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References

1. Muir AJ, Bornstein JD, Killenberg PG; Atlantic Coast Hepatitis Treatment Group. Peginterferon alfa-2b and RBV for the treatment of chronic hepatitis C in blacks and non-Hispanic whites. N Engl J Med 2004;350:2265-2271.
2. Conjeevaram HS, Fried MW, Jeffers LJ, Terrault NA, Wiley-Lucas TE, Afdhal N, et al. Peginterferon and ribavirin treatment in African American and Caucasian American patients with hepatitis C genotype 1. Gastroenterology 2006;131:470-477.
3. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 2009;461:399-401.
4. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and RBV therapy for chronic hepatitis C. Nat Genet 2009;41:1105-1109.
5. Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML. IL28B is associated with response to chronic hepatitis C interferon-alpha and RBV therapy. Nat Genet 2009;41:1100-1104.
6. Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, et al. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature 2009;461:798-801.
7. Rauch A, Kutalik Z, Descombes P, Cai T, Di Iulio J, Mueller T, et al; Swiss Hepatitis C Cohort Study; Swiss HIV Cohort Study. Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study. Gastroenterology 2010;138: 1338-1345.
8. Thompson AJ, Muir AJ, Sulkowski MS, Ge D, Fellay J, Shianna KV, et al. Interleukin-28B polymorphism improves viral kinetics and is the strongest pretreatment predictor of sustained virologic response in hepatitis C virus-1 patients. Gastroenterology 2010;139:120-129.
9. Mangia A, Thompson AJ, Santoro R, Piazzolla V, Tillmann HL, Patel K, et al. An interleukin-28B polymorphism determines treatment response of patients with hepatitis C genotypes 2 or 3 who do not achieve a rapid virologic response. Gastroenterology 2010;139:821-827.
10. Herrmann E, Neumann AU, Schmidt JM, Zeuzem S. Hepatitis C virus kinetics. Antivir Ther 2000;5:85-90.
11. Howell CD, Thompson AJ, Ryan K, Zambeeli S, Gorden A, Fried M, et al. IL28 genetic variation associated with early viral kinetics and SVR in HCV genotype 1: The ViraHepC Study. J Hepatol 2010; 52(Suppl. 1):S451.
12. Neumann AU, Bibert S, Haagmans B, Soulier A, Negro F, Lagging M. IL28B polymorphism is significantly correlated with IFN anti-viral effectiveness already on first day of pegylated interferon-α and ribavirin therapy of chronic HCV infection. J Hepatol 2010;52(Suppl. 1):S468.
13. Ank N, West H, Bartholdy C, Eriksson K, Thomsen AR, Paludan SR. Lambda interferon (IFN-lambda), a type III IFN, is induced by viruses and IFNs and displays potent antiviral activity against select virus infections in vivo. J Virol 2006;80:4501-4509.
14. Kotenko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, Shah M, et al. IFN-λs mediate antiviral protection through a distinct class II cytokine receptor complex. Nat Immunol 2003;4;69-77.
15. Sheppard P, Kindsvogel W, Xu W, Henderson K, Schlutsmeyer S, Whitmore TE, et al. IL-28, IL-29 and their class II cytokine receptor IL-28R. Nat Immunol 2003;4:63-68.
16. Marcello T, Grakoui A, Barba-Spaeth G, Machlin ES, Kotenko SV, MacDonald MR, et al. Interferons alpha and lambda inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. Gastroenterology 2006;131:1887-1898.
17. Robek MD, Boyd BS, Chisari FV. Lambda interferon inhibits hepatitis B and C virus replication. J Virol 2005;79:3851-3854.
18. Ank N, Iversen MB, Bartholdy C, Staeheli P, Hartmann R, Jensen UB, et al. An important role for type III interferon (IFN-lambda/IL-28) in TLR-induced antiviral activity. J Immunol 2008;180:2474-2485.
19. Muir AJ, Shiffman ML, Zaman A, Yoffe B, de la Torre A, Flamm S, et al. Phase 1b study of pegylated interferon lambda 1 with or without RBV in patients with chronic genotype 1 hepatitis C virus infection. Hepatology 2010;52:822-832.
20. Coccia EM, Severa M, Giacomini E, Monneron D, Remoli ME, Julkunen I, et al. Viral infection and Toll-like receptor agonists induce a differential expression of type I and lambda interferons in human plasmacytoid and monocyte-derived dendritic cells. Eur J Immunol 2004;34:796-805.
21. Doyle SE, Schreckhise H, Khuu-Duong K, Henderson K, Rosler R, Storey H, et al. Interleukin-29 uses a type 1 interferon-like program to promote antiviral responses in human hepatocytes. Hepatology 2006;44:896-906.
22. Honda M, Sakai A, Yamashita T, Nakamoto Y, Mizukoshi E, Sakai Y, et al; Hokuriku Liver Study Group. Hepatic ISG expression is associated with genetic variation in interleukin 28B and the outcome of IFN therapy for chronic hepatitis C. Gastroenterology 2010;139:499-509.
23. Urban TJ, Thompson AJ, Bradrick S, Fellay J, Schuppan D, Cronin KD, et al. IL28B genotype is associated with differential expression of intrahepatic interferon-stimulated genes in chronic hepatitis C patients. Hepatology 2010; doi:10.1002/hep.23912.
24. Fellay J, Thompson AJ, Ge D, Gumbs CE, Urban TJ, Shianna KV, et al. ITPA gene variants protect against anaemia in patients treated for chronic hepatitis C. Nature 2010;464:405-408.
25. LabCorp. LabCorp Service Announcement. http://people.genome.duke.edu/. . ./L8464-0710-Service-Announcement-FINAL.pdf. Accessed November 5, 2010.
26. Zineh I, Lesko LJ. Pharmacogenetics in medicine: barriers, critical factors and a framework for dialogue. Per Med 2009;6:359-361.
27. O'Brien TR. Inteferon-alfa, interferon-λ and hepatitis C. Nat Genet 2009;41:1048-1050.

