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α-Fetoprotein Is a Surrogate Marker for Predicting Treatment Failure in Telaprevir-Based Triple Combination Therapy for Genotype 1b Chronic Hepatitis C Japanese Patients With the *IL28B* Minor Genotype

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Even when treated with telaprevir-based triple therapy, some patients fail to achieve a sustained virological response. This study identified factors related closely to treatment failure. A total of 146 Japanese genotype 1b chronic hepatitis C patients were enrolled in this prospective, multicenter study and received a 24-week regimen of triple therapy. The end-of-treatment response rate was significantly lower in patients with the interleukin 28B (*IL28B*) (rs8099917) non-TT genotype (85.2%) than in those with the TT genotype (100%, $P=0.0002$). Multiple logistic regression analysis identified high α-fetoprotein levels as an independent factor related to non-end-of-treatment response in patients with the non-TT genotype. A cut-off value of 20 ng/ml was determined for a non-end-of-treatment response; sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy were 75.0%, 95.7%, 75.0%, 75.0%, and 92.6%, respectively. Multiple logistic regression analysis for a sustained virological response identified the *IL28B* TT genotype, low α-fetoprotein levels, non-responders, and a rapid virological response. The sustained virological response rate was significantly lower in patients with the non-TT genotype (59.3%) than in those with the TT genotype (96.7%, $P<0.0001$). In patients with the non-TT genotype, α-fetoprotein was the most significant predictor for non-sustained virological response by univariate analysis. A cut-off value of 7.4 ng/ml α-fetoprotein was determined for non-sustained virological response; sensitivity, specificity, PPV, NPV, and accuracy were 63.6%, 87.5%, 77.8%, 77.8%, and 77.8%, respectively. For the non-TT patients, serum α-fetoprotein levels may be a surrogate marker for predicting treatment failure in telaprevir-based therapy for genotype 1b chronic hepatitis C. ***J. Med. Virol.***

KEY WORDS: HCV; telaprevir; α-fetoprotein; *IL28B*

INTRODUCTION

Even when treated with standard care consisting of peginterferon (Peg-IFN) and ribavirin including an extended 72-week treatment course, a sustained virological response was achieved only in approximately 40–53% of genotype 1 chronic hepatitis C

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patients [Manns et al., 2001; Fried et al., 2002; McHutchison et al., 2009a; Toyoda et al., 2012; Tsubota et al., 2012; Yoshizawa et al., 2013]. In aging patients in Japan, improving the sustained virological response rate through more effective treatment methods is required urgently to reduce mortality from liver failure and hepatocellular carcinoma.

Of the many drugs under investigation, the most promising are direct-acting antiviral agents, such as nonstructural (NS) 3/4A protease inhibitors [Pawlotsky, 2013]. Telaprevir, a NS3/4A serine protease inhibitor, was approved in the United States, Canada, the European Union (EU), and Japan in 2011. In treatment-naïve genotype 1 chronic hepatitis C patients, telaprevir-based triple combination therapy for a shortened period was reported to remarkably improve the sustained virological response rate compared with Peg-IFN and ribavirin alone [Hézode et al., 2009; McHutchison et al., 2009b; Kumada et al., 2012]. In treatment-experienced patients, the effect of telaprevir-based therapy was prescribed according to the patient's response to previous Peg-IFN and ribavirin combination therapy [McHutchison et al., 2010; Chayama et al., 2011; Muir et al., 2011; Zeuzem et al., 2011; Akuta et al., 2012, 2013; Hayashi et al., 2012].

Pivotal genome-wide association studies demonstrated that genetic variations near the interleukin 28B (*IL28B*) gene (rs8099917 and rs12979860) are associated strongly with treatment outcome of Peg-IFN and ribavirin combination therapy [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009]. These genetic variations appear to be strong predictors of the sustained virological response to telaprevir-based triple therapy as well as Peg-IFN and ribavirin combination therapy [Akuta et al., 2010, 2013; Chayama et al., 2011; Bota et al., 2013; Furusyo et al., 2013; Muir, 2013]. Additionally, fibrosis of liver likely influences treatment outcome. Particularly, presence of cirrhosis decreased the sustained virological response rate, even in telaprevir-based therapy [McHutchison et al., 2010; Jacobson et al., 2011]. Furthermore, the importance of a rapid virological response, defined as undetectable serum hepatitis C virus (HCV) RNA at treatment week 4, and extended rapid virological response, defined as undetectable serum HCV RNA at both treatment weeks 4 and 12, were also reported as significant predictors of telaprevir-based treatment outcome [Chayama et al., 2011; Jacobson et al., 2011; Sherman et al., 2011; Furusyo et al., 2013].

Indeed, telaprevir-based triple combination therapy remarkably improves the sustained virological response rate in chronic hepatitis C patients with the difficult-to-treat HCV genotype 1. However, some patients still fail to achieve a sustained virological response. Adverse events occurred more frequently and were more severe in patients treated with telaprevir-based therapy than in those treated with Peg-IFN and ribavirin alone [Hézode et al., 2009; McHutchison et al., 2009b, 2010; Zeuzem et al., 2011; Kumada et al., 2012]. Additionally, telaprevir-based therapy is very costly. In clinical practice, predictive factors of treatment failure are necessary for preventing unnecessary treatment as well as physical and economic burdens. This prospective, multicenter study was conducted to identify factors related closely to treatment failure in telaprevir-based triple combination therapy for genotype 1b chronic hepatitis C patients.

PATIENTS AND METHODS

Patients

Between December 2011 and May 2012, 146 Japanese genotype 1b-monoinfected chronic hepatitis C patients were enrolled in this study at Shinmatsudo Central General Hospital, Nippon Medical School Chiba Hokusoh Hospital, Jikei University School of Medicine Katsushika Medical Center, Jikei University School of Medicine Kashiwa Hospital, and Nippon Medical School Hospital. Inclusion criteria for the study included persistently positive sera for HCV RNA for >6 months as determined using quantitative real-time polymerase chain reaction (PCR) (COBAS AmpliPrep/COBAS TaqMan HCV test, Roche Diagnostics, Tokyo, Japan), age of 18–75 years, and body weight >35 kg at the time of entry into the study. Exclusion criteria included: (1) decompensated cirrhosis, (2) positive for hepatitis B surface antigen or antibody against human immunodeficiency virus, (3) previous or current development of hepatocellular carcinoma, (4) co-existence of other liver diseases such as autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, Wilson disease, and alcoholic liver disease, (5) renal disease or creatinine clearance $\leq$50 ml/min at baseline, (6) hemoglobin level <12 g/dl, white blood cell count <2,000/μl, neutrophil count <1,500/μl, and platelet count $<8.0 \times 10^4$/μl at baseline, (7) depression, schizophrenia or its history, and history of suicide attempt, (8) pregnancy in progress or planned during the study period for either partner. For all patients, liver biopsy was conducted within 12 months of enrollment and the presence or absence of cirrhosis was established according to the Metavir score [The French METAVIR Cooperative Study Group, 1994].

Patient profiles are shown in Table I. In this study, all treatment-experienced patients were treated with Peg-IFN and ribavirin combination therapy. Patients in this study were categorized as relapsers (HCV RNA undetectable at the end of treatment and then positive in follow up), partial responders ($\geq 2 \log_{10}$ IU/ml reduction in HCV RNA at week 12 but never undetectable), null responders ($<2 \log_{10}$ U/ml reduction in HCV RNA at week 12). In this study, partial responders and null responders were analyzed as non-responders.

All patients were treated with Peg-IFN-α-2b, ribavirin, and telaprevir triple therapy. Telaprevir (Telavic;

