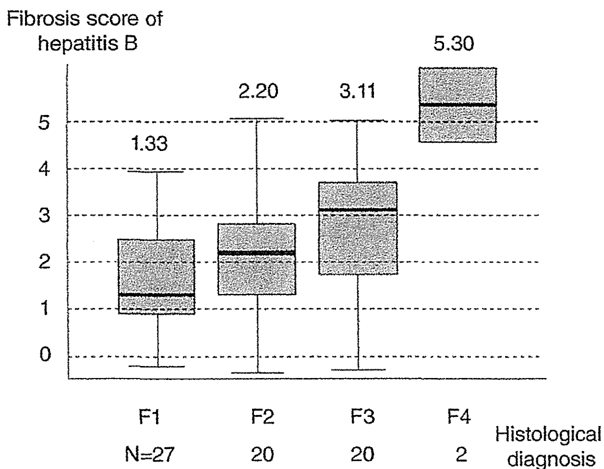

Figure 3 Box and whisker plots of fibrotic score of each group of histological fibrosis in the validation dataset. The fibrosis score of hepatitis B was generated by the function, $z = 1.40 \times ln$ (type IV collagen 7S) (ng/mL) $- 0.017 \times$ (platelet count) ($\times 1000^3/mm^3$) $+ 1.24 \times ln$ (tissue inhibitor of matrix metalloproteinase-2) (ng/mL) $+ 1.19 \times ln$ (α-2-macroglobulin) (mg/dL) $- 9.15$.

As many as 227 patients with chronic hepatitis B were analyzed in this study, who had been diagnosed as having chronic hepatitis or cirrhosis by liver biopsy performed in experienced liver units in Japan. To obtain the most suitable equation approximating histological fibrotic stage, multivariate analysis was performed using two demographic parameters (age and sex) and 21 hematological and biochemical markers with or without logarithmic transformation. They included many kinds of fibrosis markers: α-2-macroglobulin, haptoglobin concentration, haptoglobin typing, apolipoprotein A1, hyaluronic acid, TIMP-1, TIMP-2, procollagen III peptide and type IV collagen 7S Multiple regression analysis finally generated a first-degree polynomial function consisting of four variables: type IV collagen 7S, platelet count, TIMP-2 and α2-macroglobulin. A constant numeral (−9.15) was finally adjusted in the regression equation in order to obtain fitted figures for a fibrotic stage of F1–F4. From the magnitude of the standardized partial regression coefficient of individual variable in the function, platelet count demonstrated the most potent contribution toward the prediction of liver fibrosis. Type IV collagen 7S and *ln* (TIMP-2) proved to be the second and third distinctive power in the model, respectively.

The FSB was sufficiently fitted to actual fibrotic stages with certain overlapping as is usually found in histological ambiguity judged by pathologists. Because judgment of fibrosis in chronic hepatitis often shows a transitional histological staging, pathological examination cannot always make a clear-cut diagnosis discriminating F1–F4. Considering the limitation of the pathological difficulty in differentiating the four continuous disease entities, the obtained regression function showed satisfactory high accuracy rates in the prediction of liver disease severity. The FSB can provide one or two decimal places (e.g. 3.2 or 3.24) and the utility of the score is possibly higher than the mere histological stage of F1–F4. The reproducibility was confirmed by the remaining 67 patients' data obtained from the other six hospitals. Although the validation data were collected from a different geographic area and different chronological situation, the FSB showed similar results in prediction of histological staging.

The FSB seemed a very useful quantitative marker in evaluating fibrotic severity of hepatitis B patients without invasive procedures and without any specialized ultrasonography or magnetic resonance imaging. The FSB also has an advantage of measurement, in which old blood samples are available for retrospective assessment of varied clinical settings: for example, old sera from 20 years prior to the time of initial liver biopsy, or paired sera before and after long-term antiviral therapy. These kinds of retrospective assessments of fibrotic staging will be valuable in estimating a long-term progression of liver disease, in evaluating efficacy of long-term medication or other medical intervention, or in making a political judgment from the viewpoints of socioeconomic efficacy.

The score can be calculated for any patients with chronic HBV infection. Although this multiple regression model dealt with appropriate logarithmic transformation for non-normal distribution parameters, the regression analysis was based on a linear regression model. Very slight fibrosis can be calculated as less than 1.00, which is commonly found to a slight degree in chronic hepatitis with tiny fibrotic change as F0. Very severe fibrosis might be calculated as more than 4.00, which is an imaginary and nonsense number in the scoring system of fibrosis. The FSB is, however, very useful and valuable in a real clinical setting: estimation of severity of liver fibrosis in an outpatient clinic, evaluation of the natural progression of a patient's fibrosis over 10 years and assessment of a long-term administration of interferon in patients with chronic hepatitis B from the viewpoint of fibrotic change. Recent development of new nucleoside/nucleotide analogs requires evaluation for long-term histological advantage, for aggravation of hepatitis stage during viral and biochemical breakthrough caused by HBV mutation, and even for

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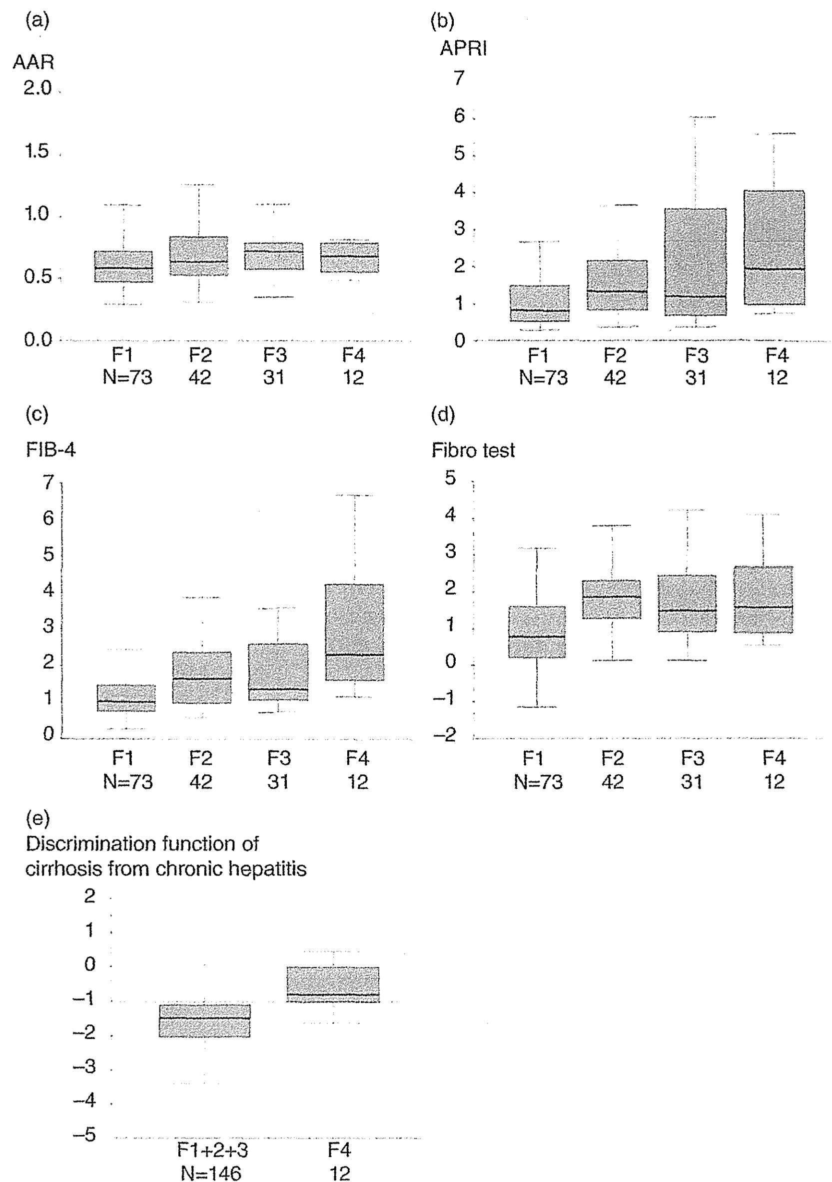

