Single-nucleotide polymorphisms in GALNT8 are associated with the response to interferon therapy for chronic hepatitis C

Rikita Nakano,1,2 Toshiro Maekawa,1 Hiromi Abe,3 Yasufumi Hayashida,1 Hidenori Ochi,1,3 Tatsuhiko Tsunoda,4 Hiromitsu Kumada,5 Naoyuki Kamatani,6 Yusuke Nakamura7 and Kazuaki Chayama1,3

Correspondence
Kazuaki Chayama
chayama@mba.ocn.ne.jp

1Laboratory for Digestive Diseases, RIKEN Center for Genomic Medicine, Hiroshima, Japan
2Pharmacology Research Laboratories, Drug Research Division, Dainippon Sumitomo Pharma Co., Osaka, Japan
3Department of Gastroenterology and Metabolism, Applied Life Sciences, Institute of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan
4Laboratory for Medical Informatics, RIKEN Center for Genomic Medicine, Yokohama, Japan
5Department of Hepatology, Toranomon Hospital, Tokyo, Japan
6Laboratory for International Alliance, RIKEN Center for Genomic Medicine, Yokohama, Japan
7Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan

New anti-hepatitis C virus (HCV) therapeutics developed recently are more effective and lead to improvements in sustained viral response. However, interferon (IFN) monotherapy is still used to a limited extent for fear of adverse effects. This study investigated host genetic factors affecting the IFN response in patients with chronic hepatitis C (CHC). Using a two-step design, a large-scale association screening including 1088 Japanese CHC patients treated with IFN was performed employing ~70 000 gene-based single-nucleotide polymorphisms (SNPs). Replication was tested in an independent Japanese cohort of 328 patients. Fine-mapping and functional analyses were also performed. Through two-step screening, it was found that rs2286580 in intron 6 of the gene encoding N-acetylgalactosaminyltransferase 8 (GALNT8) on chromosome 12 was significantly associated with a sustained viral response (combined $P=3.9\times 10^{-6}$, odds ratio 1.52, 95% confidence interval 1.29–1.82). The association was replicated in an additional cohort of 328 Japanese patients. In subgroup analysis, GALNT8 variants were associated with treatment outcome independently of HCV genotype. By contrast, the outcome of pegylated IFN and ribavirin combined therapy was not affected by the SNP. Fine-mapping analysis revealed that the association peak was at rs10849138 in intron 6 of GALNT8. Allele-specific transcription analysis demonstrated that GALNT8 expression was upregulated by an unfavourable allele of the variant. A luciferase reporter assay demonstrated that overexpression of GALNT8 attenuated IFN-a-induced gene transcription via the IFN-stimulated response element. These results suggest that GALNT8 variants contribute to the response to IFN therapy against CHC, providing a new insight into antiviral mechanisms of IFN.

INTRODUCTION

Type I interferons – alpha interferon (IFN-a) and beta interferon – have been widely used as antiviral agents for hepatitis C virus (HCV) infection. Although the improved efficacy of pegylated (PEG)-IFN-a plus ribavirin combined therapy is well recognized, it was shown that >50% of patients infected with HCV genotype 1b and ~20% of those with genotype non-1b still failed to eradicate the virus (NIH Consensus Statement on Management of Hepatitis C, 2002). Addition of telaprevir to therapy with PEG-IFN and ribavirin is a new strategy that improves the eradication rate of HCV genotype 1. However, telaprevir is
associated with increased rates of adverse effects including rash and anaemia (Hézode et al., 2009; McHutchison et al., 2009), which can be severe enough to necessitate discontinuation of treatment.

Both viral (e.g. HCV genotype and serum HCV RNA levels) and host factors (e.g. age, sex, race, liver fibrosis and obesity) have been shown to be associated with the outcome of IFN therapy. Several genetic factors including cytokine-, chemokine- and IFN-stimulated genes have been reported to influence the response to IFN therapy. We have reported that polymorphism in the gene encoding mitogen-activated protein kinase-activated protein kinase 3 (MAPKAPK3) is significantly associated with IFN therapy in patients infected with HCV genotype 1b (Tsukada et al., 2009). Recently, a positive association of a polymorphism in the interleukin IL28B gene with the outcome of PEG-IFN plus ribavirin therapy has been reported (Ge et al., 2009), and this association has been replicated by other investigators (Rauch et al., 2010; Suppiah et al., 2009; Tanaka et al., 2009; Thomas et al., 2009).