TABLE I. Characteristics of Patients

Variables	All	Naïve	Relapsers	Partial responders	Null responders
Number of patients	146	62	58	16	10
Gender, male/female (male%)	65/81 (44.5%)	28/34 (45.2%)	21/37 (36.2%)	12/4 (75.0%)	4/6 (40.0%)
Age (years)	57.4 (10.2)	55.1 (11.6)	59.4 (8.9)	56.6 (9.6)	60.3 (5.6)
Body weight (kg)	60.0 (11.9)	60.3 (11.7)	58.5 (11.6)	62.8 (12.9)	62.1 (12.0)
Body mass index (kg/m^2)	23.2 (3.4)	23.5 (3.5)	22.7 (3.1)	23.3 (3.3)	24.2 (3.7)
Absence or presence of cirrhosis (non-cirrhosis/cirrhosis) (cirrhosis %)	107/39 (26.7%)	47/15 (24.2%)	44/14 (24.1%)	11/5 (31.3%)	5/5 (50.0%)
rs8099917 (TT/TG/GG) (TT %)	92/52/2 (63.0%)	46/16/0 (74.2%)	40/17/1 (69.0%)	6/10/0 (37.5%)	0/9/1 (0.0%)
rs1127354 (CC/CA/AA)	123/22/1	50/11/1	50/0	14/2/0	9/1/0
Core amino acid substitution 70 (wild-type/mutant-type)	97/49	44/18	40/18	11/5	2/8
Core amino acid substitution 91 (wild-type/mutant-type)	100/46	44/18	40/18	10/6	6/4
ISDR of NS5A (wild-type/non-wild-type)	123/23	48/14	52/6	13/3	10/0
White blood cells (/μl)	5,032 (1,466)	5,241 (1,305)	4,803 (1,613)	5,105 (1,457)	4,941 (1,318)
Hemoglobin (g/dl)	14.0 (1.5)	14.2 (1.5)	13.6 (1.4)	14.7 (1.4)	14.0 (1.4)
Platelets (×10^4%L)	17.6 (5.2)	17.9 (5.1)	17.5 (5.5)	17.8 (5.0)	15.9 (3.6)
Aspartate aminotransferase (IU/L)	53.7 (38.5)	54.8 (36.5)	51.8 (44.7)	47.1 (14.3)	68.1 (34.8)
Alanine aminotransferase (IU/L)	61.7 (54.0)	66.2 (52.7)	56.8 (60.1)	54.9 (27.5)	73.2 (52.3)
γ-Glutamyl-transpeptidase (IU/L)	51.4 (62.3)	59.9 (59.6)	58.2 (50.8)	67.2 (76.0)	114.4 (87.7)
Albumin (g/dl)	4.2 (0.4)	4.1 (0.3)	4.2 (0.4)	4.2 (0.4)	4.2 (0.4)
Low density lipoprotein-cholesterol (mg/dl)	101.0 (29.4)	103.8 (31.7)	101.5 (27.1)	92.6 (22.9)	93.1 (31.7)
Fasting plasma glucose (mg/dl)	100.5 (22.8)	105.2 (23.7)	105.4 (23.8)	104.9 (19.4)	109.1 (14.1)
Homeostasis model assessment-insulin resistance	4.0 (5.9)	4.0 (6.7)	3.7 (4.9)	3.7 (4.6)	6.0 (6.9)
α-Fetoprotein (ng/ml)	11.3 (22.9)	13.8 (26.0)	6.5 (7.3)	4.4 (2.2)	34.6 (48.2)
HCV RNA ($\log_{10}$ IU/ml)	6.4 (0.8)	6.5 (0.8)	6.3 (0.9)	6.5 (1.0)	6.6 (0.3)
Initial dose of Peg-IFN (μg/kg)	1.5 (0.2)	1.5 (0.1)	1.5 (0.2)	1.5 (0.2)	1.6 (0.2)
Initial dose of ribavirin (mg/kg)	11.1 (1.9)	11.1 (1.8)	10.6 (1.9)	11.8 (1.6)	12.3 (0.9)
Initial daily dose of telaprevir (1,500/2,250 mg)	67/79	24/38	33/25	5/11	5/5
The administration intervals of telaprevir (q8h/q12q)	98/48	38/24	41/17	11/5	8/2

Data are expressed as numbers or mean (standard deviation).
ISDR, interferon sensitivity-determining region; HCV, hepatitis C virus; Peg-IFN, peginterferon.

Mitsubishi Tanabe Pharma, Osaka, Japan) was administered every 8 hr after meals (q8h) at 500 or 750 mg or every 12 hr after meals (q12h) at 750 mg or 1,125 mg. The initial daily dose of telaprevir (1,500 mg per day or 2,250 mg per day) and administration intervals (q8h or q12h) were determined by each attending physician according to age, gender, body weight, and hemoglobin level. Peg-IFN-α-2b (Peg-Intron. MSD, Tokyo, Japan) was injected subcutaneously at a median dose of 1.5 μg/kg/week. Ribavirin (Rebetol, MSD) dose was adjusted by body weight (600 mg for <60 kg; 800 mg for ≥60 to <80 kg; and 1,000 mg for ≥80 kg, and in the case of hemoglobin <13 g/dl at start of therapy, ribavirin was reduced by 200 mg), based on the guidelines of the Ministry of Health, Labor and Welfare of Japan. The drug was administered orally after breakfast and dinner. Triple therapy was given for 12 weeks, followed by an additional 12 weeks of Peg-IFN-α-2b and ribavirin combination therapy (T12PR24). Each drug was reduced appropriately or withdrawn when a serious adverse event was suspected of developing or if a serious adverse event occurred during the treatment course.

In addition, treatment was stopped for patients who had HCV RNA >3 $\log_{10}$ IU/ml at week 4 or detectable at week 12 or those showing a 2 $\log_{10}$ IU/ml increase from the lowest level during therapy because these patients had a low likelihood of achieving a sustained virological response and a high risk of developing antiviral resistance.

Adherence to Peg-IFN was calculated based on an initial weekly dose and that to ribavirin was calculated based on an initial daily dose. Adherence to telaprevir was defined as 100% when 2,250 mg was given per day for 12 weeks, which is the recommended daily dose.

Virological response was analyzed on an intent-to-treat basis. Treatment efficacy was evaluated based on HCV RNA negativity at the end of treatment (end-of-treatment response) and a successful endpoint

of treatment was a sustained virological response for patients showing undetectable HCV RNA for 24 weeks after cessation of treatment. In patients with relapse, HCV RNA levels became undetectable by the end-of-treatment but became positive during the follow-up period. In patients with viral breakthrough, HCV RNA became undetectable during the treatment period but then became positive before the end of the treatment period. In patients with non-response, HCV RNA was always detected during therapy. Furthermore, a rapid virological response was defined as undetectable HCV RNA at week 4 of starting treatment, while an extended rapid virological response was defined as undetectable HCV RNA at both weeks 4 and 12 of starting treatment.

All patients provided written informed consent. This study protocol was prepared following ethics guidelines established in conformity with the 2008 Declaration of Helsinki and was approved by the Ethics Committee of Shinmatsudo Central General Hospital, Nippon Medical School, and Jikei University (Approval numbers: 2012001, 523029, and 23-246, respectively).

Measurement of HCV RNA

HCV genotype was determined by direct sequencing followed by phylogenic analysis of the NS5B region [Simmonds et al., 1996]. The antiviral effects of the therapy on HCV were assessed by measuring serum HCV RNA levels. In this study, HCV RNA levels during treatment were evaluated at least once every 4 weeks before, during, and after therapy. HCV RNA concentrations were determined using the COBAS AmpliPrep/CABAS TaqMan HCV test (Roche Diagnostics). The linear dynamic range of the assay was 1.2–7.8 $\log_{10}$ IU/ml, and undetectable samples were defined as negative.

Detection of Amino Acid Substitution in Core and NS5A Regions of HCV Genotype 1b

Core amino acid substitutions at positions 70 and 91 were determined according to a previously described method [Akuta et al., 2007a]. Core amino acid substitutions at position 70 were defined as wild-type (arginine) or mutant-type (glutamine or histidine), and core amino acid substitutions at position 91 were defined as wild-type (leucine) or mutant-type (methionine). Additionally, substitutions at amino acids 2290–2248 of the NS5A region (interferon-sensitivity determining region, ISDR) were determined using a previously described method [Enomoto et al., 1996]. Amino acid substitutions in ISDR were defined as wild-type (0 or 1) or non-wild-type ($\geq$2).

Single Nucleotide Polymorphism Genotyping

Genomic DNA was extracted from whole blood using the MagNA Pure LC and a DNA Isolation Kit (Roche Diagnostics). Genetic polymorphisms, rs8099917 near the *IL28B* gene [Suppiah et al., 2009; Tanaka et al., 2009] and rs1127354 at the inosine triphosphatase (*ITPA*) gene [Fellay et al., 2010], were genotyped by real-time detection PCR using the TaqMan SNP Genotyping Assays and the 7500Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The rs8099917 genotypes were classified into two categories, including TT (major genotype) and non-TT genotype (minor genotype: TG or GG), while the rs1127354 genotypes were classified into two categories, including CC (major genotype) and non-CC genotype (minor genotype: CA or AA).

Statistical Analysis

Continuous variables are expressed as the mean and standard deviations. Categorical data were analyzed using the chi-squared test and Fisher's exact test, while continuous data were analyzed using the nonparametric Mann–Whitney *U* test and Mann–Whitney *U* test with Bonferroni's correction. Univariate and multiple logistic regression analysis were used to identify factors that significantly contributed to rapid virological response, end-of-treatment response (or non-end-of-treatment response), and sustained virological response (or non-sustained virological response). Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated. All *P* values for statistical tests were two tailed, and values of <0.05 were considered statistically significant. Variables that achieved statistical significance ($P < 0.05$) according to univariate analysis were entered into multiple logistic regression analysis to identify significant independent predictive factors of rapid virological response, end-of-treatment response or non-end-of-treatment response, and sustained virological response or non-sustained virological response.

Receiver-operating characteristics (ROC) analyses were performed to determine cut-off values for sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy for predicting treatment outcomes. Statistical analysis was performed using SPSS version 17.0 (IBM-SPSS, Chicago, IL).

RESULTS

Characteristics According to Previous Treatment Response

Characteristics of patients involved in the study are shown in Table I. The distribution of the *IL28B* SNP genotype was the most significantly different across the four categories of previous treatment response. The proportion of the TT genotype was 74.2% (46 of 62) in treatment-naïve patients, 69.0% (40 of 58) in relapsers, 37.5% (6 of 16) in partial responders, and 0% (0 of 10) in null responders ($P = 0.0001$). The α-fetoprotein levels were also significantly different as follows: null (34.6 ± 48.2 ng/ml) versus naïve (13.8 ± 26.0 ng/ml; $P = 0.0019$); null

versus relapsers (6.5 ± 7.3 ng/ml; $P = 0.0368$); and null versus partial (4.4 ± 2.2 ng/ml; $P = 0.0029$). The presence of cirrhosis was higher in partial responders and null responders than in treatment-naïve and relapsers, though not statistically significant.