Figure 4 Previously published fibrosis scores. (a) Aspartate aminotransferase/alanine aminotransferase ratio (AAR),[19] (b) aspartate aminotransferase-to-platelet ratio index (APRI),[20] (c) FIB-4,[21] (d) FibroTest[22] and (e) discrimination function of cirrhosis from hepatitis in Japanese patients.[23]

the best management of patients with chronic hepatitis B. The FSB seems one of the ideal methods of approximating the fibrotic stage of chronic hepatitis B. Repeated measurement is quite suitable for patients with an unestablished treatment or trial, every 1 or 2 years, for example. Because the current regression function was generated from the data of HBV-related chronic liver disease, this equation would not be suitable for the recognition of hepatitis C virus-related chronic liver disease, alcoholic liver disease, and other congenital or autoimmune liver diseases. To recognize the latter diseases, other studies of individual diseases must be performed.

We compared the usefulness of the FSB with that of other fibrosis scores.[19–23] The more simple and less expensive AAR or APRI could not estimate fibrotic stages with poor correlation coefficients of 0.199 and 0.265, which are much lower than the coefficient of the FSB of 0.625. FibroTest, which contained three costly fibrosis markers (α-2-macroglobulin, haptoglobin and apolipo-

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protein A1), also showed a low correlation coefficient of 0.330, suggesting that its usefulness was limited in HBV positive oriental patients. Although FIB-4 demonstrated the best coefficient of 0.412 among the fibrosis scores, significant overlaps were found between neighboring stages and obtained scores were not coordinated for real histological classification.

In conclusion, the FSB was a useful and reliable biomarker for prediction of liver fibrosis in patients with chronic HBV infection. The FSB is expected to be introduced and utilized in varied kinds of studies and trials. Its accuracy and reproducibility require further validation using higher numbers of patients in several countries other than Japan.

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

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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 $P=3.9\times10^{-6}$, 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-α-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.

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INTRODUCTION

Type I interferons – alpha interferon (IFN-α) and beta interferon – have been widely used as antiviral agents for hepatitis C virus (HCV) infection. Although the improved efficacy of pegylated (PEG)-IFN-α 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

Three supplementary figures and three tables are available with the online version of this paper.

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 *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 $P=3.9\times10^{-6}$; 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 $P=7.8\times10^{-6}$; OR 1.49; 95 % CI 1.32–1.78) exceeded the significance level of $P<1.36\times10^{-5}$ 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 ($P=3.3\times10^{-3}$) (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 ($P=2.2\times10^{-8}$) 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, $P=2.3\times10^{-4}$, and OR=1.71 $P=5.0\times10^{-4}$, 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.

Table 1. Summary of screening and replication studies

ND, Not determined.

rs2286580	SRs				NRs				Allele G (%)		OR (95 % CI)	*P*	P_{het}
	CC	CG	GG	ND	CC	CG	GG	ND	SRs	NRs			
Screening 1	16	36	13	0	60	46	10	2	47.7	28.4	2.29 (1.47–3.59)	2.3×10^{-4}*	
Screening 2	154	204	59	0	232	210	45	1	38.6	30.8	1.41 (1.16–1.72)	4.9×10^{-4}*	
Replication	26	52	16	0	107	98	26	3	44.7	32.5	1.68 (1.19–2.38)	3.3×10^{-3}*	
Screening stage combined											1.52 (1.29–1.82)	3.9×10^{-6}†	0.051‡
All stages combined											1.56 (1.32–1.82)	5.3×10^{-8}†	0.13‡

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

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, $P=1.3 \times 10^{-3}$). 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).