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Forum Minireview

Novel Findings for the Development of Drug Therapy for Various Liver Diseases: Genetic Variation in *IL-28B* Is Associated With Response to the Therapy for Chronic Hepatitis C

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Abstract. Hepatitis C infection is a global health problem. Spontaneous viral clearance was observed in approximately 30% of individuals with acute infection. In the therapy using a combination of pegylated interferon-α and ribavirin, approximately 50% of chronic hepatitis C patients infected with high viremia of hepatitis C virus infection (HCV) genotype 1 reached a sustained viral response. These findings were strongly expected to reflect variations of the host genome. To reveal genetic effects against viral clearance or treatment response, four independent groups applied a genome-wide association study (GWAS) to HCV infection. These groups almost simultaneously reported a strong association of interleukin (IL)-28B polymorphisms with viral clearance or final decision of HCV therapy. The discovered single nucleotide polymorphisms (SNPs) also revealed the enigma that the viral clearance rate was dependent on ethnic type. The significant SNPs are useful for prediction prior to treatment because of the strong association with clinical outcome. In addition, the unexpected results revealed by GWAS could promote the development of a novel drug related to IL-28B. Herein, we present current understanding in regard to the relationship between host variations and clinical outcome of hepatitis C.

Keywords: hepatitis C virus, genome-wide association study, interleukin-28, interferon-λ, single nucleotide polymorphism, liver disease

1. Introduction

Chronic infection with hepatitis C virus (HCV) presents a significant health problem worldwide with approximately 3% of the world population, that is, more than 170 million people. Only 20% – 30% of HCV-infected individuals recover spontaneously. The remaining 70% – 80% going on to develop chronic infection have a significant risk for progressive liver fibrosis and subsequent liver cirrhosis (LC) and hepatocellular carcinomas (HCC) (1). Successful treatment of chronic hepatitis C would reduce the morbidity and mortality of patients because around 8% of patients progressing to LC will develop HCC annually (2).

Spontaneous clearance following acute infection occurs in some cases for reasons that remain unclear, and previous studies report that 50% – 85% of patients progress to chronicity. The relationship between race and spontaneous viral clearance following acute infection have been reported (3 – 6). These characteristics based on ethnic types would suggest the effect of a host genetic factor on HCV infection.

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2. HCV treatment and the response

The current standard of care for the HCV infections comprises pegylated interferon (PEG-IFN)-α 2a or 2b plus ribavirin (RBV). Successful treatment, termed "sustained virological response (SVR)", was defined by an HCV RNA negative after 6 months of completing therapy, whereas a transient viral response (TVR) was defined as a reappearance of HCV RNA in serum after treatment was discontinued in a patient who had undetectable HCV RNA during the therapy or on completion of the therapy (Fig. 1). A non-viral response (NVR) was defined as cases with detectable viremia after and during treatment. The standard therapy is effective in only 42% – 52% of patients with HCV genotype 1 in the US and Europe (7 – 9). A significant difference in response to PEG-IFN&RBV therapy between ethnicities were reported: the SVR achievement of African Americans was only approximately 20% – 28% compared to 40% – 52% in Caucasian patients with genotype 1 infection (10 – 12) and 57% vs. 82% for genotype 2/3 (13). The current therapies are limited by expensive, ineffectiveness in part of the patients, and numerous potentially severe side effects, which cause dose reduction and/or premature termination of treatment. Additionally, premature withdrawal from IFN-based therapy (14) was necessary for 10% – 14% of the patients, leading to failure of the HCV therapy.

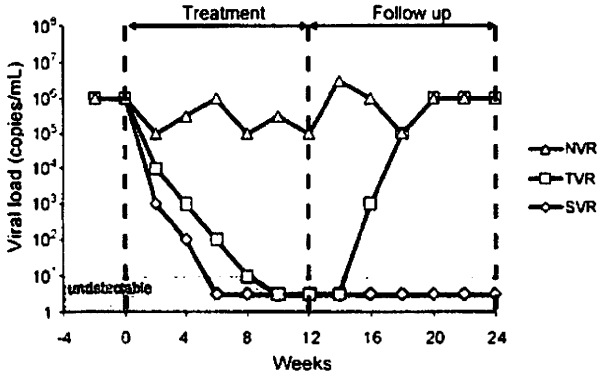

Fig. 1. Representative changes of HCV viral load in patients treated with PEG-IFN&RBV combination therapy. The response type under the therapy of PEG-IFN&RBV is divided into three groups: SVR, TVR, or NVR. SVR is defined as successful treatment, which is HCV RNA negative after 6 months of completing therapy. TVR is defined as a transiently negative for HCV RNA during treatment. However, after the end of therapy, HCV RNA of the patients reappears with impaired liver function in TVR. NVR is defined as a constitutive high viremia during and after treatment. SVR, sustained viral response; TVR, transient viral response; NVR, non-viral response

3. Viral factors associated with HCV therapy

To prevent treatment failure in such patients, we must identify the predictive factors leading to treatment failure as well as production of severe side effects in the clinic. Previous studies have reported that viral titer, mutations, or gene expression levels of innate immunity could be prediction factors for NVR using clinical specimens of chronic hepatitis C patients. Several viral factors such as genotype 1 (HCV-1), high baseline viral load, viral kinetics during treatment, and amino acid pattern in the IFN sensitivity–determining region have been found to be significantly associated with the outcome by a number of independent studies (15 – 17). Accumulated data have provided strong evidence that approximately 20% of patients with HCV genotype 1 have NVR to PEG-IFN&RBV. The reliable prediction for NVR would allow avoidance of side effects and reduce the cost of treatment in the 20% of patients with HCV-1 before starting the treatment.

4. Host factors associated with response to PEG-IFN&RBV therapy

Several host factors related to viral clearance have been reported based on clinical features or laboratory data, for example, gender, age <40 years, low HCV RNA level prior to treatment, lack of liver cirrhosis, and HCV genotypes 2/3 (18, 19). As for host genetic factor, candidate gene approaches have been adopted to identify host factors related to clinical outcomes, single nucleotides polymorphisms (SNPs), copy number variation (CNV), or insertion/deletion of genes. The approach could latently find weak associations and show significant differences because only one or a limited number of SNPs or gene loci are detected in candidate genes. The focused approach, however, contains the restraint to detect crucial factors. In detail, the selection of candidate regions for genetic study depends on the researcher's knowledge or the present data of the gene pathway.

In contrast, a recent genome-wide association study (GWAS) approach using high-throughput genotyping technology usually for SNPs, ranging from 300,000 to 900,000 SNPs in each sample, is able to detect strong association factors affecting disease susceptibility and drug response without any a-priori hypotheses on causative SNPs apart from the hypotheses (20, 21). On the basis of the GWAS, four independent groups assessed the role of genetic variation on response to PEG-IFN&RBV combination therapy for chronic hepatitis C patients, and the data was reported in a short-term (21 – 24). In all cases, the conclusive finding was that polymorphisms in or near the *IL-28B* gene strongly de-

termined the outcome of HCV therapy.