According to previous treatment response, among the 62 treatment-naïve patients, 55 (88.7%) achieved a sustained virological response, 3 (4.8%) relapsed, 3 (4.8%) had viral breakthrough, and 1 (1.6%) showed a non-response. Among the 58 relapsers, 54 (93.1%) achieved a sustained virological response, 3 (5.2%) relapsed, and 1 (1.7%) had viral breakthrough. Among the 16 partial responders, 12 (75.0%) achieved a sustained virological response and 4 (25.0%) relapsed. Among the 10 null responders, none (0%) achieved a sustained virological response, 7 (70.0%) relapsed, 1 (10.0%) had viral breakthrough, and 2 (20%) showed non-response (Fig. 1). For the *IL28B* SNP genotype, among the 92 patients with the TT genotype, 89 (96.7%) achieved a sustained virological response and 3 (3.3%) relapsed. Among the 54 patients with the non-TT genotype, 32 (59.3%) achieved a sustained virological response, 14 (25.9%) relapsed, 5 (9.3%) had viral breakthrough, 3 (5.6%) showed non-response (Fig. 2).

Factors Associated With Rapid Virological Response

The rate of rapid virological response was 82.2% (120 of 146 patients). All rapid virological response patients also achieved an extended rapid virological response. According to univariate analysis, previous relapse ($P = 0.0087$), high platelet counts ($P = 0.0481$), and low aspartate aminotransferase ($P = 0.0156$), alanine aminotransferase ($P = 0.0486$), α-fetoprotein ($P = 0.0300$), and HCV RNA ($P = 0.0001$) were significant positive predictors for rapid virological response. Being previous non-responders was a significant negative predictor for rapid virological response ($P = 0.0118$). The rate of rapid virological response was higher in patients with the *IL28B* TT genotype (84.8%, 78 of 92) than in those with the non-TT genotype (77.8%, 42 of 54, respectively), although the values were not significantly different ($P = 0.2855$). Multiple logistic regression analysis revealed that low HCV RNA levels ($P = 0.0002$, OR = 0.16, 95% CI = 0.06–0.42) and previous non-responders ($P = 0.0121$, OR = 0.25, 95% CI = 0.09–0.74) were independent predictors of rapid virological response.

Factors Associated With End-of-Treatment Response

A total of 138 of 146 (94.5%) patients achieved end-of-treatment response. For the *IL28B* genotype, end-of-treatment response was achieved in all 92 (100%) patients with the TT genotype and 46 of 54 (85.2%) patients with the non-TT genotype ($P = 0.0002$). Surprisingly, all patients with the TT genotype showed a favorable end-of-treatment response. Only patients with the non-TT genotype failed in end-of-treatment response. Therefore, factors associated with failed end-of-treatment response (non- end-of-treatment response) were explored in patients with the non-TT genotype alone. High aspartate aminotransferase ($P = 0.0139$), alanine aminotransferase ($P = 0.0085$), γ-glutamyl-transpeptidase ($P = 0.0499$), and α-fetoprotein ($P = 0.0011$) were significantly associated with non-end-of-treatment response. For the non-end-of-treatment response rate, the presence of cirrhosis (5 of 16 patients, 31.2%) was significantly higher than the absence of cirrhosis (3 of 38, 7.9%; $P = 0.0413$). Multiple logistic regression analysis only identified high α-fetoprotein levels as an independent predictor for non-end-of-treatment response

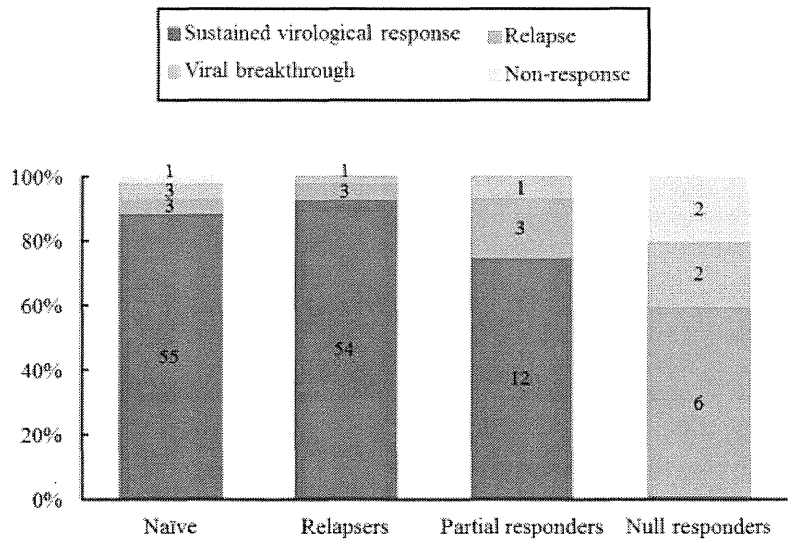

Fig. 1. Virological outcome according to previous treatment response.

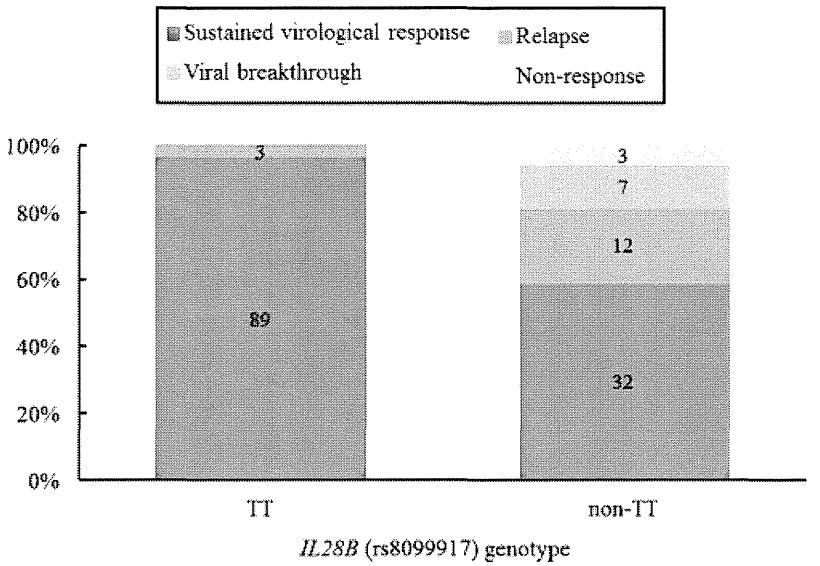

Fig. 2. Virological outcome according to the *IL28B* (rs8099917) genotype.

($P = 0.0146$, OR = 1.05, 95% CI = 1.01–1.09; Table II). The association between α-fetoprotein levels and non-end-of-treatment response was determined based on ROC curve analysis (Fig. 3). The area under the ROC curve was 0.864; the cut-off value of 20 ng/ml revealed the maximum likelihood of discriminating between end-of-treatment responses and non-end-of-treatment responses. The rate of non-end-of-treatment response was significantly higher in patients with high α-fetoprotein levels (≥20 ng/ml; 6 of 8, 75.0%) than in those with low α-fetoprotein levels (<20 ng/ml, 2 of 46, 4.3%; $P < 0.0001$, OR = 66.0, 95% CI = 7.78–559.58; Suppl. Fig. S1). The sensitivity, specificity, PPV, NPV, and accuracy of the cut-off value for non-end-of-treatment response were 75.0%, 95.7%, 75.0%, 95.7%, and 92.6%, respectively.

Factors Associated With Sustained Virological Response

A sustained virological response was achieved in 121 of 146 (82.9%) patients. For the *IL28B* genotype, a sustained virological response was achieved in 89 of 92 patients (96.7%) with genotype TT and 32 of 54 patients (59.3%) with genotype non-TT ($P < 0.0001$). All three patients with the TT genotype who failed to show a sustained virological response discontinued treatment (renal dysfunction at 4 weeks in a previous relapser, acute pancreatitis at 7 weeks in a treatment-naïve patient, and renal dysfunction at 10 weeks in a partial responder) and thereafter relapsed. All had cirrhosis.

According to univariate analysis, the following factors were associated with a sustained virological response: previous relapsers ($P = 0.0012$), absence of cirrhosis ($P = 0.0006$), *IL28B* genotype TT ($P < 0.0001$), high white blood cell counts ($P = 0.0404$), platelet counts ($P = 0.0192$), and low density lipoprotein-cholesterol ($P = 0.0178$), low γ-glutamyl-transpeptidase ($P = 0.0201$) and α-fetoprotein ($P = 0.0030$), core amino acid substitutions at position 70 wild-type ($P = 0.0032$), achievement of rapid virological response ($P < 0.0001$), longer treatment duration ($P = 0.0364$), and high dosage adherence of ribavirin ($P = 0.0413$). Being previous non-responders was a significant negative predictor for sustained virological response ($P < 0.0001$). Multiple logistic regression analysis identified the following 4 independent factors: *IL28B* TT genotype ($P = 0.0001$, OR = 36.51, 95% CI = 5.86–227.51), low α-fetoprotein levels ($P = 0.0045$, OR = 0.96, 95% CI = 0.93–0.99), previous non-responders ($P = 0.0147$, OR = 0.15, 95% CI = 0.03–0.69), and achievement of a rapid virological response ($P = 0.0261$, OR = 1.25, 95% CI = 1.25–33.59) (Table III).