In this report, we have described the results of a large-scale case–control association analysis for responsiveness to IFN therapy in chronic hepatitis C (CHC) using gene-based single-nucleotide polymorphisms (SNPs). We identified common variants in the single-nucleotide polymorphisms (SNPs). We identified common variants in the N-acetylgalactosaminyltransferase 8 (GALNT8) gene that were significantly associated with treatment outcome. Moreover, we have provided functional evidence suggesting that GALNT8 may modulate signal transduction of IFN-α-mediated antiviral activity.

RESULTS

Association analysis

In the first screening using 65 sustained responders (SRs) and 118 non-responders (NRs), the success rate of genotyping was 90.3% (72 739 SNPs). After quality-control filtering, 58 809 autosomal SNPs remained. The distribution of association P values (Manhattan plot) of the first screening is shown in Fig. S1 (available in JGV Online), and the quantile–quantile plots in Fig. S2. To identify and correct for possible population stratification, the genomic control method was used (Lewis, 2002). The calculated genomic control λ was 1.04, indicating that the effect of population stratification was minimal (Devlin & Roeder, 1999). The top 100 SNPs are listed in Table S1. The quantile–quantile plot indicated a number of SNPs showing a stronger association than would be expected by chance but that were not statistically significant. We selected 689 SNPs having P<0.01 under the allelic model for the second screening, consisting of 417 SRs and 488 NRs. After the two-step screening, we found that SNP rs2286580 [combined P=3.9 × 10^{-6}; odds ratio (OR) 1.52; 95% confidence interval (CI) 1.29–1.82] located in intron 6 of GALNT8 and rs2267552 in intron 9 of NADH dehydrogenase (ubiquinone) 1α subcomplex, subunit 9 (NDUFA9) (combined P=7.8 × 10^{-6}; OR 1.49; 95% CI 1.32–1.78) exceeded the significance level of P<1.36 × 10^{-5} using the joint analysis (Skol et al., 2006) (Table 1). They were both on chromosome 12 and were in strong linkage disequilibrium (LD) with each other (r^2=0.81) (Fig. 1). This association was validated in an independent replication cohort consisting of 94 SRs and 234 NRs (P=3.3 × 10^{-3}) (Table 1). The remaining SNPs carried to the second screening eventually showed a lack of association.

Fine mapping

According to the genotype data of Japanese individuals from the Phase II HapMap database, rs2286580 and rs2267552 are located within the same LD block (defined by Gabriel et al., 2002) on chromosome 12p13, spanning about 104 kb (Fig. 1, lower panel). Fourteen tag SNPs were selected from 127 SNPs within the LD block surrounding rs2286580 using HapMap JPT data (minor allele frequency >0.05, r^2>0.8; de Bakker et al., 2005) and were genotyped in all subjects enrolled in the study. An additional 25 SNPs, which were in the same bin as rs2286580 (r^2>0.8), were also genotyped and analysed because rs2286580 had the lowest P value among these tag SNPs. Finally, the most significant association (P=2.2 × 10^{-6}) was observed at rs10849138 in intron 6 of the GALNT8 gene (Fig. 1, upper panel, and Table 2). We also examined associations with respect to haplotype. None of the haplotypes showed stronger associations than the single-marker association of rs10849138 (data not shown).

