^{1}H NMR (300 MHz, $CDCl_3$) δ 10.22 (s, 1H), 8.26 (d, J = 8.7 Hz, 2H), 7.63 (d, J = 8.7 Hz, 2H), 7.42 (s, 1H), 6.71 (s, 1H), 5.31 (s, 2H), 3.94 (s, 3H), 2.96 (q, J = 7.5 Hz, 2H), 1.23 (t, J = 7.5 Hz, 3H).

4.3.2.14. Compound 10 (N-[(E)-[3-methoxy-4-[(4-nitrophenyl)methoxy]phenyl]methylideneamino]-5-nitro-1-benzofuran-2-carboxamide). Compound **16** (100 mg, 0.388 mmol) was suspended in toluene, and compound **19a** (122 mg, 0.425 mmol) and sodium acetate (36 mg, 0.44 mmol) were added. The resultant solution was stirred at 80 °C for 20 h. After cooling in air, the solution was filtered, and washed with water and toluene to give compound **10** (114 mg, 0.232 mmol, 60% yield, 100% purity).

^{1}H NMR (300 MHz, DMSO-d6) δ 8.83 (d, J = 2.4 Hz, 1H), 8.44 (s, 1H), 8.35 (dd, J = 9.0 Hz, J = 2.4 Hz, 1H), 7.27 (d, J = 8.7 Hz, 2H), 7.95 (d, J = 9.0 Hz, 1H), 7.91 (s, 1H), 7.73 (d, J = 8.7 Hz, 2H), 7.40 (s, 1H), 7.26–7.10 (m, 2H), 5.34 (s, 2H), 3.87 (s, 3H).

MS calcd. for $C_{24}H_{18}N_4O_8$ $(M+H)^+$ 491.11, found 490.8.

4.3.2.15. Compound 11 (N-[(E)-[5-methoxy-2-methyl-4-[(4-nitrophenyl)methoxy]phenyl]methylideneamino]-5-nitro-1-benzofuran-2-carboxamide). Compound **16** (116 mg, 0.450 mmol) was suspended in toluene (3 mL), and compound **19d** (149 mg, 0.495 mmol) and sodium acetate (41 mg, 0.50 mmol) were added. The resultant solution was stirred at room temperature for 1 h, at 80 °C for 5 h and at 100 °C for 4 h. After cooling in air, the solution was filtered and suspended in dimethoxyethane at 80 °C for 1 h. The mixture was then cooled in air, filtered and washed with dimethoxyethane to give compound **11** (122 mg, 0.242 mmol, 54% yield, 95.5% purity).

^{1}H NMR (300 MHz, DMSO-d6) δ 8.83 (s, 1H), 8.76 (s, 1H), 8.36 (d, J = 9.0 Hz, 1H), 8.27 (d, J = 7.8 Hz, 2H), 7.95 (d, J = 9.0 Hz, 1H), 7.90 (s, 1H), 7.72 (d, J = 7.8 Hz, 2H), 7.43 (s, 1H), 6.98 (s, 1H), 5.31 (s, 2H), 3.83 (s, 3H), 2.56–2.37 (s, 3H).

MS calcd. for $C_{25}H_{20}N_4O_8$ $(M+H)^+$ 505.13, found 504.8.

4.3.2.16. Compound 12 (N-[(E)-[2-ethyl-5-methoxy-4-[(4-nitrophenyl)methoxy]phenyl]methylideneamino]-5-nitro-1-benzofuran-2-carboxamide). Compound **16** (100 mg, 0.388 mmol) was suspended in toluene, and compound **19e** (134 mg, 0.425 mmol) and sodium acetate (35 mg, 0.43 mmol) were added. The resultant solution was stirred at 80 °C for 16 h. After cooling in air, the obtained solid was filtered and suspended in dimethoxyethane (4 mL). The resultant solution was stirred at 80 °C for 16 h. After cooling in air, the mixture was filtered and washed with dimethoxyethane to give compound **12** (128 mg, 0.247 mmol, 64% yield, 100% purity).