Factors Associated With Non-Sustained Virological Response

Patients with the *IL28B* TT genotype showed extremely high sustained virological response rate (96.7%). Therefore, factors associated with a failed sustained virological response (non-sustained virological response) were explored in patients with the non-TT genotype alone. According to univariate analysis, previous non-responders ($P = 0.0093$), presence of cirrhosis ($P = 0.0137$), low white blood cell counts ($P = 0.0324$) and platelet counts ($P = 0.0046$), and high aspartate aminotransferase ($P = 0.0022$), alanine aminotransferase ($P = 0.0055$), and α-fetoprotein ($P = 0.0002$) were associated positively with a non-sustained virological response. Being a previous relapsers was a significant negative predictor of non-sustained virological response ($P = 0.0178$). However,

TABLE II. Factors Associated With Non-End-of-Treatment Response in Patients With the *IL28B* SNP Non-TT Genotype

	Simple	Multiple		
Variable	*P*-Value	OR	95% CI	*P*-Value
Host-related factor				
Age (year)	0.6173			
Gender, male vs. female	0.7058			
Body weight (kg)	0.9418			
Body mass index (kg/m^2)	0.4954			
Cirrhosis absence vs. presence	0.0413			
Treatment-naïve	0.2174			
Relapsers	0.2447			
Partial responders	0.3264			
Null responders	0.1561			
rs1127354 CC vs. CA+AA	0.5769			
White blood cells (/μl)	0.8646			
Hemoglobin (g/dl)	0.8359			
Platelets (×10^4/μl)	0.0860			
Aspartate aminotransferase (IU/L)	0.0139			
Alanine aminotransferase (IU/L)	0.0085			
γ-Glutamyl-transpeptidase (IU/L)	0.0499			
Albumin (g/dl)	0.1064			
Low density lipoprotein-cholesterol (mg/dl)	0.1765			
Fasting plasma glucose (mg/dl)	0.2892			
Homeostasis model assessment-insulin resistance	0.9798			
α-Fetoprotein (ng/ml)	0.0011	1.05	1.01–1.09	0.0146
Virus-related factor				
HCV RNA ($\log_{10}$ IU/ml)	0.0621			
Core amino acid substitution 70 wild-type vs. mutant-type	1.0000			
Core amino acid substitution 91 wild-type vs. mutant-type	0.7019			
ISDR of NS5A non-wild-type vs. wild type	1.0000			
Treatment-related factor				
The administration intervals of telaprevir q8h vs. q12h	0.6966			
Initial daily dose of telaprevir 2,250 mg vs. 1,500 mg	1.0000			
Duration of therapy (weeks)	0.6264			
Adherence of Peg-IFN (%)	0.9895			
Adherence of ribavirin (%)	0.2053			
Adherence of telaprevir (%)	0.7575			

HCV, hepatitis C virus; ISDR, interferon sensitivity-determining region.

multiple logistic regression analysis did not identify any significant independent factors associated with non-sustained virological response. Since the high α-fetoprotein levels was the most significant predictor by univariate analysis, the association between α-fetoprotein levels and non-sustained virological response was determined using ROC curve analysis (Fig. 4). The area under the ROC curve was 0.798, and a cut-off value of 7.4 ng/ml yielded maximum likelihood of discrimination between non-sustained virological response and sustained virological response ($P = 0.0001$, OR = 12.25, 95% CI = 3.14–47.77). The rate of non-sustained virological response was significantly higher in patients with high α-fetoprotein levels (>7.4 ng/ml; 14 of 18, 77.8%) than in those with low α-fetoprotein levels (≤7.4 ng/ml; 8 of 36, 22.2%; Suppl. Fig. S2). The sensitivity, specificity, PPV, NPV, and accuracy of the cut-off value for non-sustained virological response were 63.6%, 87.5%, 77.8%, 77.8%, and 77.8%, respectively.

Regarding the presence or absence of cirrhosis, the rate of non-sustained virological response was significantly higher in patients with cirrhosis (11 of 16, 68.8%) than in those without cirrhosis (11 of 38, 28.9%; $P = 0.0137$). The sensitivity, specificity, PPV, NPV, and accuracy of presence of cirrhosis for non-sustained virological response were 50.0%, 84.4%, 68.8%, 71.1%, and 70.4%, respectively. The α-fetoprotein levels was significantly higher in patients with cirrhosis than in those without cirrhosis (37.4 ± 45.7 ng/ml vs. 5.7 ± 4.5 ng/ml, $P = 0.0001$).

DISCUSSION

Multiple logistic regression analysis revealed the *IL28B* genotype as the most significant factor predicting a sustained virological response to telaprevir-based triple combination therapy. The contribution of the *IL28B* genotype to a sustained virological response agreed with the results of previous studies conducted in Japan [Akuta et al., 2010, 2013; Chayama et al., 2011; Furusyo et al., 2013]. In this study, the sustained virological response rate was very high (96.7%) in patients with the *IL28B* TT genotype and numerically higher than values reported previously [Akuta et al., 2010, 2013; Chayama

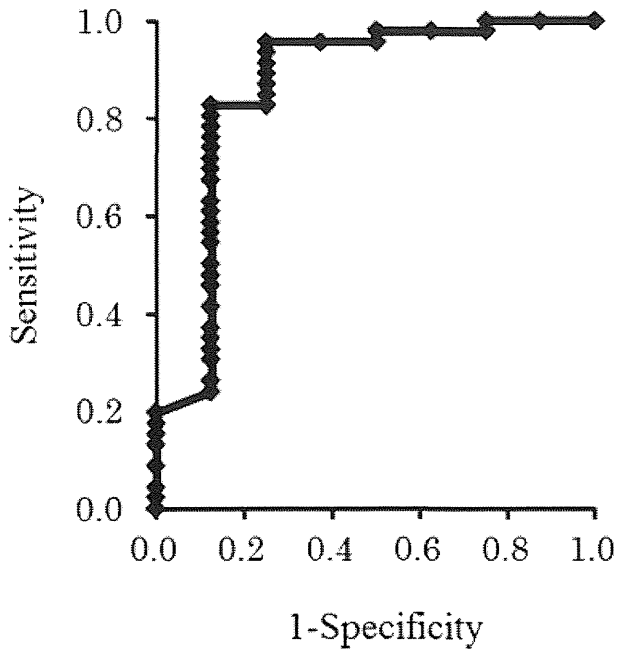

Fig. 3. The receiver operating characteristics (ROC) analysis for predicting non-end-of-treatment response to telaprevir-based triple combination therapy in patients with the *IL28B* non-TT according to serum α-fetoprotein levels. The area under the ROC curve was 0.864.

et al., 2011; Furusyo et al., 2013]. Notably, all patients with the favorable *IL28B* TT genotype achieved end-of-treatment response. A few TT genotype patients who discontinued prematurely treatment and experienced treatment failure may have achieved a sustained virological response if they had completed treatment as scheduled. Interestingly, rapid virological response did not significantly differ between patients with the TT and non-TT genotypes, despite distinct differences in the rates of end-of-treatment response and sustained virological response. Even if patients with the TT genotype failed a rapid virological response, they may have achieved a sustained virological response with a shortened 24-week treatment. Taken together, Japanese patients with the favorable TT genotype showed the strongest likelihood of achieving a sustained virological response to telaprevir-based therapy.

In contrast, the sustained virological response rate decreased to approximately 60% in patients with the unfavorable *IL28B* non-TT genotype. However, telaprevir-based therapy substantially increased the rate from approximately 14–24% [Hayes et al., 2011; Kurosaki et al., 2011; Toyoda et al., 2012; Yoshizawa et al., 2013] in Peg-IFN and ribavirin therapy to approximately 60%. Therefore, this study focused on patients with the non-TT genotype alone and investigated which factors contributed to treatment failure in the relatively unfavorable patient group. Interestingly, α-fetoprotein was associated closely with the failure of both end-of-treatment and sustained virological response. Furthermore, the cut-off value was determined using ROC analysis to discriminate between treatment success and failure.

α-Fetoprotein, known as a single-stranded glycoprotein, belongs to the albuminoid gene family, which includes albumin and the vitamin D-binding protein. α-Fetoprotein is produced in the embryonic yolk sac and fetal liver; therefore, the serum α-fetoprotein level in the fetus remains high until birth. Serum α-fetoprotein level decrease by 10 ng/ml within several weeks after birth and remain at low concentrations throughout life [Takikawa and Suzuki, 2002]. However, reactivation of α-fetoprotein production in adults occurs during liver regeneration and hepatocarcinogenesis [Liaw et al., 1986]. Therefore, serum α-fetoprotein is measured routinely as a tumor marker of hepatocellular carcinoma [Tyson et al., 2011]. However, α-fetoprotein levels are sometimes elevated in patients with chronic HCV infection who do not have hepatocellular carcinoma [Bayati et al., 1998; Goldstein et al., 1999; Chu et al., 2001]. Patients with increased α-fetoprotein levels have a very high risk of developing hepatocellular carcinoma in chronic HCV infection [Kumada et al., 2010]. Although advanced liver fibrosis is generally associated with high α-fetoprotein levels [Bayati et al., 1998; Hu et al., 2004; Chen et al., 2007], elevated α-fetoprotein levels are a risk factor for hepatocellular carcinoma, irrespective of fibrosis stage [Tateyama et al., 2011]. In Peg-IFN and ribavirin therapy for chronic hepatitis C, high pretreatment α-fetoprotein levels independently predict a lower sustained virological response [Males et al., 2007; Akuta et al., 2007b]. The increased serum α-fetoprotein levels are ascribed to hepatic damage with selective transcriptional activation of the α-fetoprotein gene [Taketa, 1990].