HCV genotype-stratified analysis

Because each sample set except for the first set of the screening stage contained various HCV genotypes, subjects were stratified according to HCV genotype to evaluate whether GALNT8 variants were associated with treatment outcome independently of HCV genotypes. We found that rs10849138 (C/G) was significantly associated within subjects chronically infected with HCV genotypes 1b and 2a (OR=1.51, P=2.3 × 10^{-4}, and OR=1.71 P=5.0 × 10^{-4}, respectively), whereas subjects chronically infected with HCV genotype 2b showed the same tendency but this was not significant (OR=1.60, P=0.064). No heterogeneity was observed in the ORs among HCV genotypes for the SNP by the Breslow–Day test. The estimated combined OR by the Mantel–Haenszel method was 1.58 (95% CI 1.33–1.86) (Table 3). We further genotyped rs3792323 of MAPKAPK3, which we have reported previously in subjects treated with IFN monotherapy (Tsukada et al., 2009) (Table S2). The impacts of GALNT8 and MAPKAPK3 were comparable in genotype 1b, whereas in non-1b genotypes, GALNT8 seemed to have a stronger effect on treatment than MAPKAPK3. In contrast, IL28B showed the most significant association in genotype 1b.
Multivariate logistic regression analysis

Multivariate logistic regression analysis was used to adjust the effects of various known factors on IFN treatment outcome. The association of rs10849138 remained significant after adjusting for sex, viral load before treatment, HCV genotype and IL28B variant (OR=1.67, 95% CI 1.22–2.28, \( P=1.3\times10^{-3} \)). Thus, the GALNT8 variant was found to be an independent predictor of IFN treatment outcome for CHC subjects, irrespective of the HCV genotype (Table 4).

Table 1. Summary of screening and replication studies

<table>
<thead>
<tr>
<th>rs2286580</th>
<th>SRs</th>
<th>NRs</th>
<th>Allele G (%)</th>
<th>OR (95% CI)</th>
<th>( P )</th>
<th>( P_{net} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC</td>
<td>CG</td>
<td>GG</td>
<td>ND</td>
<td>CC</td>
<td>CG</td>
</tr>
<tr>
<td>Screening 1</td>
<td>16</td>
<td>36</td>
<td>13</td>
<td>0</td>
<td>60</td>
<td>46</td>
</tr>
<tr>
<td>Screening 2</td>
<td>154</td>
<td>204</td>
<td>59</td>
<td>0</td>
<td>232</td>
<td>210</td>
</tr>
<tr>
<td>Replication</td>
<td>26</td>
<td>52</td>
<td>16</td>
<td>0</td>
<td>107</td>
<td>98</td>
</tr>
<tr>
<td>Screening stage combined</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All stages combined</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Under the allelic model.
†Mantel–Haenszel test.
‡Breslow–Day heterogeneity test.

Fig. 1. LD mapping and probabilities for SNPs around the GALNT8 locus. The lower panel depicts the haplotype structure from Phase II HapMap JPT genotype data. Dark grey indicates regions with high \( r^2 \) values and light grey indicates regions with low \( r^2 \) values. The upper panel shows \( P \) value plots of case–control association results. \( P \) values by \( \chi^2 \) test under the allelic model are plotted on a log_{10} scale. ○ and ● represent SNPs in the first screening study (screening 1) and in the fine-mapping analysis using all subjects (screening 1, screening 2 and replication), respectively.
To evaluate whether the GALNT8 variant also affected the viral response to PEG-IFN plus ribavirin combination therapy, another cohort of 526 SRs and 222 NR subjects who were treated with PEG-IFN plus ribavirin was examined. However, among these patients there was no significant association between the rs10849138 genotype and treatment outcome in multivariate analysis (Table S3).

**Expression of GALNT8 in normal human tissues**

Semi-quantitative RT-PCR analysis revealed that GALNT8 was expressed ubiquitously in the tissues examined (Fig. S3).

GALNT8 expression was approximately 1000-fold lower than β-actin expression (Fig. S3).

**Allele-specific gene transcript quantification analysis**

As rs10849138 is located within an intron, we performed allele-specific gene transcription quantification using a TaqMan genotyping probe for rs1468556 (G/T, exon 8) and PBMCs from four healthy human volunteers who were heterozygous for both SNPs, rs1468556 and rs10849138 (C/G, intron 6). These two SNPs were in almost complete LD,
with a D' value of >0.99. Thus, these subjects were theoretically expected to have two haplotypes consisting of an unfavourable haplotype 1 (rs10849138 G/rs1468556 G) and a favourable haplotype 2 (rs10849138 C/rs1468556 T). As shown in Fig. 2, expression of the IFN ineffective haplotype 1 was approximately 1.5-fold higher than that of the IFN-effective haplotype 2. These results suggested that GALNT8 variants affect transcriptional regulation in an allele-specific manner.