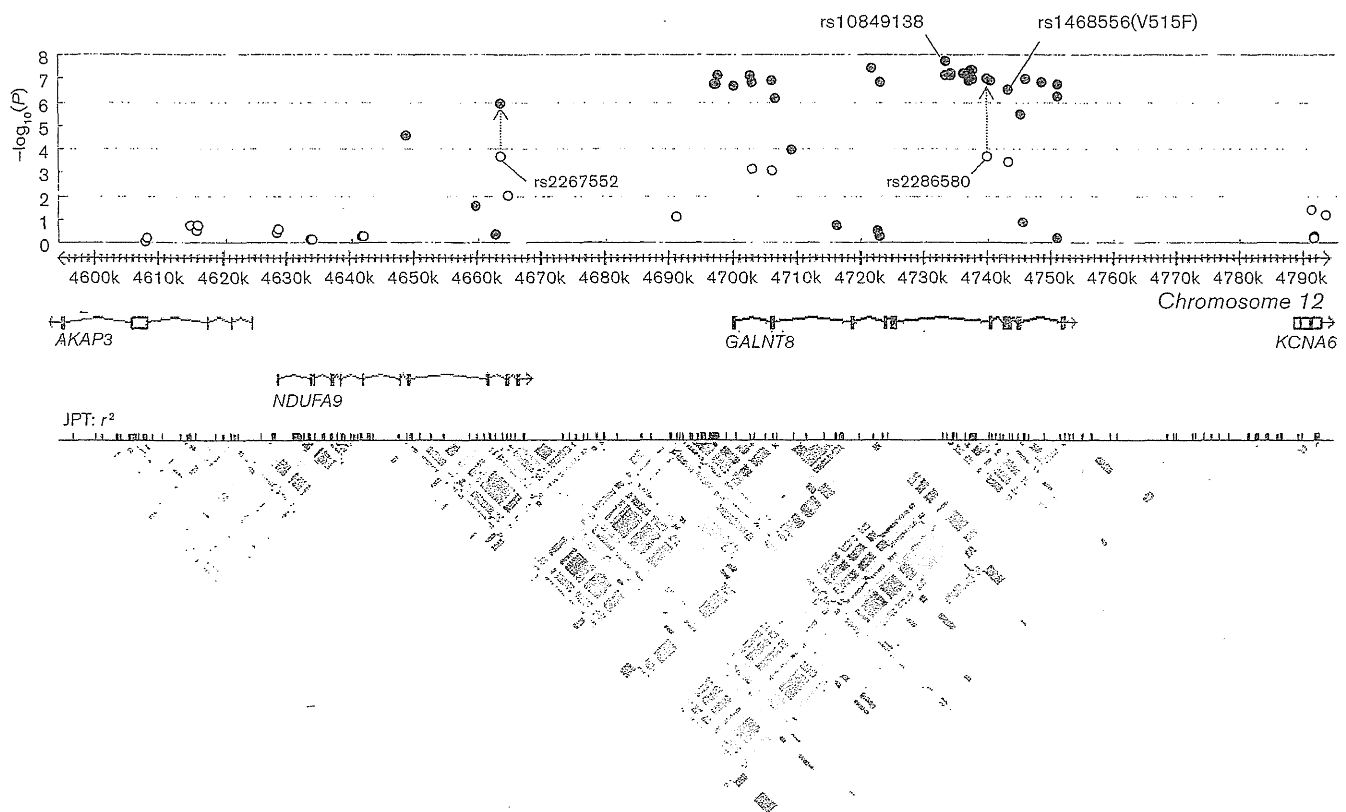

Fig. 1. LD mapping and probabilities for SNPs around the *GALNT8* locus. The lower panel depicts the haplotype structure from Phase II HapMap JPT genotype data. Dark grey indicates regions with high r^2 values and light grey indicates regions with low r^2 values. The upper panel shows *P* value plots of case–control association results. *P* values by χ^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.

Table 2. Summary of fine-mapping studies

SNP	Gene	Structure	aa	Chromosome position*	P value†	r^2‡	D'‡
rs7312558	*NDUFA9*	Intron 7		4648724	3.1×10^{-5}	0.53	0.92
rs12230096	*NDUFA9*	Intron 8		4659656	3.0×10^{-2}	0.05	1.00
rs10849118	*NDUFA9*	Intron 9		4662753	4.7×10^{-1}	0.05	0.86
rs2267552	*NDUFA9*	Intron 9		4663725	1.3×10^{-6}	0.80	0.93
rs10849130				4697179	1.9×10^{-7}	0.94	0.99
rs10849131				4697289	1.8×10^{-7}	0.94	0.99
rs12296205				4697676	8.0×10^{-8}	0.94	0.99
rs10849133	*GALNT8*	Exon 1	D53Y	4700261	2.3×10^{-7}	0.94	0.99
rs3782740	*GALNT8*	Intron 1		4702637	8.4×10^{-8}	0.94	1.00
rs3782741	*GALNT8*	Intron 1		4703041	1.7×10^{-7}	0.95	1.00
rs2286578	*GALNT8*	Exon 2	L132L	4706143	1.3×10^{-7}	0.94	1.00
rs9300304	*GALNT8*	Intron 2		4706599	7.8×10^{-7}	0.94	0.99
rs11063319	*GALNT8*	Intron 2		4709286	1.2×10^{-4}	0.44	0.97
rs11063324	*GALNT8*	Intron 2		4716302	2.3×10^{-1}	0.12	1.00
rs3782742	*GALNT8*	Intron 3		4721816	4.0×10^{-8}	0.98	1.00
rs17176851	*GALNT8*	Intron 3		4722773	3.6×10^{-1}	0.10	1.00
rs17783475	*GALNT8*	Intron 3		4723009	5.9×10^{-1}	0.04	1.00
rs4766286	*GALNT8*	Intron 3		4723276	1.7×10^{-7}	0.93	1.00
rs10849138	*GALNT8*	Intron 6		4733569	2.2×10^{-8}	–	–
rs10849139	*GALNT8*	Intron 6		4733618	7.9×10^{-8}	0.99	1.00
rs7311675	*GALNT8*	Intron 6		4734338	7.5×10^{-8}	1.00	1.00
rs7311694	*GALNT8*	Intron 6		4734378	6.2×10^{-8}	1.00	1.00
rs7315211	*GALNT8*	Intron 6		4734515	7.4×10^{-8}	1.00	1.00
rs7975978	*GALNT8*	Intron 6		4736415	6.3×10^{-8}	0.97	0.99
rs2359466	*GALNT8*	Intron 6		4737099	8.2×10^{-8}	0.97	0.99
rs2359467	*GALNT8*	Intron 6		4737402	1.3×10^{-7}	0.97	0.99
rs7305525	*GALNT8*	Intron 6		4737631	4.5×10^{-8}	0.98	0.99
rs7316209	*GALNT8*	Intron 6		4737760	4.8×10^{-8}	0.98	0.99
rs7132030	*GALNT8*	Intron 6		4737881	1.2×10^{-7}	0.98	0.99
rs2286580	*GALNT8*	Intron 6		4740189	1.1×10^{-7}	0.96	0.99
rs2191157	*GALNT8*	Intron 7		4740624	1.4×10^{-7}	0.97	0.99
rs1468556	*GALNT8*	Exon 8	F515V	4743424	3.4×10^{-7}	0.97	0.99
rs766505	*GALNT8*	Intron 10		4745595	3.9×10^{-6}	0.73	0.98
rs766506	*GALNT8*	Intron 10		4745876	1.6×10^{-1}	0.08	1.00
rs7967117	*GALNT8*	Intron 10		4746294	1.1×10^{-7}	0.97	0.99
rs10774267	*GALNT8*	Intron 10		4748837	1.7×10^{-7}	0.97	0.99
rs17177087	*GALNT8*	Intron 10		4751141	6.8×10^{-1}	0.04	1.00
rs7973527	*GALNT8*	Intron 10		4751306	6.1×10^{-7}	0.67	0.91
rs10849143	*GALNT8*	Intron 10		4751396	1.8×10^{-7}	0.89	0.99

*Chromosome positions are based on build 36 of the human genome.
†*P* values were calculated by a χ^2 test under the allelic model.
‡LD (r^2 and D′) were measured between rs10849138 and each SNP.

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 NRs 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 lowe than β-actin expression (Fig. S3).