This study involved multiple regression and ROC analyses and is the first to demonstrate that the high levels of α-fetoprotein is a useful predictive factor for treatment failure in patients with the *IL28B* non-TT genotype by telaprevir-based triple combination therapy in clinical practice. Only one study reported that α-fetoprotein levels were related to treatment outcome in telaprevir-based therapy [Akuta et al., 2012]. The difference between the present study and the previous study is the patient group. The previous study investigated previous non-responders to Peg-IFN and ribavirin in clinical trials, while the present study investigated patients with the *IL28B* non-TT genotype in clinical practice. In the previous study, the rate of end-of-treatment response failure was significantly higher in patients with high levels of α-fetoprotein (≥10 ng/dl) than in those with low levels of α-fetoprotein (<10 ng/dl). The previous study investigated 15 non-responders, and 14 of those had the *IL28B* non-TT genotype. Therefore, these two studies appear to be similar in that patients with the *IL28B* non-TT genotype were examined. The discrepancy in cut-off value can be explained by including

TABLE III. Factors Associated With Sustained Virological Response

	Simple		Multiple		
Variable	OR	*P*-Value	OR	95% CI	*P*-Value
Host-related factor					
Age (year)	0.98	0.4171			
Gender, male vs. female	0.84	0.7008			
Body weight (kg)	1.00	0.8929			
Body mass index (kg/m^2)	0.95	0.3920			
Cirrhosis absence vs. presence	4.89	0.0006			
Treatment-naïve	2.14	0.1134			
Relapsers	4.23	0.0122			
Non-responders	0.09	<0.0001	0.15	0.03–0.69	0.0147
rs8099917 TT vs. TG+GG	20.40	<0.0001	36.51	5.86–227.51	0.0001
rs1127354 CC vs. CA+AA	0.41	0.2554			
White blood cells (/μl)	1.00	0.0404			
Hemoglobin (g/dl)	1.30	0.0902			
Platelets (×10^{4}μl)	1.13	0.0192			
Aspartate aminotransferase (IU/L)	0.99	0.0783			
Alanine aminotransferase (IU/L)	1.00	0.2192			
γ-Glutamyl-transpeptidase (IU/L)	0.99	0.0201			
Albumin (g/dl)	2.95	0.0651			
Low density lipoprotein-cholesterol (mg/dl)	1.02	0.0178			
Fasting plasma glucose (mg/dl)	1.02	0.1291			
Homeostasis model assessment-insulin resistance	0.97	0.4013			
α-Fetoprotein (ng/ml)	0.97	0.0030	0.96	0.93–0.99	0.0045
Virus-related factor					
HCV RNA ($\log_{10}$ IU/ml)	0.81	0.4525			
Core amino acid substitution 70 wild-type vs. mutant-type	3.84	0.0032			
Core amino acid substitution 91 wild-type vs. mutant-type	1.93	0.1438			
ISDR of NS5A non-wild-type vs. wild type	2.41	0.2554			
Treatment response factor					
Rapid virological response + vs. −	7.05	<0.0001	6.48	1.25–33.59	0.0261
Treatment-related factor					
The administration intervals of telaprevir q8h vs. q12h	0.76	0.5694			
Initial daily dose of telaprevir 2,250 mg vs. 1,500 mg	1.11	0.8161			
Duration of therapy (weeks)	1.10	0.0364			
Adherence of Peg-IFN (%)	1.02	0.0899			
Adherence of ribavirin (%)	1.02	0.0413			
Adherence of telaprevir (%)	1.00	0.7031			

HCV, hepatitis C virus; ISDR, interferon sensitivity-determining region; Peg-IFN, peginterferon.

treatment-naïve patients and relapsers and the difference in baseline α-fetoprotein levels. Baseline α-fetoprotein levels in the present study were higher compared to those in the previous study (15.1 ± 29.0 ng/dl vs. 10.5 ± 11.2 ng/dl).

In this study, the sustained virological response rate was significantly lower in patients with the *IL28B* non-TT genotype. Therefore, this study focused on patients with the non-TT genotype alone and attempted to identify factors contributing to treatment failure in patients with the unfavorable *IL28B* genotype. As a result, high levels of α-fetoprotein may be a surrogate marker for predicting non-sustained virological response as well as non-end-of-treatment response in patients with the *IL28B* non-TT genotype. Although the populations and aims of the studies differed, the cut-off value of α-fetoprotein levels (7.4 ng/ml) for non-sustained virological response in this study were surprisingly similar (7.3 ng/ml) for high risk of hepatocellular carcinoma in chronic hepatitis C patients before interferon therapy [Asahina et al., 2013]. The results suggest that patients with baseline α-fetoprotein levels with a high risk of hepatocarcinogenesis experience difficulty in achieving a sustained virological response. Even when treated with telaprevir-based triple combination therapy, high α-fetoprotein concentrations appeared to attenuate successful treatment outcome.

This study also found that α-fetoprotein was more useful as a predictor of non-sustained virological response than the presence of cirrhosis. Although liver biopsy remains the gold standard for evaluating fibrosis, there are several limitations, such as invasiveness, variability in sampling and pathological interpretation, and high cost. However, measuring serum α-fetoprotein is easy, inexpensive, non-invasive, and superior in quantitative capability. Thus, measuring serum α-fetoprotein is simpler and more useful than histological evaluation by liver biopsy for identifying patients with treatment failure in telaprevir-based therapy, particularly for the *IL28B* non-TT genotype. However, in the patients who had high

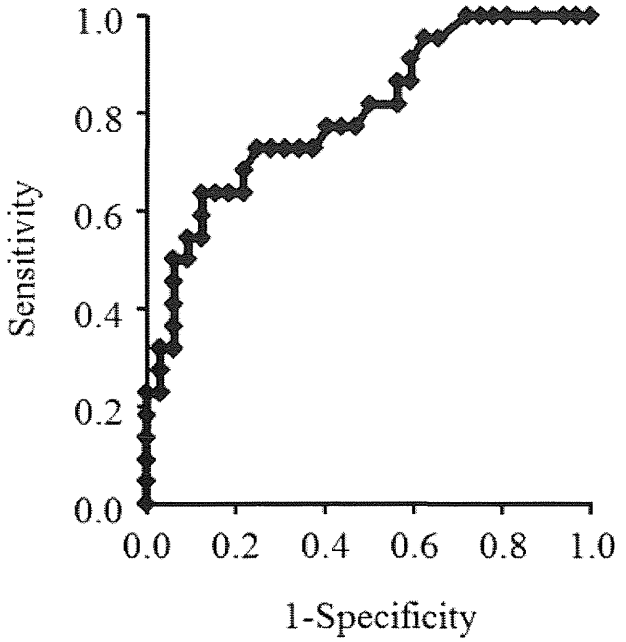

Fig. 4. The receiver operating characteristics (ROC) analysis for predicting non-sustained virological response to telaprevir-based triple combination therapy in patients with the *IL28B* genotype non-TT according to serum α-fetoprotein levels. The area under the ROC curve was 0.798.

levels of α-fetoprotein, the population ratio of cirrhosis would be high. Therefore, that would be a main reason that α-fetoprotein could be a surrogate marker for treatment failure.

In this study, the proportions of the *IL28B* genotype were different mostly based on previous treatment response. In treatment-experienced patients, the proportions of the unfavorable *IL28B* non-TT genotype increased with refractoriness to treatment. In the REALIZE study, the proportions of the unfavorable *IL28B* (rs12979860) non-CC genotype (CT/TT) were increased similarly, at 72.9% in relapsers, 86.9% in partial responders, and 94.0% in null responders [Pol et al., 2013]. Treatment outcome is associated clearly with previous treatment response. The close relationship between these factors may arise largely from the proportions of the unfavorable *IL28B* genotype. Furthermore, α-fetoprotein levels and the frequency of cirrhosis in null responders were higher compared to those in others. There may be close relationship among these unfavorable factors. Thus, previous null responders showed an extremely poor response, even to telaprevir-based therapy. These patients also had a high risk of developing hepatocellular carcinoma. Hence, a new combination therapy with potent direct-acting antiviral agents with or without interferon is needed urgently.

There were some limitations to this study. First, the number of patients was too small to identify factors contributing to treatment failure conclusively. In particular, the number of non-responders was only 26. Second, telaprevir-resistant variants were not analyzed. Resistant variants were reported to occur in 56% of HCV genotype 1b patients who did not achieve a sustained virological response [Sullivan et al., 2013]. Therefore, resistance variants should be identified in patients with treatment failure. Third, this study regimen was limited in T12PR24. Only 24-week telaprevir-based triple combination therapy is allowed by the Japanese National Insurance System. In the US, Canada, and EU, triple combination therapy is administered for either 12 or 36 additional weeks after Peg-IFN and ribavirin, according to the response-guided regimen based on early viral response in each category: treatment-naïve patients and previous relapsers or partial responders and null responders.

In conclusion, this prospective, multicenter study of telaprevir-based triple combination therapy for Japanese genotype 1b chronic hepatitis C patients showed that the *IL28B* SNP genotype is the most important pretreatment factor for predicting a sustained virological response and indicated that high levels of α-fetoprotein could be a surrogate marker for predicting treatment failure in patients with the *IL28B* non-TT genotype. Further large-scale prospective studies are necessary to confirm these findings and to facilitate development of more rational and effective therapeutic regimens.

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SUPPORTING INFORMATION

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Review Article

Genome-Wide Association Study Reveals Host Genetic Factors for Liver Diseases

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Abstract

A number of disease-associated genetic markers for common liver diseases have been identified using genome-wide association studies (GWASs). The GWAS strategy is based on genome-wide single-nucleotide polymorphism typing technologies, which are now commercially available, accompanied by statistical methods to identify host genetic factors that are associated with target diseases or complex genetic traits. One of the most striking features of the GWAS strategy is the ability to identify unexpected disease-associated genetic markers across the entire human genome. Here, we describe the technological aspects of the GWAS strategy with examples from actual GWAS reports related to hepatitis research, including drug response for patients with chronic hepatitis C, susceptibility to primary biliary cirrhosis, and hepatitis-B-related hepatocellular carcinoma.