^{1}H NMR (300 MHz, DMSO-d6) δ 8.82 (s, 1H), 8.80 (s, 1H), 8.36 (d, J = 9.3 Hz, 1H), 8.27 (d, J = 7.8 Hz, 2H), 7.95 (d, J = 9.0 Hz, 1H), 7.90 (s, 1H), 7.73 (d, J = 7.8 Hz, 2H), 7.45 (s, 1H), 6.97 (s, 1H), 5.32 (s, 2H), 3.83 (s, 3H), 2.71 (q, J = 7.5 Hz, 2H), 1.61 (t, J = 7.5 Hz, 3H). MS calcd. for $C_{26}H_{22}N_4O_8$ $(M+H)^+$ 519.14, found 518.7.

4.3.2.17. Compound 13 (N-[(E)-[3-methoxy-5-nitro-4-[(4-nitrophenyl)methoxy]phenyl]methylideneamino]-5-nitro-1-benzofuran-2-carboxamide). Compound **16** (100 mg, 0.388 mmol) was suspended in toluene (2 mL), and compound **19c** (142 mg, 0.427 mmol) and sodium acetate (35 mg, 0.43 mmol) were added. The resultant solution was stirred at room temperature for 15 h and at 80 °C for 5 h. After cooling in air, the obtained solution was filtered and washed with water and toluene to give compound **13** (148 mg, 0.276 mmol, 71% yield, 95.9% purity).

^{1}H NMR (300 MHz, DMSO-d6) δ 8.80 (d, J = 2.1 Hz, 1H), 8.53 (s, 1H), 8.33 (dd, J = 9.0 Hz, J = 2.1 Hz, 1H), 8.26 (d, J = 9.0 Hz, 2H), 7.94 (d, J = 9.0 Hz, 1H), 7.92 (s, 1H), 7.78 (s, 1H), 7.73 (s, 1H), 7.70 (d, J = 9.0 Hz, 2H), 5.31 (s, 2H), 4.00 (s, 3H).

MS calcd. for $C_{24}H_{17}N_5O_{10}$ $(M+H)^+$ 536.1, found 535.8.

Acknowledgments

This work was supported by Health and Labor Sciences Research Grants for Research on Hepatitis, as well as by Grants-in-Aid for research on hepatitis and BSE, from the Ministry of Health, Labor and Welfare and by a contract research fund for the Program of Funding Research Centers for Emerging and Reemerging Infectious Diseases, from the Ministries of Education, Culture, Sports, Science, and Technology, Japan. This work was supported by the RIKEN Structural Genomics/Proteomics Initiative (RSGI), the National Project on Protein Structural and Functional Analyses, and the Targeted Proteins Research Program (TPRP), the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan. We thank Dr. Shunta Sasaki, from Structure Based Drug Design Research, for the MS data analysis.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.09.023.

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Research Article

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Pre-treatment prediction of response to pegylated-interferon plus ribavirin for chronic hepatitis C using genetic polymorphism in *IL28B* and viral factors

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Background & Aims: Pegylated interferon and ribavirin (PEG-IFN/RBV) therapy for chronic hepatitis C virus (HCV) genotype 1 infection is effective in 50% of patients. Recent studies revealed an association between the *IL28B* genotype and treatment response. We aimed to develop a model for the pre-treatment prediction of response using host and viral factors.

Methods: Data were collected from 496 patients with HCV genotype 1 treated with PEG-IFN/RBV at five hospitals and universities in Japan. *IL28B* genotype and mutations in the core and IFN sensitivity determining region (ISDR) of HCV were analyzed to predict response to therapy. The decision model was generated by data mining analysis.

