (1%) had haplotype differences, but none showed differences between the two genotyping methods. For patients with HCV genotype 1, the prevalence of responders in the major homozygous type was 83.3%, and that of non-responders in the minor heterozygous/homozygous type was 72.5%. A convenient *IL28B* genotyping method using the Invader Plus assay could be useful to predict the treatment outcome in clinical practice.**

Key words interferon, *IL28B*, single nucleotide polymorphism genotyping, invader plus assay.

Hepatitis C virus (HCV) infection results in cirrhosis and hepatocellular carcinoma (HCC) worldwide (1). Pegylated interferon-α with ribavirin (PEG-IFN-α/RBV) is currently the most dominant therapy for chronic HCV infection, but roughly 50% of patients with genotype 1b, the most common in Japan, are not able to achieve a sustained virological response (SVR) determined by the serum HCV-RNA level 24 weeks after treatment (2, 3). In addition, this therapy often leads to side effects, such as flu-like symptoms, depression and anemia(4); therefore, it is valuable to predict the particular response before treatment with PEG-IFN-α/RBV to avoid these side-effects and to avoid ineffective therapy.

Not only viral factors (genotype and viral mutation), but also host factors influence the therapeutic outcome. Age, sex, body mass index and histological grade are considered to determine the individual's treatment regimen and outcome (5, 6, 7). Recently, it has been reported through a genome-wide association study (GWAS) of patients with genotype 1 HCV that single nucleotide polymorphisms (SNPs) located near the *IL28B* gene are strongly associated with a response to PEG-IFN-α/RBV

Correspondence
Yasuhito Tanaka, Department of Virology & Liver unit, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya 467-8601, Japan.
Tel: +81 52 853 8191; fax: +81 52 842 0021; email: ytanaka@med.nagoya-cu.ac.jp

Received 19 January 2012; revised 29 January 2012; accepted 1 February 2012.

List of Abbreviations: GWAS, genome-wide association study; HCV, hepatitis C virus; NPV, negative predictive value; NVR, null virological response; PEG-IFN-α, pegylated interferon α; PPV, positive predictive value; RBV, ribavirin; SVR, sustained virological response; TVR, transient virological response.

Table 1. Primers and allele probes used for Invader Plus genotyping of *IL28B* single nucleotide polymorphisms (SNPs)

SNP		(5'-3')
rs8099917	Forward primer	TCA TCC CTC ATC CCA CTT CTG GAA CA
	Reverse primer	CGG GCC ATC TGT TTC CTG CTG
	Major allele probe	agg cca cgg acg AAT TGC TCA CAG AAA GGA A
	Minor allele probe	cgc gcc gag gCA TTG CTC ACA GAA AGG A
	Invader oligo	GCT ACC AAA CTG TAT ACA GCA TGG TTC CAA TTT GGG TGA t
rs12979860	Forward primer	GGA TGG GTA CTG GCA GCG C
	Reverse primer	AGG CGC CTC TCC TAT GTC AGC
	Major allele probe	cgc gcc gag gCG AAC CAG GGT TGA AT
	Minor allele probe	agg cca cgg acg TGA ACC AGG GTT GAA TT
	Invader oligo	CCA GGG AGC TCC CCG AAG GCG a

† Lowercase letters in each probe indicate 5'flap region.

therapy in Japanese (8), European (9), and a multi-ethnic population (10, 11). In particular, the two outstanding SNPs, rs12979860 and rs8099917 (located ~3 kb and 8 kb upstream of *IL28B*, respectively) have been found in strong association with the treatment response (8, 10). The minor allele frequency of rs8099917 was significantly higher in the null virological response (NVR) group compared with the virological response group. By taking advantage of *IL28B* typing, it may be possible to predict a NVR as well as a SVR in order to tailor the most suitable treatment regimens.

In this study, the "Invader Plus genotyping assay *IL28B* SNP" test kit including primers and probe setting was developed and compared with the usual TaqMan probe assay for genotyping *IL28B* SNPs and the pre-treatment prediction of the response to PEG-IFN-α/RBV therapy in HCV infection.

MATERIALS AND METHODS

Patients

DNA samples were obtained from 512 Japanese chronic hepatitis C patients, after informed consent, recruited from NHO Nagasaki Medical Center, Nagoya City University Hospital, Nagoya Daini Red Cross Hospital, and Kawasaki Medical University Hospital in Japan. Of them, the data of 90 patients were also used in a previous paper (15). All of the subjects had undergone a standard course of PEG-IFN-α/RBV therapy and 316 patients had their virological response status established before this study, of which 272 patients had HCV genotype 1. The NVR, transient virological response (TVR) and SVR were defined 24 weeks after PEG-IFN-α/RBV therapy, as previously described (8). This study classified the response outcome in two categories; responders (including those with SVR and TVR) and non-responders (including patients with NVR).

Informed consent was obtained from each patient and the study protocol 22 conformed to the ethics guidelines of the Declaration of Helsinki and was approved by 23 the institutional ethics review committee.

Samples for assay validation

To assess intra-assay and inter-assay precision and both coefficients of variation (CV%), three different genotype samples (major, minor, and hetero), two positive (major and minor) and one negative control were run in triplicate during three runs (three different days). The limit of detection was evaluated by analyzing three different genotype samples using two different DNA concentrations (1 ng, and 0.3 ng per assay) with the same three controls.

Invader Plus assay

The Invader Plus assay, which combines polymerase chain reaction (PCR) and the Invader reaction, was performed using the LC480II (Roche Diagnostics, Basel, Switzerland). The enzymes used in Invader Plus are native Taq polymerase (Promega, Fitchburg, WI, USA) and cleavase enzyme (Third Wave Technologies, Madison, WI, USA). Primers and allele probes newly designed for Invader Plus genotyping of *IL28B* SNPs are shown in Table 1 (Third Wave Japan, Tokyo, Japan). The reaction is configured to use PCR primers with a melting temperature (Tm) of 72°C and an Invader detection probe with a target-specific Tm of 63°C. The invader oligonucleotide overlaps the probe by one nucleotide, forming at 63°C an overlap flap substrate for the cleavase enzyme. The first step in Invader Plus is PCR target amplification, in which the reaction is subjected to 18 cycles of a denaturation step (95°C for 15 s) and hybridization and extension steps (70°C for 1 min). At the end of PCR cycling, the reaction mixture is