Introduction

Numerous single-nucleotide polymorphisms (SNPs) have been discovered and deposited in public databases (e.g. the National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov], Ensembl [http://asia.ensembl.org/index.html], and the MEXT Integrated Database Project [http://dbcls.rois.ac.jp]) through international SNP discovery projects such as the Human Genome Project,[1] the International HapMap project (http://hapmap.ncbi.nlm.nih.gov/index.html.en), and the 1000 Genomes project (www.1000genomes.org). Together with the development of technologies for large-scale SNP genotyping, genome-wide association studies (GWASs) using hundreds of thousands of SNPs allow the identification of candidate genetic loci for multifactorial diseases. Disease-associated SNPs have also been deposited in public databases, such as the database of Genotypes and Phenotypes (www.ncbi.nlm.nih.gov/gap). Moreover, a number of SNPs have been reported to be associated with complex genetic traits, such as body mass index,[2] height,[3] and hair thickness.[4] In the National Human Genome Research Institute (NHGRI) GWAS catalog (www.genome.gov), more than 8,800 trait- or disease-associated SNPs with genome-wide significance ($p<5\times10^{-8}$) have been archived from a total of 1,551 published GWAS (through March, 2013).[5]

Here, we describe a GWAS strategy to identify disease-associated SNPs, including SNP genotyping technologies for both the GWAS stage and the following replication stage. Based on this GWAS strategy, we have identified associations of genetic variations with diseases related to hepatitis B and C viruses (HBV and HCV), including drug response in patients with chronic HCV infection,[6] susceptibility to primary biliary cirrhosis (PBC),[7] and HBV-related hepatocellular carcinoma (HCC).[8]

Technologies for GWAS and replication analysis

A number of SNP typing methods have been used to analyze a single SNP, or SNPs at multiple sites of a template or templates simultaneously. Most of the methods employ single or multiple site-specific amplifications and a genotyping step based on various types of chemical reactions, including Sanger direct sequencing, 5′ exonuclease fluorescence-based assay (TaqMan),[9] pyrosequencing,[10] DigiTag2 assay,[11] single-base extension,[12] and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF).[13]

Together with technology developments in large-scale SNP genotyping, the most recent versions of commercially available genotyping platforms allow the simultaneous analysis of more than one million SNPs across the whole genome in a single experiment. Two platforms are commercially available and widely used for genome-wide SNP typing: Affymetrix SNP GeneChip arrays[14] and Illumina BeadArray genotyping technology.[15] The number of SNPs embedded in both platforms has been gradually increasing since 2003, when the first commercial genome-wide SNP genotyping platform was released by Affymetrix.[16] The first platform of the Affymetrix GeneChip Mapping 10K Array included 14,548 SNPs, which enabled the performance of whole-genome linkage analyses and was indeed used to identify a disease-associated missense mutation in the *HOXD10* gene with Charcot–Marie-Tooth disease through a family-based linkage study.[17] The current versions of the commercial platforms from Affymetrix and Illumina include more than 900,000 SNPs (Genome-Wide Human SNP Array 6.0) and 4.3 million SNPs (HumanOmni5-Quad BeadChip), respectively. A newly released genome-wide SNP typing platform, named the

Keywords: GWAS; Hepatitis B infection; Hepatitis C infection; Primary biliary cirrhosis; HLA-DP; Hepatocellular carcinoma; Host genetic factors.
Abbreviations: GWAS, genome-wide association study; SNP, single nucleotide polymorphism; HBV, hepatitis B virus; HCV, hepatitis C virus; PBC, primary biliary cirrhosis; HCC, hepatocellular carcinoma; PCR, polymerase chain reaction; HLA, human leukocyte antigen; CHB, chronic hepatitis B.
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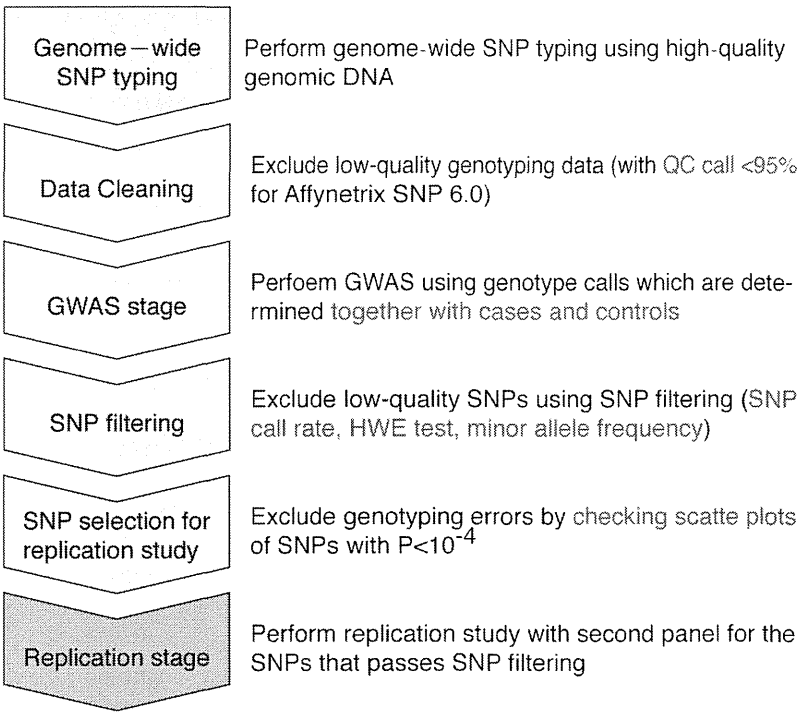

Fig. 1. GWAS strategy from genome-wide SNP typing to replication analysis.

Affymetrix Axiom Genome-Wide ASI 1 Array, has a probe set for SNPs (including rare and common variants) that are optimized for Asian populations. These platforms open a new approach for researchers to perform GWASs with hundreds of thousands of SNPs, allowing the identification of candidate genetic loci for multifactorial diseases.

In 2002, the first GWAS using 92,788 gene-based SNPs was reported by a Japanese group (RIKEN), which identified the lymphotoxin-α gene as being associated with susceptibility to myocardial infarction.[18] The RIKEN group developed its own platform to perform a GWAS based on the Invader assay[19] with multiplex polymerase chain reaction (PCR).[20] Since 2002, the number of published genome-wide associations with genome-wide significance ($p<5\times10^{-8}$) has increased annually, reaching 1,551 publications in the NHGRI GWA catalog (through March, 2013).[5]

For a replication study following a GWAS stage, several candidate genetic regions that have been detected in the initial GWAS need to be analyzed. Suitable platforms for replication analyses have the ability to perform multiplex detections in a single reaction, such as the mini-sequencing (SNaPshot) technique,[21] chip-based genotyping by mass spectrometry (Sequenom),[22] and the DigiTag2 assay.[11] The DigiTag2 assay is our own technology for multiplex SNP typing, and represents a simple and cost-effective approach by combining multiplex PCR to enrich genetic regions including the target SNPs with an oligonucleotide ligation assay to determine the genotype of the target locus. For a single locus analysis the TaqMan assay would be more commonly used to determine the genotype of the target locus, as opposed to conventional Sanger sequencing, which is more commonly used when a large number of samples need to be analyzed.

Hepatitis research based on GWAS

In a GWAS, two groups of participants are compared to detect the "association(s)" of certain variants with a particular trait by examining differences in allele and/or genotype frequency of all SNPs, which exist across the entire genome. GWAS enables the effective detection of associated variations in strong linkage disequilibrium with the causal variants and genes, and the following replication analysis and high-density mapping identify the causal variants and genes using an independent set of participants with a larger number of samples. However, the association of SNPs with low minor allele frequency (below 1–5%; known as rare variants) would be difficult to detect in a SNP-based GWAS because of insufficient statistical power due to the limitation of sample number.[23] Fig. 1 outlines the GWAS strategy from whole-genome SNP typing to replication analysis.

The emerging strategy of GWAS has revealed disease-causing alleles, or variants that lead to susceptibility to complex polygenic diseases with small additive or multiplicative effects on the disease phenotype. For example, a recent GWAS and subsequent meta-analyses in populations of European descent identified human leukocyte antigen (HLA) and 21 non-HLA susceptibility loci, most of which are involved in interleukin (IL)-12/IL-12 receptor (IL-12R) signaling, tumor necrosis factor (TNF)/toll-like receptor (TLR)–nuclear factor (NF)-κB signaling, and B-cell differentiation in the development of PBC.[24–27] PBC is a chronic cholestatic liver disease characterized by chronic non-suppurative destructive cholangitis of the intrahepatic small bile ducts. A high concordance rate in monozygotic twins and familial clustering of patients with PBC indicates the involvement of strong genetic factors in the development of PBC.[28] To

Table 1. Replication analysis of Japanese samples for SNPs associated with PBC in previous studies, and two newly identified loci (*TNFSF15* and *POU2AF1*).