Allele-specific gene transcript quantification analysis

As rs10849138 is located within an intron, we performe allele-specific gene transcription quantification using TaqMan genotyping probe for rs1468556 (G/T, exon 8 and PBMCs from four healthy human volunteers who wer heterozygous for both SNPs, rs1468556 and rs10849138 (C G, intron 6). These two SNPs were in almost complete LD

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

rs10849138 (C/G)	SRs			NRs			OR (95 % CI)*	P*	P_{het}†
	CC	CG	GG	CC	CG	GG			
HCV 1b	38	120	84	63	250	291	1.51 (1.21–1.87)	2.30×10^{-4}	
HCV 2a	42	140	90	10	66	72	1.71 (1.26–2.32)	5.00×10^{-4}	0.8
HCV 2b	9	32	15	9	35	35	1.60 (0.97–2.63)	6.40×10^{-2}	
Combined‡							1.58 (1.33–1.86)	1.00×10^{-7}	

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

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 4. Predictors for SRs in IFN monotherapy

Variable	Simple			Multiple (n=1065)	
	n	OR (95 % CI)	P	OR (95 % CI)	P
Sex (male/female)	1416	1.30 (1.04–1.62)	2.1×10^{-2}	1.64 (1.20–2.22)	1.6×10^{-3}
Age	1409	0.97 (0.88–1.07)*	5.2×10^{-1}		
BMI	772	0.97 (0.93–1.02)	2.2×10^{-1}		
Fibrosis stage (F0–F2/F3–F4)	1144	1.29 (0.92–1.81)	1.3×10^{-1}		
Log viral load	1072	7.46 (5.68–9.79)	2.2×10^{-47}	7.66 (5.69–10.31)	5.0×10^{-41}
HCV (non-1b/1b)	1416	3.61 (2.89–4.50)	3.0×10^{-29}	3.41 (2.54–4.60)	5.3×10^{-16}
rs8099917 (G/T)†	1413	0.44 (0.33–0.57)	2.2×10^{-9}	0.34 (0.23–0.49)	1.3×10^{-8}
rs10849138 (C/G)†	1407	1.58 (1.34–1.85)	2.5×10^{-8}	1.56 (1.25–1.95)	8.0×10^{-5}
rs3792323 (A/T)†	1416	0.91 (0.78–1.06)	5.1×10^{-1}		

*Per 10-year increase.
†Each genotype is coded as (0, 1, 2) assuming additive effects.

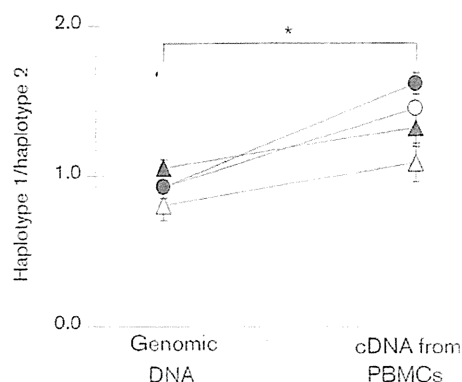

Fig. 2. Allele-specific gene expression quantification. SNP rs1468556 (T/G) in exon 8, which was in almost complete LD with rs10849138 (C/G), was selected as a probe. The relative abundance of RNA transcribed from each allele was evaluated in PBMCs from normal human volunteers who were heterozygous for both SNPs. Genomic DNA was used as a control for equal biallelic representation. *GALNT8* mRNA expression from haplotype 1 (rs10849138 G/rs1468556 G) was approximately 1.5-fold higher than that from haplotype 2 (rs10849138 C/rs1468556 T). Data are represented as means ± SD in triplicate assays. Each symbol represents an individual subject. $^*P<0.05$.

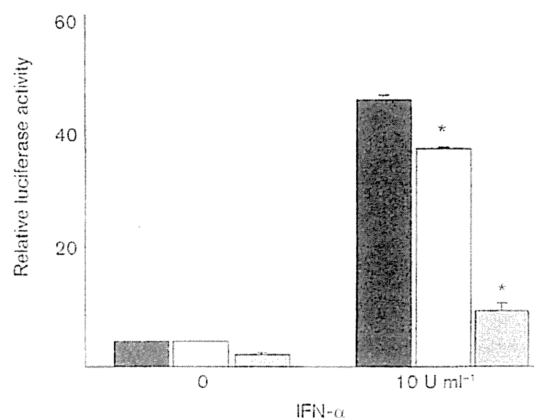

Fig. 3. Attenuation of GALNT8 in ISRE-mediated gene expression and anti-HCV activity by IFN-α. A *GALNT8*-expressing plasmid was co-transfected with ISRE reporter plasmid into Huh7 cells. After 48 h, cells were stimulated with IFN-α for 24 h followed by a dual-luciferase assay. GALNT8 significantly inhibited IFN-α-induced luciferase activities. Filled bar, mock vector; open bar, pEF-DEST-GALNT8; shaded bar, pEF-DEST-SOCS1. Data represent the means ± SD of triplicate assays. $^*P<0.05$ compared with mock.

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* ($P=2.2\times10^{-8}$; 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 ($r^2=0.94$ 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.

GALNT family proteins catalyse the initial key step of mucin-type *O*-glycan biosynthesis (Ten Hagen *et al.*, 2003). *O*-Glycan is involved in various cellular process, including cell motility (Carlow *et al.*, 2009), cell growth and death (Julien *et al.*, 2005), cell-to-cell communication (Song *et al.*, 2008), immune functions (Priatel *et al.*, 2000) and cancer metastatic potential (Bresalier *et al.*, 1996). Taken together with these previous findings, our results suggested that there might be an involvement of alterations in *O*-glycan synthesis.

Among patients treated with PEG-IFN plus ribavirin combination therapy, multivariate analysis showed that the association between the *GALNT8* variant and treatment outcome was not significant (Table S3). The reason for this difference is unclear. One plausible explanation is that the effect of GALNT8 is rather modest, so that the SNP does not influence outcome when a more effective therapy is used.

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 (Kamatani *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 6×10^6 U IFN intramuscularly every day for 8 weeks, followed by the same dose twice a week for 16 weeks, with a total dose of 5.28×10^8 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/; 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-Invader 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_017417.1): 5′-CGTTTCTTCCAGAA-TGGCACT-3′ and 5′-CAATCAGTTGTCCCACATAGAGC-3′ and β-actin (GenBank accession no. NM_007393): 5′-GTGGGCCGCCCT-AGGCACCAG-3′ and 5′-CTCTTTGATGTCACGCACGATTTC-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 minimal essential medium (Sigma-Aldrich) with 10 % FBS, 100 U penicillin ml^{-1} and 100 ng streptomycin ml^{-1} at 37 °C under 5 % CO_2.