Luciferase reporter assay

To evaluate the functional importance of GALNT8 in the IFN signalling pathway, we overexpressed GALNT8 (GenBank accession no. NM_017417.1) in Huh7 cells and evaluated the effect of GALNT8 on the IFN-stimulated response element (ISRE), which is an essential promoter element regulating type I IFN-induced antiviral activity. Overexpression of GALNT8 significantly attenuated IFN-α-induced luciferase activity (Fig. 3). These results suggested that GALNT8 can repress IFN-α-induced gene transcription via the ISRE.

DISCUSSION

Through a large-scale association analysis using gene-based SNPs followed by LD mapping, we identified GALNT8 variants as being associated with the outcome of IFN therapy for Japanese CHC patients. To the best of our knowledge, this study is the first to show an association of GALNT8 polymorphisms with IFN response. GALNT8 is a member of the O-linked UDP-N-acetylgalactosamine (GalNAc) glycosyltransferase (ppGalNTase) family, which transfers GalNAc to serine and threonine residues on target proteins in the Golgi apparatus and thus participates in the biosynthesis of mucin-like O-glycan. To date, a total of 20 human GALNT isoforms have been identified, namely GALNT1–14 and GALNTL1–6 (Tarp & Clausen, 2008; Ten Hagen et al., 2003). These isoforms have shown different tissue distributions and substrate specificities (Kingsley et al., 2000; Tian & Ten Hagen, 2006). GALNT8 encodes a type II membrane protein of 637 aa that is widely expressed in human tissues and is 45–60% identical to the other mammalian ppGaNTase (http://www.proteinatlas.org/; White et al., 2000). According to

Table 3. Stratified analysis of GALNT8 polymorphism according to HCV genotype in patients treated with IFN monotherapy

<table>
<thead>
<tr>
<th>rs10849138 (C/G)</th>
<th>HCV 1b</th>
<th>HCV 2a</th>
<th>HCV 2b</th>
<th>Combined†</th>
<th>OR (95% CI)*</th>
<th>P*</th>
<th>Phet‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SRs</td>
<td>NRs</td>
<td>SRs</td>
<td>NRs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>CG</td>
<td>GG</td>
<td>CC</td>
<td>CG</td>
<td>GG</td>
<td>CC</td>
<td>CG</td>
</tr>
<tr>
<td>38</td>
<td>120</td>
<td>84</td>
<td>63</td>
<td>250</td>
<td>291</td>
<td>1.51 (1.21–1.87)</td>
<td>2.30 × 10⁻⁴</td>
</tr>
<tr>
<td>42</td>
<td>140</td>
<td>90</td>
<td>10</td>
<td>66</td>
<td>72</td>
<td>1.71 (1.26–2.32)</td>
<td>5.00 × 10⁻⁴</td>
</tr>
<tr>
<td>9</td>
<td>32</td>
<td>15</td>
<td>9</td>
<td>35</td>
<td>35</td>
<td>1.60 (0.97–2.63)</td>
<td>6.40 × 10⁻²</td>
</tr>
</tbody>
</table>

*Under the allelic model.
‡Breslow–Day test.
‡Mantel–Haenszel test.