Gene name	SNP	OR	95% CI	P-value
Significant associations with PBC				
TNFSF15	rs4979462	1.57	1.76–1.40	1.85×10^{-14}
POU2AF1	rs4938534	1.38	1.55–1.23	3.27×10^{-8}
IKZF3	rs9303277	1.44	1.63–1.28	3.66×10^{-9}
CD80	rs2293370	1.48	1.68–1.29	3.04×10^{-9}
IL7R	rs6890853	1.47	1.69–1.28	3.66×10^{-8}
Suggestive associations with PBC				
NFKB1	rs7665090	1.35	1.52–1.21	1.42×10^{-7}
STAT4	rs7574865	1.35	1.52–1.19	1.11×10^{-6}
Marginal associations with PBC				
CXCR5	rs6421571	1.42	1.75–1.16	0.0004
TNFAIP2	rs8017161	1.22	1.38–1.08	0.0006
MAP3K7IP1(TAB1)	rs968451	1.29	1.52–1.10	0.0009
rs6974491	rs2717948	1.33	1.66–1.07	0.005
DENND1B	rs12134279	1.14	1.33–0.98	0.0405
No apparent associations with PBC				
rs11117432	rs8062669	1.21	1.52–0.96	0.0521
IL12RB2/SCHIP1	rs3790567	1.12	1.28–0.98	0.0540
RPS6KA4	rs538147	1.12	1.28–0.98	0.0554
TNFRSF1A	rs1800693	1.12	1.30–0.97	0.0607
CLEC16A	rs12924729	1.10	1.28–0.94	0.1197
MMEL1	rs3748816	1.07	1.20–0.95	0.1256
PLCL2	rs1372072	1.07	1.20–0.95	0.1396
SPIB	rs3745516	1.08	1.27–0.92	0.1803
IRF5/TNPO3	rs4728142	1.08	1.30–0.90	0.2027
RAD51L1	rs911263	1.07	1.30–0.89	0.2353
IL12A	rs6441286	1.02	1.15–0.91	0.3422

identify susceptibility loci for PBC in the Japanese population, we conducted a GWAS and subsequent replication study using a total of 1,327 PBC patients and 1,120 healthy controls.[7] In addition to the most significant susceptibility region at *HLA*, two significant susceptibility loci (*TNFSF15* and *POU2AF1*) with *p*-values $<5 \times 10^{-8}$ were identified (Table 1). Moreover, of the 21 non-HLA susceptibility loci that were identified in populations of European descent, three loci (*IKZF3*, *CD80*, and *IL7R*) showed significant associations and two loci (*NFKB1* and *STAT4*) showed suggestive associations with PBC in the Japanese population. Five other loci (*CXCR5*, *TNFAIP2*, *MAP3K7IP1*, rs6974491, and *DENND1B*) also showed marginal associations ($p<0.05$) with PBC in the Japanese population (Table 1). These results indicate that additional important disease pathways (via *TNFSF15* and *POU2AF1*) – differentiation to T-helper 1 (Th1) cells (via *TNFSF15*, *CD80*, *IL12*, *IL12R*, and *STAT4*; Fig. 2), B-cell differentiation (via *POU2AF1*, *CXCR5*, *SPIB*, *IL7R*, and *IKZF3*), and NF-κB signaling – in addition to the previously reported disease pathways have a role in the development of PBC in Japanese populations.

In another study that aimed to identify host genetic factors related to drug response to pegylated interferon-α plus ribavirin treatment for HCV infected patients, comparatively small number of samples were analyzed in a GWAS, including samples from 154 Japanese HCV patients undergoing pegylated interferon-α/ribavirin treatment, 78 null virologic responders, and 64 virologic responders.[6] Despite the small number of samples in the GWAS in comparison with other studies in European descendants (European American,[29] Australian,[30] and Swiss[31]), the same disease-causing locus of *IL28B* was identified with the strongest association in the Japanese population. In general, the number of samples affects the statistical power of detection in a GWAS. Moreover, false-positive associations can increase when low-quality genotype data are incorporated in the analysis, presumably caused by accidental errors in genotyping steps or low-quality genomic DNA. The Japanese GWAS was able to successfully identify the risk factors in a small number of samples because: (1) *IL28B* is a strong host risk factor for drug response in Asian and white populations; and (2) quality controls were used in sample collection in terms of clinical characteristics, and the genotype data were checked for quality.[14]

As for HBV-related HCC, a GWAS using chronic HBV carriers with and without HCC in five independent Chinese samples found that one SNP (rs17401966) in *KIF1B* was associated with susceptibility to HBV-related HCC.[32] Moreover, in the most recent report on this topic, genetic variants in the *STAT4* and *HLA-DQ* genes were identified as

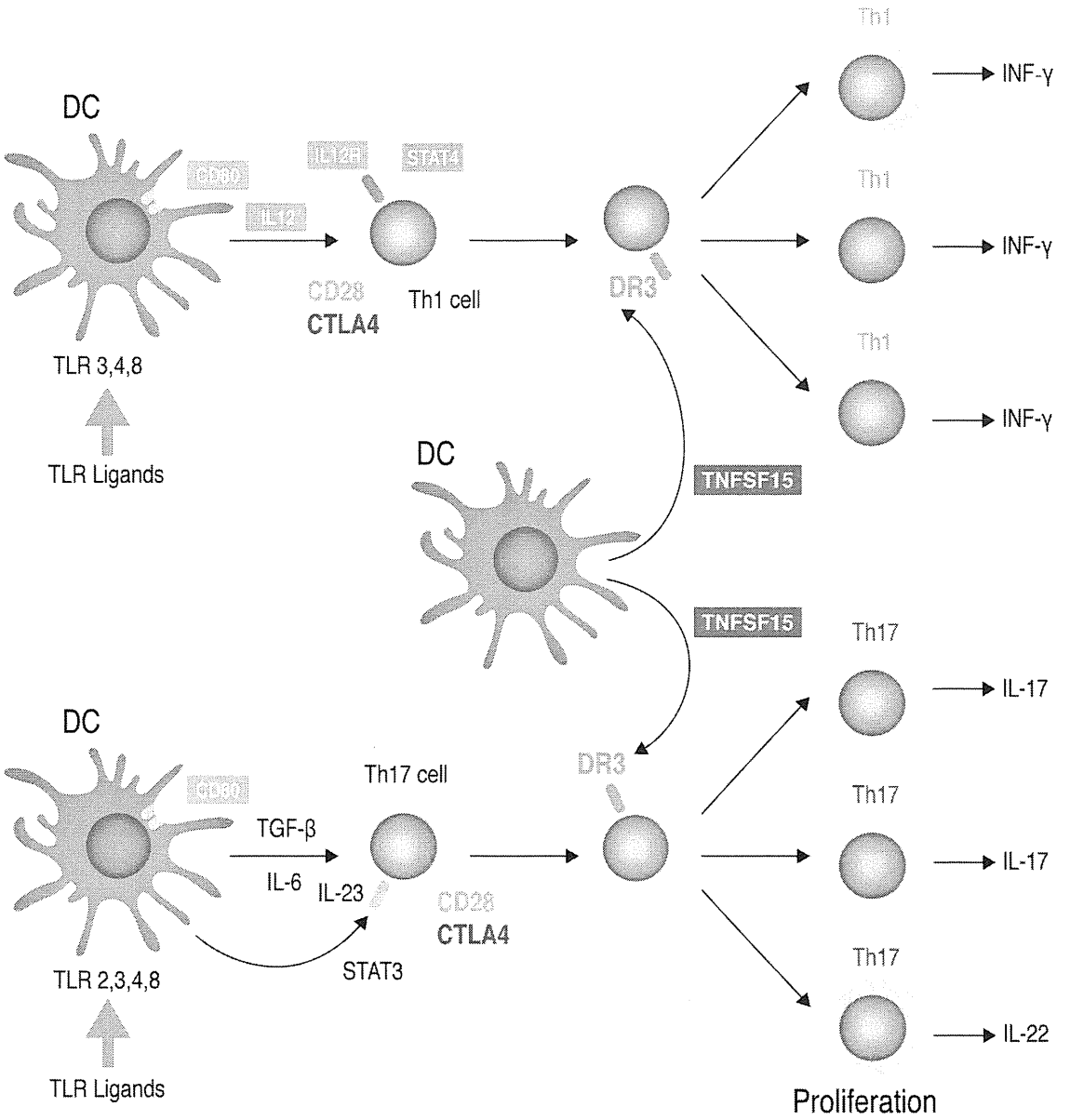

Fig. 2. T-cell proliferation via *TNFSF15* and other related genes. A proportion of susceptibility genes associated with PBC (*CD80*, *IL12A*, *IL12RB2*, *STAT4*, and *TNFSF15*) are related to T-cell proliferation via both Th1 and Th17 cells.

genetic susceptibility loci for HBV-related HCC in the Chinese population.[33] We performed SNP genotyping of rs17401966 on the *KIF1B* gene in Japanese, Korean, and Hong Kong populations for the purpose of replication analysis of a previous GWAS report.[8] We first examined two independent Japanese HBV-related HCC populations and healthy controls, including 179 patients and 769 controls from Biobank Japan, and142 patients and 251 controls from various hospitals. We did not detect any associations between rs17401966 and HCC in the Japanese population. We also detected no association of the SNP with HBV-related HCC in Korean and Hong Kong populations using 164 patients and 144 controls, and 94 patients and 187 controls, respectively. In a recent report from another group, no significant association of the *KIF1B* gene was observed in HBV-related HCC patients of Saudi Arabian ethnicity.[34] These results may be explained by genetic diversity among the Chinese, Japanese, Korean, Hong Kong, and Saudi Arabian populations. The complexity of multivariate interactions in the pathogenesis of HCC may lead to difficulties in identifying the gene(s) associated with HBV-related HCC.