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, 5×10^3 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

Table 5. Characteristics of the subjects with CHC in this study

Characteristic	First screening set			Second screening set			Replication set		
	SRs	NRs	*P* value	SRs	NRs	*P* value	SRs	NRs	*P* value
n	65	118		417	488		94	234	
Female (%)	14 (21.5)	45 (38.1)	0.022*	144 (34.5)	187 (38.3)	0.24*	37 (39.4)	103 (44.0)	0.44*
Age in years (mean)	55.4 (9.9)	56.0 (10.5)	0.69†	54.8 (12.1)	56.0 (10.2)	0.13†	54.9 (13.2)	53.5 (10.7)	0.34†
Body mass index (mean)	22.9 (2.8)	23.1 (2.5)	0.80†	22.9 (3.1)	23.2 (2.9)	0.19†	23.2 (3.1)	23.3 (3.3)	0.35†
HCV genotype (*n*)						4.6×10^{-20}‡			4.4×10^{-10}‡
1a				3	2		0	1	
1b	65	118		142	316		36	175	
2a				226	111		46	38	
2b				46	59		12	20	
HCV RNA (*n*)§			2.2×10^{-13}*			8.9×10^{-31}*			
1.6×10^{-11}*									
High	14	92		107	294		9	63	
Low	42	19		236	106		58	31	
No data	9	7		74	88		27	140	
Fibrosis (*n*)			0.52‖			0.23‖			0.81‖
F0	0	1		5	3		1	5	
F1	32	65		185	207		22	56	
F2	17	34		120	148		17	52	
F3	1	10		26	38		15	27	
F4	1	5		15	26		3	7	
No biopsy	14	3		66	66		36	87	

*χ^2 test.
†Student's *t*-test.
‡χ^2 test between HCV genotypes 1b and non-1b.
§Low HCV RNA level: 100 KIU ml^{-1} by Amplicor Monitor assay and 1.0 mEq ml^{-1} by branched-chain DNA assay.
‖χ^2 test between F0–F1 and F2–F4.

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 µl 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 µl 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 χ^2 test was used to compare genotype frequencies at each SNP. Deviations from Hardy–Weinberg equilibrium were tested by using a χ^2 test. Other data were analysed using Student's *t*-test or a χ^2 test as appropriate. Definition of LD block, haplotype frequency, calculation of pairwise LD indices r^2 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.

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Original Article

Effects of branched-chain amino acid granules on serum albumin level and prognosis are dependent on treatment adherence in patients with liver cirrhosis

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Aim: To test if the treatment adherence to branched-chain amino acid (BCAA) granules influences the serum albumin level and prognosis in prospective 2984 patients with decompensated liver cirrhosis who were prescribed BCAA granules containing 952 mg of L-isoleucine, 1904 mg of L-leucine and 1144 mg of L-valine at 4.15 g/sachet three times a day after meals.

Methods: The primary end-point was the time to the event defined as "hospital admission due to progression of hepatic failure", and factors affecting this outcome were explored. Changes in serum albumin level were evaluated as the secondary end-point.

Results: Patients were divided into the good adherence group (those who reported to have taken "nearly all" prescribed doses) and the poor adherence group (those who reported to have taken "approximately half" or "less" doses), because such stratification was validated by treatment responses in plasma BCAA/tyrosine ratio. Factors related to the primary end-point were age, drug adherence during 6 months of study treatment, previous hepatic cancer, current clinical manifestations, previous clinical manifestations, baseline serum albumin level, platelet count and total bilirubin level. The cumulative event-free survival was significantly higher in the good adherence group. Increase in the serum albumin level was also greater in the good adherence group.

Conclusion: Higher BCAA treatment adherence better raised the serum albumin level, leading to improvement of event-free survival. These results indicate the importance of patient instruction for the adequate use of BCAA granules.

Key words: branched-chain amino acids, hepatic failure, liver cirrhosis, prognosis, serum albumin, treatment adherence

INTRODUCTION

ALTHOUGH LIVER CIRRHOSIS is caused by any of a wide variety of etiologies,[1] clinical features of the disease share complications such as ascites, edema, hepatic encephalopathy and esophageal varices.[2] Some of these complications are attributable to decreased serum concentrations of albumin and other proteins,[3–5] and oral supplemental branched-chain amino acid (BCAA) therapy with BCAA granules or BCAA-enriched nutrients is recommended, in addition to dietary treatment with adequate protein and energy intake, for the management of these complications.[6–8]

Branched-chain amino acid granules are used for the improvement of hypoalbuminemia in patients with decompensated liver cirrhosis,[4,9–11] and several studies have demonstrated their efficacy in reducing complications of liver cirrhosis.[4,12–14] Furthermore, a reduction in

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the risk of hepatic cancer is also reported in patients taking BCAA granules.[12,15–17]

On the other hand, the patients' treatment adherence was not so favorable owing to the size of individual doses and unpleasant taste, causing interruption of treatment[13] or reduction of doses.[4] Although serum albumin level has been shown to improve in a dose-dependent manner based on the prescribed BCAA doses,[10] no studies have investigated exactly how treatment adherence may influence the serum albumin level and prognosis of patients with liver cirrhosis.

We conducted the present analysis to evaluate how treatment adherence may affect the serum albumin level and prognosis in a prospective cohort of 5042 patients with liver cirrhosis who had started BCAA treatment at a fixed dose of three sachets/day in a preceding study.[18]

METHODS

Study design and protocol

THIS WAS A multicenter prospective observational study to determine the incidence of adverse events, including hepatocellular carcinoma (HCC) and cirrhosis-related events, under the actual condition of treatment in patients with decompensated liver cirrhosis who were prescribed BCAA granules between June 2003 and December 2006,[18] and were further followed up thereafter.

A total of 5042 patients with decompensated liver cirrhosis, who presented hypoalbuminemia despite adequate dietary intake, were enrolled in this study at 929 medical institutions in Japan. These patients were p.o. administrated BCAA granules containing 952 mg of L-isoleucine, 1904 mg of L-leucine and 1144 mg of L-valine (Livact Granules, Ajinomoto Pharmaceutical, Tokyo, Japan) at 4.15 g/sachet three times a day after meals.

Patient flow is shown in Figure 1. Of the 5042 patients enrolled, the medical records were not available for 222 patients, and 123 patients were lost to follow up after the initial hospital visit. Thus, the remaining 4697 patients constituted the prospective cohort. Patients meeting any of the following criteria were then excluded, and the remaining 2984 patients were subjected to the analysis: (i) a baseline serum albumin level higher than 3.5 g/dL; (ii) a baseline serum total bilirubin level of 3.0 mg/dL or higher; (iii) unknown duration of study observation; (iv) baseline dosage of prescribed BCAA granules other than three sachets/day; or (v) unknown BCAA treatment adherence for 6 months after the start of study observation.