5. Study design of four studies for GWAS

Ge et al. and Suppiah et al. studied genetic variants associated with SVR to PEG-IFN&RBV therapy in individuals infected with HCV genotype 1 (21, 22). The former examined genetic factors associated with treatment response in patients from the IDEAL trial (Individualized Dosing Efficacy vs. flat dosing to Assess optimaL pegylated interferon therapy) (25), a large randomized controlled trial involving Caucasian, American-African, and Hispanic individuals in North America (n = 1137) (Table 1). The latter study group analyzed Caucasians consisting of 293 Australian individuals (Northern European ancestry) with HCV genotype 1 and also validates an independent replication cohort consisting of 555 Europeans from the UK, Germany, Italy, and Australia. These two study groups mainly investigated GWAS in Caucasians and analyzed host factors associated with SVR.

Tanaka et al. studied host factors associated with the response to PEG-IFN&RBV treatment in 142 Japanese patients with chronic hepatitis C of HCV genotype 1 for GWAS and prepared an independent replication cohort of 172 Japanese (Table 1) (24). In this study, patients were divided into three groups, SVR, TVR, or NVR. NVR vs. virological responder (VR) consisting of SVR and TVR was used for the predication of NVR factors. The data set of SVR vs. non-SVR (TVR and NVR) was constructed to discover the host factor related to SVR (Fig. 1).

Rauch et al. investigated 465 Caucasians infected with HCV genotypes 1, 2, 3, or 4 to reveal genetic variations associated with response to the combination therapy (23). A case control study was designed to detect genetic variations related to SVR in European individuals. Three study groups, except Suppiah et al., selected patients receiving at least 80% of the recommended treatment dose to emphasize genetic associations.

6. Identification of strongly significant SNPs associated with PEG-IFN&RBV therapy

Ge et al. identified a genetic polymorphism (rs12979860) near the *IL-28B* gene on chromosome 19, also known as IFN-λ3 (Fig. 2). Individuals with the CC genotype showed the association with an approximately two-fold change in response to PEG-IFN&RBV treatment compared with those with the TT genotype, both among patients of European ancestry ($P = 1.06 \times 10^{-25}$) and African-Americans ($P = 2.06 \times 10^{-3}$). An important finding in the study is the strong correlation between being a carrier of this SNP and SVR rates in diverse ethnic groups, which is significantly more frequent in European-Americans and Asian populations than in African-Americans. Approximately 23% – 55% of Africans (<40% of African-Americans) carry advantageous C-allele frequency of rs12979860, compared with approximately 53% – 85% of Europeans (<70% of European-

Table 1. Four GWAS groups studying host factor related to the response to HCV therapy

Study (Ref. No.)	Ge et al. (22)	Suppiah et al. (21)	Tanaka et al. (24)	Rauch et al. (23)
Region	**Northern America**	**Northern Europe, Australia**	**Japan**	**Switzerland**
Ancestry	Caucasian/ African/ Hispanic	Caucasian	Japanese	Caucasian
GWAS size	**871/ 191/ 75**	**293**	**142**	**465**
Replication	No replication	555	172	No replication
Case/ control	**SVR vs. non-SVR**	**SVR vs. non-SVR**	**SVR vs. non-SVR SVR&TVR vs. NVR**	**SVR vs. non-SVR**
Adherence	Over 80% adherent to PEG-IFN&RBV during the first 12 weeks of therapy	Not controlled	Over 80% adherent to PEG-IFN&RBV during the first 12 weeks of therapy	Over 80% adherent to PEG-IFN&RBV during the first 12 weeks of therapy
HCV genotype	**1**	**1**	**1**	**1, 2, 3, 4**
Significant SNPs	rs12979860	rs8099917	rs8099917	rs8099917
***P* value**	1.37×10^{-28}	9.25×10^{-9}	1.18×10^{-10}*	3.11×10^{-8}
OR (95% CI)	3.1 (2.1 – 4.7)	1.98 (1.57 – 2.52)	12.1 (6.5 – 22.4)*	5.19 (2.9 – 9.3)
Platform	**Illumina610-quad**	**IlluminaCNV370-quad**	**Affymetrix SNPs 6.0**	**Illumina Human 1M-duo, Human Hap550/ Human610W-quad**

*The combined value in the study in comparison with SVR vs. non-SVR.

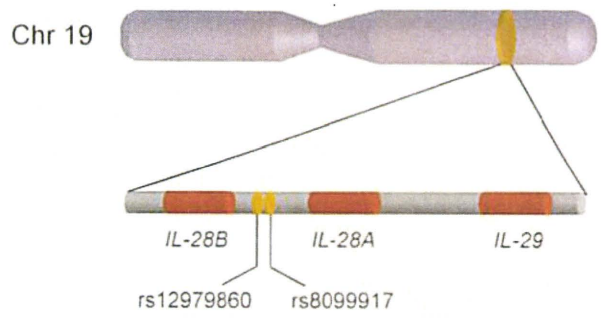

Fig. 2. *IL-28B* gene and SNPs location in chromosome 19. Four independent GWAS discovered SNPs strongly associated with the response to PEG-IFN&RBV therapy around *IL-28B* in chromosome 19. Ge et al. (22) reported rs12979860 as a strongly associated SNP, whereas Suppiah et al. (21), Tanaka et al. (24), and Rauch et al. (23) detected a statistical difference on rs8099917. These 2 SNPs are in strong linkage disequilibrium because these loci are very near to each other. The interferon lambda family consists of *IL-29* (IFN-λ1), *IL-28A* (IFN-λ2), and *IL-28B* (IFN-λ3), which are induced by type I IFN, or bacterial and viral infection.

Americans) and approximately 90% of Chinese and Japanese. Ge et al. showed that the SVR rates across different population groups displayed a striking concordance with the C-allele frequency. This SNP explained about half of the difference in response rates between African-Americans and Europeans.

Suppiah et al. and Tanaka et al. revealed the strong association of particular haplotypes of SNPs around *IL-28B* in the population of PEG-IFN&RBV therapy. The most significant SNPs in both study groups was rs8099917 (8 kb upstream of *IL-28B*) associated with SVR in European and Japanese patients (Fig. 2). Suppiah et al. also identified the association of rs8099917 in European ancestry with HCV genotype 1 based on the determination of SVR factors (combined $P = 9.25 \times 10^{-9}$, OR = 1.98, 95% CI = 1.57 – 2.52) (21). Homozygotes for the risk allele (rs8099917 G-allele) showed 2-fold higher risk of treatment failure than that of major allele homozygotes. In the gene expression assay, the minor allele of rs8099917 tended to suppress mRNA levels of *IL-28A/B*.