In a previous report that studied 179 Japanese individuals with chronic HBV infection (CHB) and 934 control participants, a GWAS identified significant associations of CHB with a region including *HLA-DPA1* and *HLA-DPB1*.[35] The same group was also conducted a second GWAS with a total of 2,667 Japanese patients with persistent HBV and 6,496 controls, which confirmed significant associations between

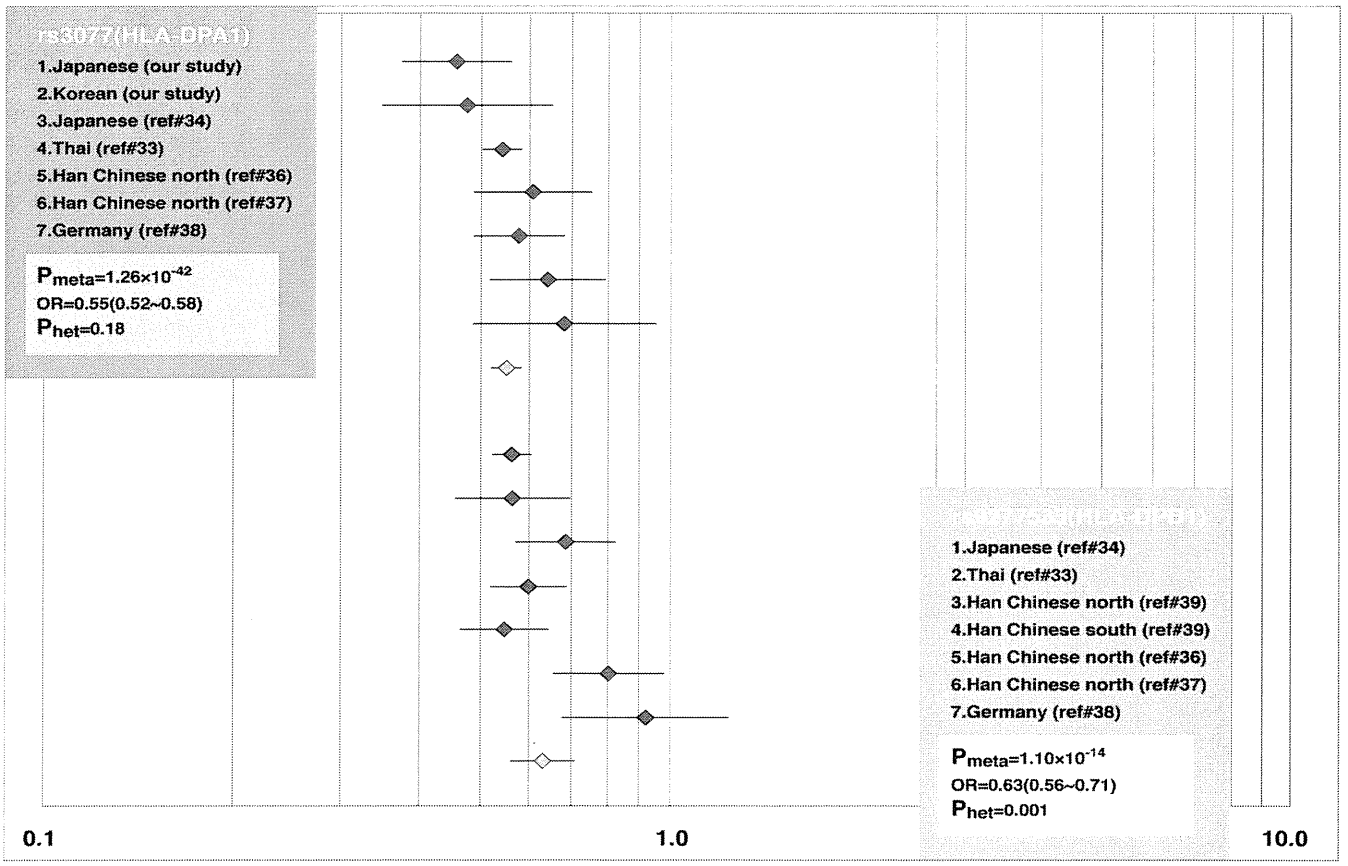

Fig. 3. Associations of *HLA-DP* with CHB and HBV clearance in Asian populations. Meta-analysis using the random-effects model across seven independent studies, including six additional published data sets, showed $Pmeta = 1.26 \times 10^{-42}$, odds ratio (OR) 0.55 for rs3077, and $Pmeta = 1.10 \times 10^{-14}$, OR 0.63 for rs9277535. Heterogeneity was tested using a general variance-based method.

the *HLA-DP* locus and CHB, in addition to associations with another two SNPs located in a genetic region including the *HLA-DQ* gene.[36] We performed a GWAS using samples from Japanese HBV carriers, healthy controls, and individuals who spontaneously resolved HBV infections (hepatitis B surface antigen [HBsAg] negative and hepatitis B core antibody [anti-HBc] positive), in order to confirm or identify the host genetic factors related to CHB and viral clearance.[37] In the subsequent replication analysis, we validated the associated SNPs in the GWAS using two independent sets of Japanese and Korean individuals. In our study, healthy controls with no clinical evidence of HBV exposure were randomly selected; therefore, HBV-resolved individuals were prepared to clearly identify the host genetic factors related to CHB or HBV clearance. An association analysis conducted with the Japanese and Korean data identified the *HLA-DPA1* and *HLA-DPB1* genes with $Pmeta = 1.89 \times 10^{-12}$ for rs3077 and $Pmeta = 9.69 \times 10^{-10}$ for rs9277542. We also found that the *HLA-DPA1* and *HLA-DPB1* genes were significantly associated with protective effects against CHB in Asian populations including Japanese, Korean, Chinese, and Thai individuals ($Pmeta = 1.26 \times 10^{-42}$ for rs3077 and $Pmeta = 1.10 \times 10^{-14}$ for rs9277535) (Fig. 3).[35–41] The SNP rs9277535 was located about 4 kb upstream from rs9277542 and showed strong linkage disequilibrium of r^2 0.955 in the HapMap JPT (Japanese in Tokyo, Japan) population. These results suggest that the associations between the *HLA-DP* locus and the protective effects against persistent HBV infection and with clearance of HBV are widely replicated in East Asian populations; however, there are few reports of GWASs in Caucasian or African populations. Further studies are necessary to clarify the pathogenesis of CHB and the mechanisms of HBV clearance, including functional analyses of the *HLA-DP* molecule.

The GWASs described above have successfully identified disease-associated genes or SNPs using different types of genome-wide SNP tying platforms. The embedded SNPs are varied among platforms by selecting the tagging SNPs and the suitable SNPs for their own genotyping strategy; however, the genome coverage among platforms revealed no differences over 60% between the HapMap CEU samples and the HapMap JPT+CHB samples.[42] Moreover, the genome coverage of the current version of the Affymetrix Genome-Wide Human SNP Array 6.0 platform has been estimated to reach 75% in the Japanese population.[14]

Conclusions

Together with technology developments, GWASs are a promising strategy with which to identify host genetic factors for multactorial diseases, including common liver diseases, and various host genetic traits. The GWAS strategy may allow researchers to identify unexpected significant associations. Recently, new strategies and emerging technologies for

massive parallel sequencing (also termed next-generation sequencing) have allowed whole-genome analysis to identify single-nucleotide variations and structural variations (including insertion, deletion, duplication, translocation, and transposition events). The costs of using these emerging technologies are currently high; therefore, common SNP-based GWASs using the genome-wide SNP analysis technologies introduced in this paper still have an important potential role in the fields of clinical and basic research.

Conflict of interest

None

Author contributions

Genotyping and statistical analyses for hepatitis studies (NN), acquisition of genotyping data on hepatitis researches (KT), manuscript writing (NN, KT), critical review (MM).

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Dynamics of serum metabolites in patients with chronic hepatitis C receiving pegylated interferon plus ribavirin: A metabolomics analysis

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ABSTRACT

Objectives. Serum samples from patients with chronic hepatitis C were subjected to metabolomics analysis to clarify the pretreatment characteristics of their metabolites and also changes in specific metabolites resulting from antiviral therapy with pegylated interferon plus ribavirin (PegIFN/RBV).

Materials/Methods. The serum levels of low-molecular-weight metabolites in the twenty patients before and 24 weeks after completion of PegIFN/RBV therapy were analyzed using capillary electrophoresis and liquid chromatography–mass spectrometry.

Results. Ten patients showed a non-virological response (NVR) and 10 achieved a sustained virological response (SVR) with eradication of viremia. The pretreatment levels of tryptophan were significantly higher in the patients of SVR than in those of NVR ($p = 0.010$). The area under the curve (AUC) value of tryptophan calculated from the receiver operating characteristic (ROC) curve for discriminating SVR from NVR was 0.84 (95% confidential interval, 0.66–1.02, $p = 0.010$). The ROC curve of multiple logistic regression model incorporating the pretreatment levels of tryptophan and γ-glutamate-arginine showed that the AUC value was highly significant (AUC = 0.92, 95% confidential interval, 0.79–1.05, $p = 0.002$). Twenty four weeks after completion of treatment, the levels of γ-glutamyl dipeptides, glutamic acid, 5-oxoproline, glucosamine and methionine sulfoxide were decreased, whereas those of 5-methoxy-3-indoleacetate, glutamine, kynurenine and lysine were increased significantly ($p < 0.05$) in both the NVR and SVR patients.

Conclusions. The pretreatment serum levels of certain metabolites including tryptophan are associated with the response to PegIFN/RBV therapy. PegIFN/RBV therapy can ameliorate the oxidative stress responsible for glutathione metabolism.

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; IFN, interferon; PegIFN/RBV, pegylated interferon plus ribavirin; SVR, sustained virological response; NVR, non-virological response; ALT, alanine aminotransferase; ROS, reactive oxygen species; CE-TOFMS, capillary electrophoresis–time-of-flight mass spectrometry; IL, interleukin; MRM, multiple reaction monitoring; PC, principal component; ROC, receiver operating characteristic; MLR, multiple logistic regression; AUC, area under the curve; IDO, indoleamine 2,3-dioxygenase.

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