Table 4. Predictors for SRs in IFN monotherapy

<table>
<thead>
<tr>
<th>Variable</th>
<th>Simple</th>
<th>Multiple (n=1065)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>1416</td>
<td>1.30 (1.04–1.62)</td>
</tr>
<tr>
<td>Age</td>
<td>1409</td>
<td>0.97 (0.88–1.07)*</td>
</tr>
<tr>
<td>BMI</td>
<td>772</td>
<td>0.97 (0.93–1.02)</td>
</tr>
<tr>
<td>Fibrosis stage (F0–F2/F3–F4)</td>
<td>1144</td>
<td>1.29 (0.92–1.81)</td>
</tr>
<tr>
<td>Log viral load</td>
<td>1072</td>
<td>7.46 (5.68–9.79)</td>
</tr>
<tr>
<td>HCV (non-1b/1b)</td>
<td>1416</td>
<td>3.61 (2.89–4.50)</td>
</tr>
<tr>
<td>rs8099917 (G/T)‡</td>
<td>1413</td>
<td>0.44 (0.33–0.57)</td>
</tr>
<tr>
<td>rs10849138 (C/G)‡</td>
<td>1407</td>
<td>1.58 (1.34–1.85)</td>
</tr>
<tr>
<td>rs3792323 (A/T)‡</td>
<td>1416</td>
<td>0.91 (0.78–1.06)</td>
</tr>
</tbody>
</table>

*Per 10-year increase.
‡Each genotype is coded as (0, 1, 2) assuming additive effects.
our semi-quantitative analysis, GALNT8 was expressed ubiquitously but at a very low level (Fig. S3), yet little is known about the function or pathophysiological significance of GALNT8.

In the present study, we found an association peak at rs10849138 in intron 6 of GALNT8 (P=2.2×10⁻⁵; Table 2) accompanying SNPs with P values within one order of magnitude. Among these, the top ten SNPs were located in introns or upstream of the gene. We hypothesized that these SNPs might affect GALNT8 expression. The results of allele-specific gene transcript quantification suggested that the unfavourable G allele of rs10849138 enhanced GALNT8 expression compared with the favourable C allele (Fig. 2).

Correspondingly, using a reporter gene assay, we showed that overexpression of GALNT8 attenuated IFN-α-induced luciferase activity in Huh7 cells. Further studies are desirable using liver specimens from patients to assess whether expression levels of GALNT8 are actually different between genotypes.

Two non-synonymous SNPs, rs10849133 (D53Y) and rs1468556 (F515V), were in strong LD with the top SNP, rs10849138 (r²=0.94 and 0.97, respectively; Table 2). These SNPs are located in the stem region (rs10849133) and the lectin domain (rs1468556) of GALNT8. Their P values did not exceed the genome-wide level of significance. Nevertheless, to exclude the contribution of these SNPs, further functional studies are desirable to examine whether these amino acid substitutions could affect protein function, including catalytic activity, localization, protein stability and substrate specificity.

Many gene polymorphisms have been reported to be associated with innate immunity and/or spontaneous viral clearance. IL28B variants were initially identified to be responsible for treatment success in hepatitis C patients receiving PEG-IFN and ribavirin therapy (Ge et al., 2009). Soon after, the IL28B variants were also found to be associated with spontaneous viral clearance (Thomas et al., 2009). In addition, there were reported to be several gene polymorphisms such as CCR5 in HCV infection (Goulding et al., 2005) and HLA-DP in HBV infection (Kamataki et al., 2009). It is desirable to clarify whether GALNT8 variants are involved in the innate immunity response...
against HCV by using spontaneous resolvers. The current standard of care is PEG-IFN plus ribavirin therapy, and directly acting antiviral therapies are becoming available. However, a non-negligible proportion of patients still undertake IFN monotherapy in Japan because both PEG-IFN plus ribavirin and directly acting antivirals such as telaprevir often cause severe side effects. Thus, the observations in this study may hold important implications for our understanding of antiviral mechanisms of IFN, as well as for the treatment of HCV patients.

In conclusion, through large-scale association analysis using gene-based SNPs and fine-mapping analysis, we identified GALNT8 variants as susceptible loci for IFN efficacy in Japanese CHC patients. Functional analyses suggested that GALNT8 attenuated IFN-α-dependent transcriptional activity via ISRE elements and that the difference in allele-specific expression levels of GALNT8 may in part be responsible for the association between GALNT8 variants and the IFN response. Our findings also indicated that GALNT8 variants may be useful markers for predicting the efficacy of IFN treatment outcome for CHC subjects independently of HCV genotypes. Further studies of GALNT8 function would be helpful to understand the modulation of antiviral activity of IFN.