Tanaka et al. identified several SNPs significantly associated with NVR to PEG-IFN&RBV therapy in the GWAS and the replication study. All significant SNPs were located near the *IL-28B* locus on chromosome 19. The SNPs, rs12980275 or rs8099917, validated in an independent replication cohort showed the strongest association (combined $P = 2.84 \times 10^{-27}$ and 2.68×10^{-32}; OR = 17.7, 95% CI = 10.0 – 31.3; OR = 27.1, 95% CI = 14.6 – 50.3, respectively) (24). Interestingly, the minor alleles of the SNPs were accumulated in NVR (minor allele frequency of NVR = 74.3% for rs12980275 and 75.0% for rs8099917). Multivariate analyses containing genetic and clinical factors revealed that rs8099917 was the strongest predictor for response to therapy ($P = 0.0001$, OR = 37.68, 95% CI = 16.71 – 83.85).

The fourth GWAS was published on the response to HCV therapy. Rauch et al. studied patients infected with HCV genotype 1, 2, 3, or 4 (23). Rauch et al. also identified several SNPs around the *IL-28B* gene on chromosome 19 (Fig. 2). The strongest association with treatment failure was found with rs8099917 ($P = 3.11 \times 10^{-8}$, OR = 5.19). Interestingly, rs8099917 did not associate with the response to PEG-IFN&RBV therapy in genotype 2 or 3 patients. The contribution of host factors to genotype 2 or 3 clearance would be low because HCV genotype 2 or 3 is likely to be eliminated by the standard therapy compared with genotype 1. In individuals infected with HCV genotypes 1 and 4, the SVR rate of the patients harboring the minor allele was 28%, whereas that of the major allele homozygotes reached 63%. However, patients infected with genotypes 2 or 3 showed high viral response rate, approximately 80%, without statistical significance between the patients and the control.

7. The influence of genetic background on the statistical analysis

For the prediction of SVR, OR of the Japanese population was much higher than that of the other populations (Table 1). Individuals harboring the risk allele of rs8099917 or rs12979860 was approximately 10% in Asia, whereas the risk allele frequency was generally over 20% in European Caucasians. Moreover, individuals with the risk allele were the major population in individuals with African ancestry. The differences of allele frequency might explain, in part, the observed discrepancy in the response rate of viral clearance and the statistical power between racial groups.

Tanaka et al. extracted the data of TVR patients to analyze the genetic background. The minor allele frequency (MAF) of the strongly associated SNPs (rs8099917, located in the intergenic region between *IL-28A* and *IL-28B*) in TVR was similar to that of the SVR population (Fig. 3) (24). The statistical analysis for SVR prediction (SVR vs. non-SVR) using the SNPs showed lower statistical power (OR = 12.1) than that of NVR prediction (NVR vs. SVR plus TVR, OR = 27.1), indicating that the significant SNPs are strongly associated with the outcome of NVR. In other words, TVR patients share similar genetic background with SVR patients, and they would achieve SVR by prolonged therapy or PEG-IFN&RBV plus protease inhibitor.

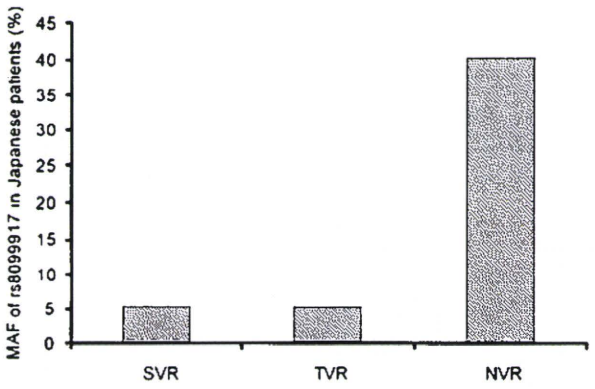

Fig. 3. Minor allele frequencies (MAF) of rs8099917 in each response type of chronic hepatitis C patients reported by Tanaka et al. (24). In the Asian population, the MAF of rs8099917 is approximately 10% according to Thomas et al. (20) and the dbSNPs international database. The MAF of chronic hepatitis C patients under a combination therapy of PEG-IFN&RBV revealed the deviation from that of the general population. The minor allele of rs8099917 accumulated in the population of NVR (approximately 40%), whereas those of SVR and TVR were occurred at lower frequency than those of the general population (approximately 5%).

8. SNPs associated with spontaneous clearance of HCV

Two study groups searched for common SNPs related to spontaneous elimination of HCV using a Caucasian cohort. Thomas et al. performed a candidate gene study on the rs12979860 SNP reported by Ge et al. to determine whether the SNP was also associated with spontaneous clearance of HCV infection (20). This study included 388 individuals with spontaneous HCV clearance and 620 with persistent HCV infection in a cohort consisting of HCV and HIV/HCV co-infected patients. The strong association of rs12979860 with spontaneous recovery was found in European and African American individuals (OR = 2.6, 95% CI = 1.9 – 3.8; OR = 3.1, 95% CI = 1.7 – 5.8, respectively) (Table 2). The association was also independent of co-infection with HIV, type of HCV transmission, and history of HBV infection.

Rauch et al. revealed the host factor associated with spontaneous clearance of HCV based on GWAS technology mounting more than 500K SNPs (23). The case-control study was designed for 347 individuals with spontaneous HCV clearance, 567 individuals with chronic hepatitis C, and 448 individuals with HCV/HIV co-infection. The significant SNP was also rs8099917 (combined $P = 6.07 \times 10^{-9}$, OR = 2.31, 95% CI = 1.74 – 3.04). The effect of HIV co-infection was also similar to that of HCV mono-infection ($P = 8.25 \times 10^{-5}$, OR = 2.16, 95% CI = 1.47 – 3.18; $P = 1.96 \times 10^{-5}$, OR = 2.49, 95% CI = 1.64 – 3.79, respectively) compared to Thomas et al. Note that rs8099917 was in strong linkage disequilibrium with rs12979860 in European and Asian individuals (26). These reports described by Thomas et al. and Rauch et al. seem to lead crucially identical results.

9. The characteristics of *IL-28B* and the IFN-λ family

IL-28B, referred to as IFN-λ3, belongs to the IFN-λ family, which consists of *IL-29*/IFN-λ1, *IL-28A*/IFN-λ2, and *IL-28B*. The *IL-28B* gene has been recently discovered and classified into type III IFN, a member of the class II cytokine family. This class II family includes type I, II, and III IFN and the *IL-10* family (*IL-10*, *IL-19*, *IL-20*, *IL-22*, *IL-24*, *IL-26*, *IL-28*, and *IL-29*). Peripheral blood mononuclear cells (PBMCs) and dendritic cells are main sources of IFN-λ (27, 28), which is induced by IFN-α, viral infection, and/or stimulations of toll-like receptors. IFN-λ behave as a interferon stimulated gene (ISG) of IFN-α, which is expressed at low levels by a broad variety of human cells, similar to IFN-α (29).