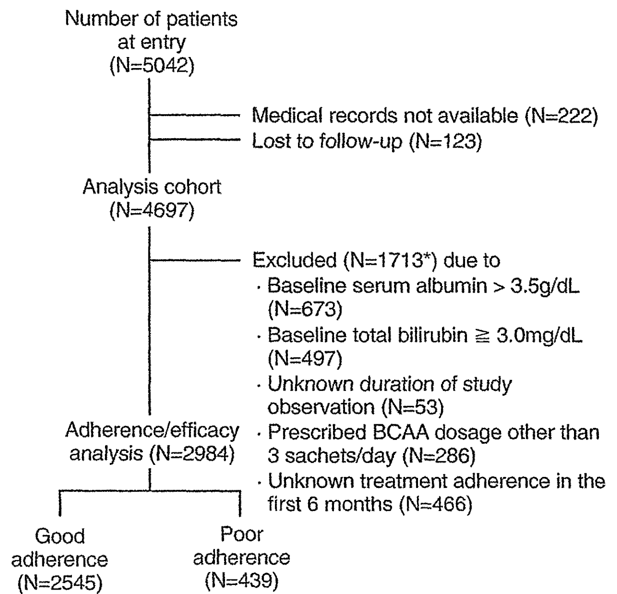

Figure 1 Patient flow. *Among the 1713 patients, 262 were excluded by meeting two or more conditions of the exclusion criteria. BCAA, branched-chain amino acid.

The patients' treatment adherence was evaluated by a questionnaire analysis at the end of the 6-month surveillance period. The questionnaire provided three answer arms who took "nearly all", "approximately half" and "less" of the prescribed dose of BCAA granules at three sachets/day. Each patient was instructed to select one of the above three answer arms that best reflected his/her drug adherence status in the preceding study period.

The primary end-point was the time to onset of the event, defined as hospital admission due to progression of hepatic failure, including ascites, edema, jaundice and hepatic encephalopathy. Changes in liver function during the 6 months were evaluated as the secondary end-point.

This study was conducted in accordance with the Japanese Good Post-Marketing Surveillance Practice.

Statistical analysis

Continuous data were expressed as mean ± standard deviation, and differences in mean values were statistically tested using paired or unpaired Student's *t*-test as appropriate. Categorical variables were compared by Wilcoxon signed rank test, Wilcoxon rank sum test or χ^2-test as required. The cumulative event-free survival rates were estimated using the Kaplan–Meier method

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and compared by log–rank test. Any risk factors contributing to the primary end-point were investigated by univariate and multivariate analyses using a Cox proportional hazards model. Data analysis was performed using JMP ver. 9.02 and SAS ver. 9.2 (both SAS Institute, Cary, NC, USA). The level of significance was assessed as two-sided $P < 0.05$.

RESULTS

Patients' characteristics and flow

OF THE PROSPECTIVE cohort consisting of 4697 patients, 1713 were excluded by meeting the exclusion criteria (Fig. 1). Among them, 673 patients had a baseline serum albumin level higher than 3.5 g/dL, 497 patients had a baseline serum total bilirubin level of 3.0 mg/dL or higher, 53 patients had an unknown duration of study observation, 286 patients were prescribed BCAA granules of a dosage other than three sachets/day, and 466 patients reported unknown treatment adherence during the 6 months of study observation. Two hundred and sixty-two patients were excluded by fulfilling two or more conditions of the exclusion criteria. Thus, the remaining 2984 patients were subjected to the adherence/efficacy analysis (Fig. 1). Clinical characteristics of these patients are shown in Table 1. The observation period ranged 6.0–47.9 months, with a median of 21.6 months.

Risk factors for the primary end-point

For the primary end-point, univariate and multivariate analyses using a Cox proportional hazards model identified the following independent factors to influence the development of the event: age, treatment adherence for the 6 months of study observation, previous hepatic cancer, current clinical manifestations, previous clinical manifestations, baseline serum albumin level, platelet count and serum total bilirubin level (Table 2).

Treatment adherence and plasma BCAA/tyrosine ratio

All these variables except treatment adherence have already been documented as risk factors in patients with liver cirrhosis.[19,20] Taking notice of treatment adherence, therefore, 2545 patients who reported to have taken "nearly all" the prescribed doses during the 6-month period comprised the good adherence group and 439 patients who reported to have taken "approximately half" or "less" of the prescribed doses during that period comprised the poor adherence group for further analysis.

Table 1 Clinical characteristics of patients

Characteristics		$n = 2984$
Sex	Male	1584 (53.1%)
	Female	1400 (46.9%)
Age (years)	20–29	1 (0.0%)
	30–39	24 (0.8%)
	40–49	165 (5.5%)
	50–59	530 (17.8%)
	60–69	1038 (34.8%)
	70–79	1024 (34.3%)
	80–89	195 (6.5%)
	>90	7 (0.2%)
	Mean ± SD	66.1 ± 10.1
Cause of liver cirrhosis	HBV	217 (7.3%)
	HCV	1755 (58.8%)
	Alcohol	487 (16.3%)
	PBC	74 (2.5%)
	AIH	63 (2.1%)
	HBV + HCV	16 (0.5%)
	HBV + alcohol	29 (1.0%)
	HCV + alcohol	92 (3.1%)
	HBV + HCV + alcohol	2 (0.1%)
	Other	57 (1.9%)
	Unknown	192 (6.4%)
Treatment adherence (during 6 months)	All	2545 (85.3%)
	Half or less	439 (14.7%)
Previous hepatic cancer	Yes	504 (16.9%)
	No	2454 (82.2%)
	Unknown	26 (0.9%)
Current clinical manifestations	Yes	1568 (52.6%)
	No	1410 (47.3%)
	Unknown	6 (0.2%)
Previous clinical manifestations	Yes	1291 (43.3%)
	No	1670 (56.0%)
	Unknown	23 (0.8%)
Diabetes	Yes	536 (18.0%)
	No	2448 (82.0%)
Serum albumin (g/dL)		3.04 ± 0.36
Platelet (×10 000/μL)		9.73 ± 6.15
AST (IU/L)		67.1 ± 62.8
ALT (IU/L)		47.8 ± 40.9
Serum total bilirubin (mg/dL)		1.30 ± 0.62
BTR		2.95 ± 1.37

For categorical variables, the number of patients and percentage are shown. For continuous variables, the mean ± SD is presented. AIH, autoimmune hepatitis; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BTR, branched-chain amino acid/tyrosine ratio; HBV, hepatitis B virus; HCV, hepatitis C virus; PBC, primary biliary cirrhosis; SD, standard deviation.