Results: The *IL28B* polymorphism correlated with early virological response and predicted null virological response (NVR) (odds ratio = 20.83, p <0.0001) and sustained virological response (SVR) (odds ratio = 7.41, p <0.0001) independent of other covariates. Mutations in the ISDR predicted relapse and SVR independent of *IL28B*. The decision model revealed that patients with the minor *IL28B* allele and low platelet counts had the highest NVR (84%) and lowest SVR (7%), whereas those with the major *IL28B* allele and mutations in the ISDR or high platelet counts had the lowest NVR (0–17%) and highest SVR (61–90%). The model had high reproducibility and predicted SVR with 78% specificity and 70% sensitivity.

Conclusions: The *IL28B* polymorphism and mutations in the ISDR of HCV were significant pre-treatment predictors of response to PEG-IFN/RBV. The decision model, including these host and viral factors may support selection of optimum treatment strategy for individual patients.

Keywords: *IL28B*; ISDR; Peg-interferon; Ribavirin; Data mining; Decision tree.
Received 14 March 2010; received in revised form 22 June 2010; accepted 7 July 2010
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Introduction

Hepatitis C virus (HCV) infection is the leading cause of cirrhosis and hepatocellular carcinoma worldwide [1]. The successful eradication of HCV, defined as a sustained virological response (SVR), is associated with a reduced risk of developing hepatocellular carcinoma. Currently, pegylated interferon (PEG-IFN) plus ribavirin (RBV) is the most effective standard of care for chronic hepatitis C but the rate of SVR is around 50% in patients with HCV genotype 1 [2,3], the most common genotype in Japan, Europe, the United States, and many other countries. Moreover, 20–30% of patients with HCV genotype 1 have a null virological response (NVR) to PEG-IFN/RBV therapy [4]. The most reliable method for predicting the response is to monitor the early decline of serum HCV-RNA levels during treatment [5] but there is no established method for prediction before treatment. Because PEG-IFN/RBV therapy is costly and often accompanied by adverse effects such as flu-like symptoms, depression and hematological abnormalities, pre-treatment predictions of those patients who are unlikely to benefit from this regimen enables ineffective treatment to be avoided.

Recently, it has been reported through a genome-wide association study (GWAS) of patients with genotype 1 HCV that single nucleotide polymorphisms (SNPs) located near the *IL28B* gene are strongly associated with a response to PEG-IFN/RBV therapy in

Please cite this article in press as: Kurosaki M et al. Pre-treatment prediction of response to pegylated-interferon plus ribavirin for chronic hepatitis C using genetic polymorphism in *IL28B* and viral factors. J Hepatol (2010), doi:10.1016/j.jhep.2010.07.037

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Table 1. Baseline characteristics of all patients, and patients assigned to the model building or validation groups.

	All patients n = 496	Model group n = 331	Validation group n = 165
Gender: male	250 (50%)	170 (51%)	80 (48%)
Age (years)	57.1 ± 9.9	56.8 ± 9.7	57.5 ± 10.2
ALT (IU/L)	78.6 ± 60.8	78.1 ± 61.4	79.7 ± 59.6
GGT (IU/L)	59.3 ± 63.6	58.9 ± 62.0	60.2 ± 66.9
Platelets (10^9/L)	154 ± 53	153 ± 52	154 ± 56
Fibrosis: F3-4	121 (24%)	80 (24%)	41 (25%)
HCV-RNA: >600,000 IU/ml	409 (82%)	273 (82%)	136 (82%)
ISDR mutation: ≤1	220 (88%)	290 (88%)	145 (88%)
Core 70 (Arg/Gln or His)	293 (59%)/203 (41%)	197 (60%)/134 (40%)	96 (58%)/69 (42%)
Core 91 (Leu/Met)	299 (60%)/197 (40%)	200 (60%)/131 (40%)	99 (60%)/66 (40%)
IL28B: Minor allele	151 (30%)	101 (31%)	50 (30%)
SVR	194 (39%)	129 (39%)	65 (39%)
Relapse	152 (31%)	103 (31%)	49 (30%)
NVR	150 (30%)	99 (30%)	51 (31%)

ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; ISDR, interferon sensitivity determining region; Arg, arginine; Gln, glutamine; His, histidine; Leu, leucine; Met, methionine; Minor, heterozygote or homozygote of minor allele; SVR, sustained virological response; NVR, null virological response.