The signal pathway of IFN-λ is initiated through a membrane receptor distinct from that of type I IFN. The receptor is composed of heterodimer molecules consisting of an IL-28RA/IFN-λR1 subunit and IL-10R2 subunit (27, 28). The IL-10R2 subunit is expressed broadly and shared by IL-10, IL-22, IL-26, and IFN-λ. Compared with the IFN-α /-β receptor, which is ubiquitously expressed, the IL-28RA receptor has a more restricted distribution. The signal transduction of IFN-λ receptor is mediated via Jak1 and Tyk2, which can induce the phos-

Table 2. Summary of associated SNPs regarding spontaneous clearance of HCV

Study (Ref. No.)	Thomas et al. (20)		Rauch et al. (23)		
SNPs	rs12979860		rs8099917		
Population	European	African	HCV mono-infection	HCV/HIV co-infection	Combined
P value	1.0×10^{-7}	1.0×10^{-4}	1.96×10^{-5}	8.25×10^{-5}	6.07×10^{-9}
OR	2.6	3.1	2.49	2.16	2.31
95% CI	1.85 – 3.84	1.75 – 5.88	1.64 – 3.79	1.47 – 3.18	1.74 – 3.04

phorylation of STAT1 and STAT2 molecules and is followed by the expression of ISG (30).

10. The antiviral effect of IFN-λ against HCV in basic studies or clinical trials

Antiviral effects of IFN-λs against HCV have been reported before the discovery of the association with the response to HCV therapy. The treatment of IFN-α, or IFN-λ1 inhibited HCV replication at similar levels at low concentrations (31). The combination treatment of IFN-α and *IL-29/28A* enhanced the antiviral effect against HCV replicon synergistically (32). In microarray analysis on ISG induction of IFN-α/β or IFN-λ1, IFN-λ1 showed a unique pattern of ISG expression compared to that of IFN-α/β (31). For example, a total of 19 genes, which were not detected in the IFN-α–treated cells, were specifically up-regulated by IFN-λ1 at the late phase of treatment, indicating the signal pathway downstream of IL-28R1 could differ from that of IFN-α and possess a important biological function, although the pattern of signal transduction currently thought to be similar to that of IFN-α R1/2 (33). Further studies are needed to elucidate the biological consequences of these differences.

As described above, HCV replication is inhibited by the antiviral effects of IFN-λ. IFN-λ might have potential as a therapeutic agent against chronic hepatitis C in patients. A pegylated IFN-λ1 has already been tried against chronic hepatitis C in phase 1B trials (34). Interestingly, sufficient antiviral effects were observed but not severe side effects. The expression pattern of the IFN-λ receptor is restricted in specific organs. The high expression of the receptor was observed in the pancreas, liver, prostate, or thyroid, whereas the central nerve system (the bone marrow or the brain) showed the low expression (27, 28). These results could explain the avoidance of severe toxicity induced by IFN-α/β.

11. Conclusions

The recent discovery revealed by GWAS technology provides the unexpected role of *IL-28B* in HCV infection. The findings could be strong evidence to enhance the development of a novel therapeutic strategy and basic studies on IFN-λs. The SNPs around the *IL-28B* gene could improve the diagnostics for the prediction of spontaneous clearance and the response to anti-HCV treatment. However, approximately 20% – 30% of the total homozygotes with the risk alleles in Caucasians and 20% of heterozygotes/homozygotes with risk alleles in the Japanese population achieved a SVR and vice versa (21 – 24), indicating that the response to a combination therapy is not inevitably restricted because of genetic factors. To improve the prediction rate, especially, host epigenetic, rare SNPs, mutations, or viral factors are eligible candidates to consider when trying to establish an adequate tailor-made therapy. Although the strongly associated SNPs may have a big impact on the type of therapy and outcome, this is the first step in the tailor-made therapy for HCV infection. Further functional studies of IFN-λs and the significant SNPs should be investigated to improve the positive predictive value using the point mutation analysis of the targeted polymorphisms (35). For applying a practical tailor-made therapy, it is also necessary to reveal the cause of exceptional cases that do not follow the *IL-28B* genotyping.

References

1 Hoofnagle JH. Course and outcome of hepatitis C. Hepatology. 2002;36:S21–S29.

2 Shiratori Y, Shiina S, Imamura M, Kato N, Kanai F, Okudaira T, et al. Characteristic difference of hepatocellular carcinoma between hepatitis B- and C-viral infection in Japan. Hepatology. 1995;22:1027–1033.

3 Grebely J, Raffa JD, Lai C, Krajden M, Conway B, Tyndall MW. Factors associated with spontaneous clearance of hepatitis C virus among illicit drug users. Can J Gastroenterol. 2007;21:447–451.

4 Thomas DL, Astemborski J, Rai RM, Anania FA, Schaefer M, Galai N, et al. The natural history of hepatitis C virus infection: host, viral, and environmental factors. Jama. 2000;284:450–456.

5 Aborsangaya KB, Dembinski I, Khatkar S, Alphonse MP, Nickerson P, Rempel JD. Impact of aboriginal ethnicity on HCV core-induced IL-10 synthesis: interaction with IL-10 gene polymorphisms. Hepatology. 2007;45:623–630.

6 Busch MP, Glynn SA, Stramer SL, Orland J, Murphy EL, Wright DJ, et al. Correlates of hepatitis C virus (HCV) RNA negativity among HCV-seropositive blood donors. Transfusion. 2006;46:469–475.

7 Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med. 2002;347:975–982.

8 Hadziyannis SJ, Sette H Jr, Morgan TR, Balan V, Diago M, Marcellin P, et al. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. Ann Intern Med. 2004;140:346–355.

9 Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, et al. 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.

10 Conjeevaram HS, Fried MW, Jeffers LJ, Terrault NA, Wiley-Lucas TE, Afdhal N, et al. Peginterferon and ribavirin treatment in African American and Caucasian American patients with hepatitis C genotype 1. Gastroenterology. 2006;131:470–477.

11 Jeffers LJ, Cassidy W, Howell CD, Hu S, Reddy KR. Peginterferon alfa-2a (40 kd) and ribavirin for black American patients with chronic HCV genotype 1. Hepatology. 2004;39:1702–1708.