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Table 2 Risk factors for the event

Explanatory variable		Univariate				Multivariate			
		Hazard ratio	*P*-value	95% CI		Hazard ratio	*P*-value	95% CI	
				Lower limit	Upper limit			Lower limit	Upper limit
Sex	Male/female	1.18	0.0625	0.99	1.40	1.18	0.0685	0.99	1.42
Age (years)		1.01	0.0190	1.00	1.02	1.02	<0.0001	1.01	1.03
Cause of liver cirrhosis	HBV (yes/no)	1.01	0.9673	0.74	1.34				
	HCV (yes/no)	0.86	0.0928	0.72	1.03				
	Alcohol (yes/no)	1.16	0.1775	0.93	1.42				
Treatment adherence (during 6 months)	Half or less/all	1.74	<0.0001	1.39	2.15	1.94	<0.0001	1.54	2.42
Previous hepatic cancer	Yes/no	1.53	<0.0001	1.25	1.86	1.76	<0.0001	1.42	2.16
Current clinical manifestations	Yes/no	2.21	<0.0001	1.84	2.65	1.66	<0.0001	1.36	2.04
Previous clinical manifestations	Yes/no	1.88	<0.0001	1.59	2.24	1.45	<0.0001	1.20	1.74
Diabetes	Yes/no	1.24	0.0488	1.00	1.52				
Serum albumin (g/dL)	Lower level	2.51	<0.0001	2.02	3.10	2.00	<0.0001	1.57	2.54
Platelet (×10 000/μL)	Lower level	1.04	<0.0001	1.02	1.06	1.03	0.0010	1.01	1.05
AST (IU/L)	Higher level	1.00	0.8840	1.00	1.00				
ALT (IU/L)	Higher level	1.00	0.0156	0.99	1.00				
Serum total bilirubin (mg/dL)	Higher level	1.68	<0.0001	1.47	1.92	1.49	<0.0001	1.29	1.72
BTR	Lower level	1.22	0.0839	0.98	1.59				

Univariate and multivariate analyses were performed using a Cox proportional hazards model, and hazard ratios, *P*-values and 95% CI of the hazard ratios are shown. For the multivariate analysis, variables were selected and determined by backwards selection ($P = 0.2$) using a model incorporating all factors except BTR. BTR was excluded from the multivariate analysis because a considerable proportion of patients lacked BTR data.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BTR, branched-chain amino acid/tyrosine ratio; CI, confidence interval; HBV, hepatitis B virus; HCV, hepatitis C virus.

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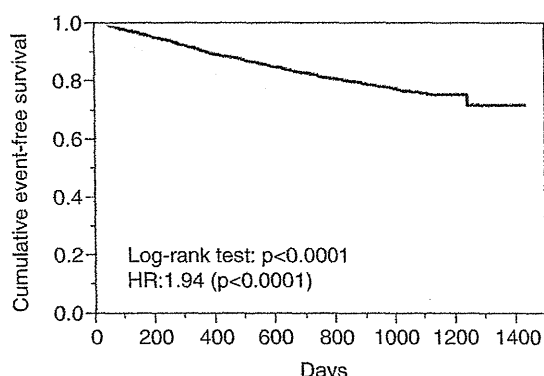
As treatment adherence was judged based on patients' self-reports, we further attempted to validate the treatment adherence by changes in the BCAA/tyrosine ratio (BTR) as an indicator reflecting true BCAA treatment adherence. Although the number of patients with BTR data was limited ($n = 185$ and 19, respectively), both absolute BTR and relative increase in BTR (increase in BTR/baseline BTR) were higher in the good adherence group (absolute BTR, 4.26 ± 0.65 for the good adherence group and 3.79 ± 0.52 for the poor adherence group; and relative increase in BTR, 0.53 ± 0.8 for the good adherence group and 0.30 ± 0.68 for the poor adherence group; $P < 0.1$ for both) at 6 months of treatment, while there was no significant difference in baseline BTR between the two groups (2.94 ± 0.49 and 2.86 ± 0.46). A comparison between the two groups was thus considered to be feasible.

Treatment adherence and event-free survival

Regarding the primary end-point, Kaplan–Meier analysis and log-rank test showed a significantly higher cumulative event-free survival rate for the good adherence group as compared with the poor adherence group (Fig. 2).



	0	200	400	600	800	1000	1200	1400
Good adherence	2545	2201	1856	1545	981	345	30	2
Poor adherence	439	301	231	182	100	32	4	0

Figure 2 Comparison of cumulative event-free survival rate by treatment adherence status. Cumulative event-free survival rates were estimated for the good adherence and poor adherence groups using the Kaplan–Meier method, and are shown along with the number of patients at risk. Two curves were compared by log-rank test, and hazard ratio (HR) was calculated by Cox proportional hazards model. (—) Good adherence; ( ) poor adherence.

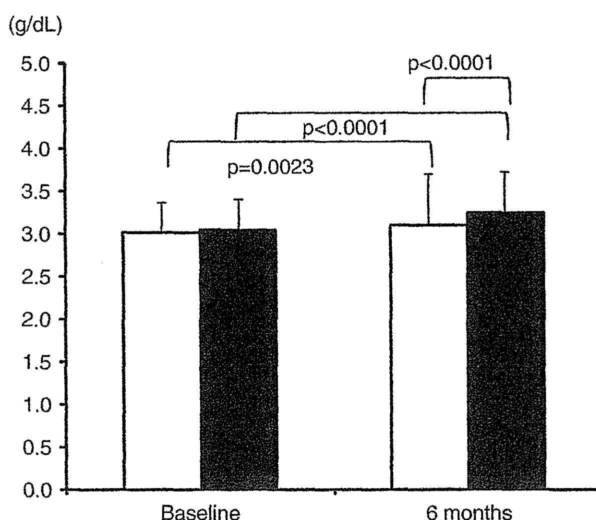


Figure 3 Comparison of serum albumin levels by treatment adherence status. Columns and bars indicate mean and standard deviation of serum albumin levels obtained at baseline and at 6 months of study treatment, respectively. Statistical assessment within each adherence group was carried out by paired Student's *t*-test. For differences between the groups at baseline and at 6 months, Student's *t*-test was conducted. (□) Poor adherence ($n = 366$); (■) good adherence ($n = 2378$).

Treatment adherence and blood biochemistry

Changes in liver function-related parameters during 6 months of the study treatment were examined for each of the good adherence and poor adherence groups. No significant difference was noted in platelet count, aspartate aminotransferase or alanine aminotransferase (ALT) between these groups. At 6 months of study treatment, serum total bilirubin level significantly increased in the poor adherence group but not in the good adherence group. Serum albumin level rose significantly in both of these groups at 6 months of study treatment, and the increase was significantly greater for the good adherence group (Fig. 3).