**METHODS**

**Subjects and study design.** We enrolled a total of 1416 Japanese patients with CHC who had been treated with IFN monotherapy at the Department of Hepatology, Toranomon Hospital, Hiroshima University Hospital, and Hiroshima University affiliated hospitals prior to 2003. All subjects had had abnormal levels of serum alanine transaminase for more than 6 months and were positive for both anti-HCV antibody and serum HCV RNA. All subjects were negative for hepatitis B surface antigen, had no evidence of other liver diseases and had not received immunosuppressive therapy before enrolment in the study. Subjects were treated with 6 × 10^8 U IFN intramuscularly every day for 8 weeks, followed by the same dose twice a week for 16 weeks, with a total dose of 5.28 × 10^9 U for IFN monotherapy. Patients were classified into the following two categories: SRs and NRs. SRs had normal alanine transaminase levels and no evidence of viraemia at 6 months after completion of IFN therapy, whereas NRs remained viraemic 6 months after completion of IFN therapy. Relapsed responders were excluded from our study. HCV RNA levels were determined by an Amplicor Monitor assay or branched-chain DNA assay (Tsukada et al., 2009). The HCV RNA level was stratified into two categories based on cut-off values, as described previously ([100 KIU ml⁻¹ by Amplicor Monitor assay and 1.0 mEq ml⁻¹ by branched-chain DNA assay; Tsukada et al., 2009]). Using biopsy specimens of liver tissue, histological staging was determined according to previously described criteria (Desmet et al., 1994).

Most of the patients studied in our previous association analysis (Tsukada et al., 2009) were included in this study. We divided the patients for IFN monotherapy into three groups, two-step screening sets and a replication set based on the time of entry into the study, except that the first group consisted of HCV genotype 1b patients only. The characteristics of patients are described in Table 5. All subjects in the present study gave written informed consent to participate in the study according to a process approved by the Ethical Committee at the SNP Research Center, Institute of Physical and Chemical Research (RIKEN), Yokohama, Japan. Genomic DNA samples were obtained from the peripheral blood of the participating patients. DNA extraction was performed using a standard phenol/chloroform protocol (Ozaki et al., 2002).

**Case–control association study.** In the screening stage, we applied a two-stage approach in which 80,592 randomly selected gene-based SNPs from the JSNP database (http://snp.ims.u-tokyo.ac.jp/japa; Haga et al., 2002) were genotyped using the first screening set (65 SRs and 118 NRs) (genotype 1b). SNPs with low genotyping success rates (<90%) were excluded from the analysis. In addition, SNPs with a low minor allele frequency (<0.01) and showing deviation from Hardy–Weinberg equilibrium (P<0.01) were also excluded. Autosomal SNPs that passed quality-control filters and had **P** values of <0.01 under the allelic model were selected and genotyped in the second screening. In the second screening, we genotyped additional samples consisting of 417 SRs and 488 NRs. The overall significance level was estimated by joint analysis (Skol et al., 2006). The association was validated in a replication cohort consisting of 94 SRs and 234 NRs.

**SNP genotyping.** The first set of the screening stage was genotyped using the high-throughput multiplex PCR-Invrier assay method (Third Wave Technologies) (Haga et al., 2002; Ohnishi et al., 2001). In the analysis of the second stage of the screening and during the replication stage, we used the multiplex PCR-based Invader assay or the TaqMan genotyping system (Applied Biosystems), as described previously (Ohnishi et al., 2001; Suzuki et al., 2003).

**Expression in normal human tissues.** Total RNA from adult human tissues (Clontech) was used to analyse mRNA expression of GALNT8 and β-actin as an internal control using a SuperScript III One-Step RT-PCR with Platinum Taq kit (Invitrogen). RT-PCR was performed according to the manufacturer’s instructions. Each reaction contained 1 μg total RNA and primers as follows: GALNT8 (GenBank accession no. NM_017417: 5'–GCTTTCTTCAGAATTGCACT-3' and 5'–CAATCAGTTGTCCCACATAGACC-3') and β-actin (GenBank accession no. NM_005739: 5'–GTTGGGCCGCCTGAAGCAACACAGC-3' and 5'–CTCTTTGATGTCACGCATTTGTC-3'). The cycling parameters were set as 94 °C for 15 s, 55 °C for 30 s and 68 °C for 30 s, with 40 cycles for GALNT8 and 30 cycles for β-actin. Aliquots (10 μl) of the 50 μl RT-PCR samples were analysed on 1.5% agarose gel.