12 Muir AJ, Bornstein JD, Killenberg PG: Peginterferon alfa-2b and ribavirin for the treatment of chronic hepatitis C in blacks and non-Hispanic whites. N Engl J Med. 2004;350:2265–2271.
13 Shiffman ML, Mihas AA, Millwala F, Sterling RK, Luketic VA, Stravitz RT, et al. Treatment of chronic hepatitis C virus in African Americans with genotypes 2 and 3. Am J Gastroenterol. 2007;102:761–766.
14 Fried MW. Side effects of therapy of hepatitis C and their management. Hepatology. 2002;36:S237–S244.
15 Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, et al. 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.
16 Pascu M, Martus P, Hohne M, Wiedenmann B, Hopf U, Schreier E, et al. Sustained virological response in hepatitis C virus type 1b infected patients is predicted by the number of mutations within the NS5A-ISDR: a meta-analysis focused on geographical differences. Gut. 2004;53:1345–1351.
17 Shirakawa H, Matsumoto A, Joshita S, Komatsu M, Tanaka N, Umemura T, et al. Pretreatment prediction of virological response to peginterferon plus ribavirin therapy in chronic hepatitis C patients using viral and host factors. Hepatology. 2008;48:1753–1760.
18 Walsh MJ, Jonsson JR, Richardson MM, Lipka GM, Purdie DM, Clouston AD, et al. Non-response to antiviral therapy is associated with obesity and increased hepatic expression of suppressor of cytokine signalling 3 (SOCS-3) in patients with chronic hepatitis C, viral genotype 1. Gut. 2006;55:529–535.
19 Gao B, Hong F, Radaeva S. Host factors and failure of interferon-alpha treatment in hepatitis C virus. Hepatology. 2004;39:880–890.
20 Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, et al. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature. 2009;461:798–801.
21 Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, et al. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet. 2009;41:1100–1104.
22 Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature. 2009;461:399–401.
23 Rauch A, Kutalik Z, Descombes P, Cai T, Di Iulio J, Mueller T, et al. Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study. Gastroenterology. 2010;138:1338–1345, 1345.e1331–e1337.
24 Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet. 2009;41:1105–1109.
25 McHutchison JG, Lawitz EJ, Shiffman ML, Muir AJ, Galler GW, McCone J, et al. Peginterferon alfa-2b or alfa-2a with ribavirin for treatment of hepatitis C infection. N Engl J Med. 2009;361:580–593.
26 Tanaka Y, Nishida N, Sugiyama M, Tokunaga K, Mizokami M. Iambda-Interferons and the single nucleotide polymorphisms: A milestone to tailor-made therapy for chronic hepatitis C. Hepatol Res. 2010;40:449–460.
27 Kotenko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, Shah NK, et al. IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. Nat Immunol. 2003;4:69–77.
28 Sheppard P, Kindsvogel W, Xu W, Henderson K, Schlutsmeyer S, Whitmore TE, et al. IL-28, IL-29 and their class II cytokine receptor IL-28R. Nat Immunol. 2003;4:63–68.
29 Siren J, Pirhonen J, Julkunen I, Matikainen S. IFN-alpha regulates TLR-dependent gene expression of IFN-alpha, IFN-beta, IL-28, and IL-29. J Immunol. 2005;174:1932–1937.
30 Donnelly RP, Sheikh F, Kotenko SV, Dickensheets H. The expanded family of class II cytokines that share the IL-10 receptor-2 (IL-10R2) chain. J Leukoc Biol. 2004;76:314–321.
31 Marcello T, Grakoui A, Barba-Spaeth G, Machlin ES, Kotenko SV, MacDonald MR, et al. Interferons alpha and lambda inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. Gastroenterology. 2006;131:1887–1898.
32 Brand S, Zitzmann K, Dambacher J, Beigel F, Olszak T, Vlotides G, et al. SOCS-1 inhibits expression of the antiviral proteins 2',5'-OAS and MxA induced by the novel interferon-lambdas IL-28A and IL-29. Biochem Biophys Res Commun. 2005;331:543–548.
33 O'Brien TR. Interferon-alfa, interferon-lambda and hepatitis C. Nat Genet 2009;41:1048–1050.
34 Muir AJ, Shiffman ML, Zaman A, Yoffe B, de la Torre A, Flamm S, et al. Phase 1b study of pegylated interferon lambda 1 with or without ribavirin in patients with chronic genotype 1 hepatitis C virus infection. Hepatology. 2010;52:822–832.
35 Sakata T, Anzai N, Kimura T, Miura D, Fukutomi T, Takeda M, et al. Functional analysis of human organic cation transporter OCT3 (SLC22A3) polymorphisms. J Pharmacol Sci. 2010;113:263–266.

The rs8099917 Polymorphism, Determined by a Suitable Genotyping Method, is a Better Predictor for Response to Pegylated Interferon- α /Ribavirin Therapy in Japanese Patients than Other SNPs Associated with IL28B

Running Title: Best Predictor for Treatment of CHC Patients

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ABSTRACT

We focused on determining the most accurate and convenient genotyping methods and most appropriate SNP among four such polymorphisms associated with IL28B in order to design tailor-made therapy for chronic hepatitis C patients. Firstly, five different methods (direct sequencing, high-resolution melting analysis (HRM), Hybridization probe (HP), InvaderPlus assay (Invader), and TaqMan SNP genotyping assay (TaqMan)) were developed for genotyping four SNPs (rs11881222, rs8103142, rs8099917 and rs12979860) associated with IL28B and their accuracy was compared in 292 Japanese patients. Next, the four SNPs associated with IL28B were genotyped by Invader in 416 additional Japanese patients and the response to PEG-IFN/RBV treatment was evaluated when the four SNPs were not in linkage disequilibrium (LD). HRM failed to genotype one of the four SNPs in five patients. In two of 287 patients, the results of genotyping rs8099917 by direct sequencing differed from the results of the other three methods. The methods of HP, TaqMan and Invader were accurate for determining the SNPs associated with IL28B. In ten of the 708 (1.4%) patients, the four SNPs were not in LD. Eight of nine (88.9%) patients whose rs8099917 was homozygous for the major allele were virological responders, even though one or more of the other SNPs were heterozygous. The methods of HP, TaqMan and Invader were suitable to determine the SNPs associated with IL28B. The rs8099917 polymorphism should be the best predictor for the response to the PEG-IFN/RBV treatment among Japanese chronic hepatitis C patients.