Japanese [6], European [7], and a multi-ethnic population [8,9]. The last three studies focused on the association of SNPs in the *IL28B* region with SVR [7–9] but we found a stronger association with NVR [6]. In addition to these host genetic factors, we have reported that mutations within a stretch of 40 amino acids in the NS5A region of HCV, designated as the IFN sensitivity determining region (ISDR), are closely associated with the virological response to IFN therapy: a lower number of mutations is associated with treatment failure [10–13]. Amino acid substitutions at positions 70 and 91 of the HCV core region (Core70, Core91) also have been reported to be associated with response to PEG-IFN/RBV therapy: glutamine (Gln) or histidine (His) at Core70 and methionine (Met) at Core91 are associated with treatment resistance [4,14]. The importance of substitutions in the HCV core and ISDR was confirmed recently by a Japanese multicenter study [15]. How these viral factors contribute to response to therapy is yet to be determined. For general application in clinical practice, host genetic factors and viral factors should be considered together.

Data mining analysis is a family of non-parametric regression methods for predictive modeling. Software is used to automatically explore the data to search for optimal split variables and to build a decision tree structure [16]. The major advantage of decision tree analysis over logistic regression analysis is that the results of the analysis are presented in the form of flow chart, which can be interpreted intuitively and readily made available for use in clinical practice [17]. The decision tree analysis has been utilized to define prognostic factors in various diseases [18–25]. We have reported recently its usefulness for the prediction of an early virological response (undetectable HCV-RNA within 12 weeks of therapy) to PEG-IFN/RBV therapy in chronic hepatitis C [26].

This study aimed to define the pre-treatment prediction of response to PEG-IFN/RBV therapy through the integrated analysis of host factors, such as the *IL28B* genetic polymorphism and various clinical covariates, as well as viral factors, such as mutations in the HCV core and ISDR and serum HCV-RNA load. In addition, for the general application of these results in clinical practice, decision models for the pre-treatment prediction of response were determined by data mining analysis.

Materials and methods

Patients

This was a multicentre retrospective study supported by the Japanese Ministry of Health, Labor and Welfare. Data were collected from a total of 496 chronic hepatitis C patients who were treated with PEG-IFN alpha and RBV at five hospitals and universities throughout Japan. Of these, 98 patients also were included in the original GWAS analysis [6]. The inclusion criteria in this study were as follows (1) infection by genotype 1b, (2) lack of co-infection with hepatitis B virus or human immunodeficiency virus, (3) lack of other causes of liver disease, such as autoimmune hepatitis, and primary biliary cirrhosis, (4) completion of at least 24 weeks of therapy, (5) adherence of more than 80% to the planned dose of PEG-IFN and RBV for the NVR patients, (6) availability of DNA for the analysis of the genetic polymorphism of *IL28B*, and (7) availability of serum for the determination of mutations in the ISDR and substitutions of Core70 and Core91 of HCV. Patients received PEG-IFN alpha-2a (180 μg) or 2b (1.5 μg/kg) subcutaneously every week and were administered a weight adjusted dose of RBV (600 mg for <60 kg, 800 mg for 60–80 kg, and 1000 mg for >80 kg daily) which is the recommended dosage in Japan. Written informed consent was obtained from each patient and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the institutional ethics review committee. The baseline characteristics are listed in Table 1. For the data mining analysis, 67% of the patients (331 patients) were assigned randomly to the model building group and 33% (165 patients) to the validation group. There were no significant differences in the clinical backgrounds between these two groups.