**Cells and cell culture.** Human hepatoma cell line HuH7 cells were purchased from the RIKEN Cell Bank (Tsukuba, Japan). HuH7 cells were cultured in Dulbecco’s modified essential medium (Sigma-Aldrich) with 10% FBS, 100 U penicillin ml⁻¹ and 100 ng streptomycin ml⁻¹ at 37 °C under 5% CO₂.

**Allele-specific transcript quantification.** Allele-specific transcript quantification was performed as described previously (Suzuki et al., 2008) with some modifications. PBMCs were collected from four normal Japanese volunteers. Total RNA was isolated using an RNeasy Plus Micro kit (Qiagen), and cDNA was synthesized with a WT-Ovation Pico RNA Amplification System (Nugen Technologies). The allelic ratio for each genomic DNA and cDNA from each individual was determined by real-time TaqMan PCR based on the standard lines plotted by using mixtures of homozygous DNA at six different ratios (2:1, 3:2, 6:5, 5:6, 2:3 and 1:2). Each sample was assayed in triplicate. Data were confirmed by two independent experiments.

**Luciferase reporter assay.** We performed a luciferase reporter assay, as described previously (Tsukada et al., 2009). In brief, 5 × 10⁵ cells in each well of a 96-well plate in Dulbecco’s modified Eagle’s medium (10% FBS without antibiotics) were co-transfected with Fugene 6 complexes (1 ng pRL-TK vector (Promega), 10 ng...
pISRE-TA-Luc Vector (Clontech), 50 ng expression vector pDEST51/mock, pDEST51/GALNT8 (GenBank accession no. NM_017417.1) or pDEST51/suppressor of cytokine signalling 1 (SOCS1) and 0.15 ml Fugene 6 (Roche Applied Science) in serum-free medium (Opti-MEM; Invitrogen) using a reverse transfection method according to the manufacturer’s instructions. At 48 h after transfection, cells were stimulated with 10 IU IFN-α (Dainippon Sumitomo Pharma) ml⁻¹ for 24 h. Cells were washed once with 100 ml PBS. Luciferase activity was determined using a Dual-luciferase Reporter Assay System (Promega) with a Centro LB 960 Luminometer (Berthold Technologies, as described in the manufacturer’s protocol. Firefly luciferase activity was normalized with Renilla luciferase activity. Analyses were carried out in triplicate, and the results were confirmed by two independent experiments.

Statistical analysis. In each stage, a χ² test was used to compare genotype frequencies at each SNP. Deviations from Hardy–Weinberg equilibrium were tested by using a χ² test. Other data were analysed using Student’s t-test or a χ² test as appropriate. Definition of LD block, haplotype frequency, calculation of pairwise LD indices r² and D’ and identification of tag SNPs were performed using Haploview 4.2 software, as described previously (Barrett et al., 2005; de Bakker et al., 2005; Wigginton et al., 2005). Simple and multiple logistic regression analyses with stepwise forward selection were performed using StatFlex V5.0 (Artech Co.) with a significance level of P<0.05 as the criterion for variable inclusion.

ACKNOWLEDGEMENTS

The authors thank the patients who agreed to participate in this study. We also thank the team members at Toranomon Hospital, Hiroshima University Hospital, and Hiroshima Liver Study Group for clinical sample collection. We thank T. Yokogi, Y. Nakagawa, H. Ishino and K. Izumoto for technical assistance; J. Sakamiya for clerical assistance; and other members of the RIKEN Center for Genomic Medicine and Hiroshima University for assistance with various aspects of this study. This study was partly supported by a grant from the Leading Project of Ministry of Education, Culture, Sports, Science and Technology Japan and by Dainippon Sumitomo Pharma Co. Ltd.

REFERENCES