Key Words: IL28B, SNP, chronic hepatitis C, tailor-made treatment, PEG-IFN/RBV

INTRODUCTION

Hepatitis C virus (HCV) infection is a global health problem, with worldwide estimates of 120-130 million carriers (1). Chronic HCV infection can lead to progressive liver disease, resulting in cirrhosis and complications including decompensated liver disease and hepatocellular carcinoma (25). The current standard-of-care treatment for suitable patients with chronic HCV infection consists of pegylated interferon alpha 2a or 2b (PEG-IFN) given by injection in combination with oral ribavirin (RBV), for 24 or 48 weeks, dependent on HCV genotype. Large-scale treatment programs in the United States and Europe showed that 42-52% of patients with HCV genotype 1 achieved a sustained virological response (SVR) (4, 8, 13), and similar results were found in Japan. This treatment is associated with well-described side effects (such as a flu-like syndrome, hematologic abnormalities and neuropsychiatric events) resulting in reduced compliance and fewer patients completing treatment (3). It is valuable to predict an individual's response before treatment with PEG-IFN/RBV to avoid these side-effects, as well as to reduce the treatment cost. HCV genotype, in particular, is used to predict the response: patients with HCV genotype 2 or 3 have a relatively high rate of SVR (70-80%) with 24 weeks of treatment, whereas those infected with genotype 1 have a much lower rate of SVR despite 48 weeks of treatment (8).

Recently, we reported from genome-wide association studies (GWAS) that several highly correlated common single nucleotide polymorphisms (SNPs), located in the vicinity of the IFN-lambda 3 (IL28B) gene on chromosome 19, are implicated in NVR (non-virological response) to PEG-IFN/RBV among patients with HCV genotype 1 (21).

At almost exactly the same time as our report, the association between response to PEG-IFN/RBV and SNPs associated with IL28B was reported from the results of GWAS by two other groups (7, 19). Determining these SNPs associated with IL28B before PEG-IFN/RBV treatment will provide extremely valuable information, because the patients predicted as NVR to PEG-IFN/RBV treatment could avoid the treatment. There are two questions to be asked before using these SNPs in clinical practice: which methods for genotyping these SNPs are efficient and which SNP is most informative in cases where the SNPs are not in linkage disequilibrium (LD). We have developed five different methods for detecting the SNPs associated with IL28B and compared their accuracy to establish the most efficient genotyping method. The response to PEG-IFN/RBV treatment was evaluated, when the SNPs associated with IL28B were not in LD, to determine the best SNP to predict the response to PEG-IFN/RBV treatment.

MATERIALS AND METHODS

Study population

Samples were obtained from 708 Japanese chronic hepatitis C patients and divided into groups of 292 (145 males, 147 females; mean age: 57.2 years) and 416 patients (194 males, 222 females; mean age: 56.6 years) for the first and second stages (Table 1). In the first stage, we focused on analyzing the effective methods for determining the genotypes of four SNPs (rs11881222, rs8103142, rs12979860, and rs8099917) associated with IL28B (Figure 1A). Figure 2 shows the location of these four SNPs in chromosome 19; rs11881222 and rs8103142 are located in the IL28B gene and

rs12979860 and rs8099917 are located downstream from IL28B. The results of genotyping the four SNPs by five different methods, described below, were compared and evaluated for consistency. For this first stage, the 292 chronic hepatitis C patients were recruited from the National Center for Global Health and Medicine, Hokkaido University Hospital, Tonami General Hospital, and Shin-Kokura Hospital in Japan (Table 1). From the results of the first stage, the InvaderPlus assay was chosen as one of the best methods to determine the genotypes of the four SNPs associated IL28B and was used for genotyping 416 patients (Figure 1B), recruited from NHO Nagasaki Medical Center, Nagoya City University Hospital, Nagoya Daini Red Cross Hospital, and Kawasaki Medical University Hospital in Japan, in the second stage (Table 1). We then focused on ten patients whose four SNPs were found in the first and second stages not to be in LD and investigated the response to PEG-IFN/RBV treatment in detail in these patients. Informed consent was obtained from each patient who participated in the study. This study was conducted in accordance with provisions of the Declaration of Helsinki.

Definition of treatment responses

Non-virological response (NVR) was defined as less than a 2-log-unit decline in the serum level of HCV RNA from the pre-treatment baseline value, within the first 12 weeks, or detectable viremia 24 weeks after treatment. Virological response (VR) was defined in this study as the achievement of sustained VR (SVR) or transient VR (TVR); SVR was defined as undetectable HCV RNA in serum 6 months after the end of treatment, whereas TVR was defined as a reappearance of HCV RNA in serum after

treatment was discontinued in a patient who had undetectable HCV RNA during the therapy or achieved more than 2-log-unit decline within the first 12 weeks after treatment.

DNA extraction

Whole blood was collected from all participants and centrifuged to separate buffy coat. Genomic DNA was extracted from the buffy coat with GENOMIX (Talent SRL, Italy).

Five different genotyping methods

Four SNPs (rs11881222, rs8103142, rs12979860 and rs8099917; shown in Figure 2) were determined in 292 patients by five different genotyping methods. We developed the five methods (direct sequencing, high-resolution melting analysis (HRM), Hybridization probe (HP), InvaderPlus® assay (Invader), and TaqMan SNP genotyping assay (TaqMan) to determine the genotypes of the rs11881222 and rs8103142 polymorphisms. We also developed four different methods (direct sequencing, HRM, HP and Invader) to determine the genotypes of the rs12979860 and rs8099917 polymorphism. The genotype of rs12979860 was also determined by the TaqMan genotyping method developed by Duke University and the genotype of rs8099917 was also determined by TaqMan® Pre-Designed SNP Genotyping Assay. Figure 3, 4 and 5 show the primers and probes for each genotyping method. Because the sequence of IL28B is very similar to those of IL28A, Il29 and a homologous sequence upstream of IL28B, we had to design the primers and probe for each method to distinguish IL28B

from the others sequencies. Firstly, primers were designed using Visual OMP Nucleic Acid software. Then, we confirmed that the candidate primers should not amplify sequences other than the target region using UCSC Genome Browser. Next, we confirmed that the amplicon was resolved as a single band, when the PCR products amplified by the primers under evaluation were electrophoresed. Finally, we had to optimize each set of primers and probe for each method (Figure 3-5 and Supplementary table).