Comparison of clinical characteristics between good adherence group and poor adherence group

Baseline clinical characteristics were compared between the good adherence group and poor adherence group as shown in Table 3. Patients of the poor adherence group showed a significantly younger age, lower proportion of

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Table 3 Clinical characteristics of patients by adherence status

Characteristics		Good adherence, $n = 2545$	Poor adherence, $n = 439$	P-value
Sex	Male	1334 (52.4%)	250 (56.9%)	$P = 0.0789$
	Female	1211 (47.6%)	189 (43.1%)	
Age (years)	20–29	1 (0.0%)	0 (0.0%)	
	30–39	16 (0.6%)	8 (1.8%)	
	40–49	135 (5.3%)	30 (6.8%)	
	50–59	445 (17.5%)	85 (19.4%)	
	60–69	894 (35.1%)	144 (32.8%)	
	70–79	888 (34.9%)	136 (31.0%)	
	80–89	161 (6.3%)	34 (7.7%)	
	>90	5 (0.2%)	2 (0.5%)	
	Mean ± SD	66.3 ± 9.9	65.2 ± 11.1	$P = 0.0344$
Cause of liver cirrhosis	HBV	184 (7.2%)	33 (7.5%)	$P = 0.0111$
	HCV	1539 (60.5%)	216 (49.2%)	
	Alcohol	393 (15.4%)	94 (21.4%)	
	PBC	59 (2.3%)	15 (3.4%)	
	AIH	52 (2.0%)	11 (2.5%)	
	HBV + HCV	13 (0.5%)	3 (0.7%)	
	HBV + alcohol	23 (0.9%)	6 (1.4%)	
	HCV + alcohol	77 (3.0%)	15 (3.4%)	
	HBV + HCV + alcohol	2 (0.1%)	0 (0.0%)	
	Other	46 (1.8%)	11 (2.5%)	
	Unknown	157 (6.2%)	35 (8.0%)	
Previous hepatic cancer	Yes	448 (17.6%)	56 (12.8%)	$P = 0.0110$
	No	2078 (81.7%)	376 (85.6%)	
	Unknown	19 (0.7%)	7 (1.6%)	
Current clinical manifestations	Yes	1321 (51.9%)	247 (56.3%)	$P = 0.1545$
	No	1218 (47.9%)	192 (43.7%)	
	Unknown	6 (0.2%)	0 (0.0%)	
Previous clinical manifestations	Yes	1094 (43.0%)	197 (44.9%)	$P = 0.6969$
	No	1432 (56.3%)	238 (54.2%)	
	Unknown	19 (0.7%)	4 (0.9%)	
Diabetes	Yes	457 (18.0%)	79 (18.0%)	$P = 0.9844$
	No	2088 (82.0%)	360 (82.0%)	
Serum albumin (g/dL)		3.04 ± 0.36	3.01 ± 0.35	$P = 0.1519$
Platelet (×10 000/μL)		9.56 ± 5.85	10.76 ± 7.63	$P = 0.0002$
AST (IU/L)		67.2 ± 66.1	66.2 ± 38.1	$P = 0.7578$
ALT (IU/L)		48.4 ± 42.7	44.2 ± 28.7	$P = 0.0518$
Serum total bilirubin (mg/dL)		1.29 ± 0.61	1.33 ± 0.66	$P = 0.2430$
BTR		2.98 ± 1.42	2.82 ± 1.07	$P = 0.4400$

For categorical variables, the number of patients and percentage are shown. For continuous variables, the mean ± SD is presented. Statistical analysis was conducted by χ^2-test or by Student's t-test.
AIH, autoimmune hepatitis; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BTR, branched-chain amino acid/tyrosine ratio; HBV, hepatitis B virus; HCV, hepatitis C virus; PBC, primary biliary cirrhosis; SD, standard deviation.

hepatitis C virus positivity and higher proportion of alcoholic cirrhosis, lower incidence of previous hepatic cancer, and higher platelet count (Table 3). Also, they tended to be male patients with lower serum ALT activity (Table 3).

DISCUSSION

THE LOTUS STUDY demonstrated that the outcome of patients with advanced liver cirrhosis was improved by the treatment with BCAA granules at three

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sachets/day, compared with the dietary treatment.[4] As utilized in that study, the recommended dosage of BCAA granules is one sachet three times a day p.o. after meals; however, some patients may not take all three sachets in a day due to problems such as treatment adherence. We therefore conducted the present prospective cohort study to examine how differences in the actual intake of BCAA granules may influence the prognosis of patients with liver cirrhosis.

Assessment of clinical characteristics of the patients included in the present study indicated that these patients shared average clinical features of liver cirrhosis in Japanese patients such as accountable etiologies.[1] Logistic analysis revealed that none of these causes was an independent risk factor for patients' outcome. Indeed, the prognosis of patients with liver cirrhosis was determined by eight factors including treatment adherence, regardless of the cause of liver cirrhosis (Table 2).

We focused on the treatment adherence among the eight independent risk factors in the present study, because the clinical significance of the other seven factors has already been described.[19,20] For this concern, patients were divided into the good adherence group (those who reported to have taken "nearly all" prescribed doses) and the poor adherence group (those who reported to have taken "approximately half" or "less" doses), because such stratification was validated by treatment responses in plasma BCAA/tyrosine ratio. Actually, 85.3% of patients reported to have taken "nearly all" three sachets of BCAA granules/day as prescribed. This result was comparable to the 86% adherence in the patients of the LOTUS study.[4] In the present study, treatment adherence was monitored longer after the first 6 months continuously, and remained similar: 81.1% for 7–12 months, 80.6% for 13–18 months and 79.7% for 19–24 months. These data indicate that treatment adherence observed for the first 6-month period was kept over longer treatment periods and, therefore, suggest that it is reasonable to monitor the treatment adherence of the first 6-month period for the long-term prognosis.

Improvement of hypoalbuminemia was reported to depend on the prescribed daily BCAA doses (8, 12 or 16 g),[10] but the present study first showed that, at the fixed prescribed dose (three sachets or 12 g/day), serum albumin level rose sufficiently only when the patient had good adherence (Fig. 3). Thus, good treatment adherence resulted in an improved serum albumin level (Fig. 3), and, consequently brought about a higher event-free survival (Fig. 2), as a decreased serum albumin level was also an independent risk factor for the patients (Table 2).

As to possible clinical factors that affect patients' BCAA adherence, we detected male sex, younger age, distribution of etiologies of liver cirrhosis, lower incidence of previous hepatic cancer, higher platelet count and lower serum ALT activities in the poor adherence group (Table 3). Among these factors, only male sex was also a possible unfavorable outcome marker (Table 2), but other factors were rather favorable or had no significance (e.g. cause of liver cirrhosis) for patients' outcome (Table 2). Such observation suggests that particular caution should be paid for drug adherence in male cirrhotics.

The limitation of such studies on advanced liver cirrhosis is the possibility that earlier development of events shortly after the start of the study influenced treatment adherence. To address this concern, we additionally performed analysis after excluding the patients who developed any event within 6 months of the study, and the cumulative event-free survival rate was still significantly higher for the good adherence group than that for the poor adherence group (hazard ratio = 1.57, $P = 0.0043$), as was the case with the analysis on the whole analysis set.

In conclusion, higher treatment adherence for BCAA is considered to be associated with an improved serum albumin level, thereby leading to improved patient outcome. These results indicate the importance of patient instruction for the adequate use of BCAA granules.

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