Direct Sequencing

PCR was carried out using 12.5 μl AmpliTaq Gold 360 Master Mix (Applied Biosystems), 10 pmol of each primer and 10ng of genomic DNA under the following thermal cycler conditions: stage 1, 94°C for 5 min; stage 2, 94°C for 30 s, 65°C for 30 s, 72°C for 45 s, for a total of 35 cycles; stage 3, 72°C for 7 min. For sequencing, 1.0 μl of the PCR products were incubated with the use of a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). After ethanol purification, the reaction products were applied to the Applied Biosystems 3130xl DNA Analyzer.

HRM analysis

HRM analysis was performed on a LightCycler 480 (LC480; Roche Diagnostics) as described previously (6, 15, 24). We designed pairs of primers flanking each SNP (Figure 3-5) to amplify DNA fragments shorter than 200 bp. PCR was performed in a 20 μl volume containing: 10 μl LightCycler 480 High-Resolution Melting Master mix

(Roche Applied Science), 4 pmol of each primer and 10 ng genomic DNA. The cycling conditions were as follows: SYBR Green I detection format; 1 cycle of 95°C for 10 min, 50 cycles of 95°C for 5 s, 60°C for 10s, and 72°C for 20 s; followed by an HRM step of 95°C for 1 min, 40°C for 1 min, 74°C for 5 s, and continuous acquisition to 90°C at 25 acquisitions per 1°C. HRM data were analyzed using the Gene Scanning Software (Roche Diagnostics).

Hybridization probe

We designed oligonucleotide primers and hybridization probes for the four SNPs (Figure 3-5). All assays were performed using the LC480 as described previously (5, 18). The amplification mixture consisted of 4 μl of 5 *X* reaction mix (LightCycler 480 genotyping master, Roche Diagnostics), 5 pmol of each oligonucleotide primer, 3.2 pmol of each oligonucleotide probe, and 10ng of template DNA in a final volume of 20 μl. Samples were amplified as follows: 45 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 10 s, and an extension at 72°C for 20 s. The generation of target amplicons for each sample was monitored between the annealing and the elongation steps at 610 and 640 nm. Samples positive for target genes were identified by the instrument at the cycle number where the fluorescence attributable to the target sequences exceeded that measured for background. Those scored as positive by the instrument were confirmed by visual inspection of the graphical plot (cycle number versus fluorescence value) generated by the instrument.

InvaderPlus assay

The InvaderPlus® assay, which combines PCR and the Invader reaction (11, 12) was performed using the LC480. The enzymes used in Invader Plus are native *Taq* polymerase (Promega Corporation,Madison, WI) and Cleavase enzyme (Third Wave Technologies,Madison, WI). The reaction is configured to use PCR primers with a melting temperature (*Tm*) of 72°C and Invader detection probe with a target-specific *Tm* of 63°C. The invader oligonucleotide overlaps the probe by one nucleotide, forming at 63°C overlap flap substrate for the Cleavase enzyme. The first step of Invader Plus is PCR target amplification, in which the reaction is subjected to 18 cycles of a denaturation step (95°C for 15s) and hybridization and extension steps (70°C for 1min). At the end of PCR cycling, the reaction mixture is incubated at 99°C for 10 min to inactivate the *Taq* polymerase. Next, the reaction temperature is lowered to 63°C for 15 to 30 min to permit the hybridization of the probe oligonucleotide and the formation of the overlap flap structure. Data were analyzed by endpoint genotyping software (Roche diagnostics).

TaqMan assay

The rs8099917 polymorphism was determined using TaqMan® Pre-Designed SNP Genotyping Assays, as recommended by the manufacturer. The TaqMan assay for determining the genotype of rs12979860 was kindly provided by Dr David B. Goldstein at Duke University. We designed primers and probes for TaqMan genotyping assays for the other two SNPs. Each genomic DNA sample (20 ng) was amplified using TaqMan

Universal PCR master mix reagent (Applied Biosystems, Foster City, CA) combined with the specific TaqMan SNP genotyping assay mix, corresponding to the SNP to be genotyped. The assays were carried out using the LC 480 (Roche Applied Science) and the following conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles: 15 sec at 95°C, and 1 min at 60°C. Data were analyzed by endpoint genotyping software (Roche diagnostics).

RESULTS

Genotyping for four SNPs associated with IL28B was failed by HRM in five cases

Figure 1A shows the patient's flow chart of the first stage. Genotyping of four SNPs (rs11881222, rs8103142, rs12979860 and rs8099917) was attempted by five different methods (direct sequencing, HRM, HP, Invader and TaqMan) in 292 patients. In five cases, one of the four SNPs could not be genotyped by HRM. Therefore, we excluded the HRM method from further study. Genotyping failures by HRM were two cases for rs11881222, two cases for rs8103142 and one case for rs8099917.

Consistencies of four different methods to determine genotypes for four SNPs associated with IL28B.

Consistencies among the results of genotyping by the remaining four methods were 100%, except for the results of rs8099917 (Table 2). For rs8099917, the results determined by direct sequencing were inconsistent with the other three methods in two cases (Table 2 and 3). HP, TaqMan, and Invader methods were accurate and reliable for genotyping the four SNPs associated with IL28B. Invader was chosen for genotyping in

the second stage, because the analysis time was the shortest and the sensitivity was the greatest of the three methods (HP, TaqMan and Invader) as reported previously (20).

Genotyping Error for rs8099917 by direct sequence due by novel SNP

In two cases, the results of genotyping for rs8099917 by direct sequencing were inconsistent with the results by the other methods (Table 3). Direct sequencing determined the genotype for rs8099917 as T/T in cases 1 and 2, however, the other three genotyping methods (HP, Invader, and TaqMan) determined the genotypes for rs8099917 as T/G in both cases. Further study using alternative primers for direct sequencing revealed that the correct genotypes were T/G and revealed a novel minor SNP present in at the forward primer binding site in these two cases (data on file) and which interfered with the PCR amplification step (Figure 3).

Distribution of haplotypes among four SNPs associated with IL28B.

In the first stage, the four SNPs were in LD in 281 (98.6%) of 285 cases and not in LD in the remaining four (1.4%). The first stage revealed five different haplotypes (haplotype 1-5; Table 4). In haplotypes 1-3, the four SNPs were in LD (haplotype 1: homozygous of major allele among 4 SNPs; n=198 [69.5%], haplotype 2: heterozygous among 4 SNPs; n=79 [27.7%], and haplotype 3: homozygous of minor allele among 4 SNPs; n=4 [1.4%]). In haplotype 4 (3 cases) rs11881222, rs8103142, rs12979860 and rs8099917 were AG, TC, CT, and TT, respectively. In haplotype 5 (one case) rs11881222, rs8103142, rs12979860 and rs8099917 were AA, TT, CT, and TT,