Laboratory and histological tests

Blood samples were obtained before therapy and were analyzed for hematologic tests and for blood chemistry and HCV-RNA. Sequences of ISDR and the core region of HCV were determined by direct sequencing after amplification by reverse-transcription and polymerase chain reaction as reported previously [4,11]. Genetic polymorphism in one tagging SNP located near the *IL28B* gene (rs8099917) was determined by the GWAS or DigiTag2 assay [27]. Homozygosity (GG) or heterozygosity (TG) of the minor sequence was defined as having the *IL28B* minor allele, whereas homozygosity for the major sequence (TT) was

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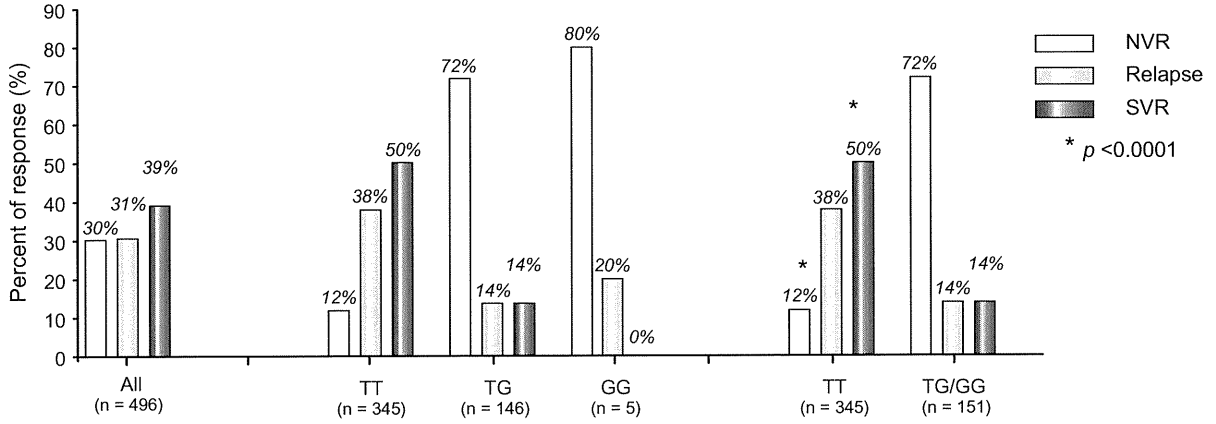

Fig. 1. Association between the IL28B genotype (rs8099917) and treatment response. The rates of response to treatment are shown for each rs8099917 genotype. The rate of null virological response (NVR), relapse, and sustained virological response (SVR) is shown. The *p* values are from Fisher's exact test. The rate of NVR was significantly higher ($p < 0.0001$) and the rate of SVR was significantly lower ($p < 0.0001$) in patients with the *IL28B* minor allele compared to those with the major allele. [This figure appears in colour on the web.]

defined as having the *IL28B* major allele. In this study, NVR was defined as a less than 2 log reduction of HCV-RNA at week 12 and detectable HCV-RNA by qualitative PCR with a lower detection limit of 50 IU/ml (Amplicor, Roche Diagnostic systems, CA) at week 24 during therapy. RVR (rapid virological response) and complete early virological response (cEVR) were defined as undetectable HCV-RNA at 4 weeks and 12 weeks during therapy and SVR was defined as undetectable HCV-RNA 24 weeks after the completion of therapy. Relapse was defined as reappearance of HCV-RNA after the completion of therapy. The stage of liver fibrosis was scored according to the METAVIR scoring system: F0 (no fibrosis), F1 (mild fibrosis: portal fibrosis without septa), F2 (moderate fibrosis: few septa), F3 (severe fibrosis: numerous septa without cirrhosis) and F4 (cirrhosis). Percentage of steatosis was quantified in 111 patients by determining the average proportion of hepatocytes affected by steatosis.

Statistical analysis

Associations between pre-treatment variables and treatment response were analyzed by univariate and multivariate logistic regression analysis. Associations between the *IL28B* polymorphism and sequences of HCV were analyzed by Fisher's exact test. SPSS software v.15.0 (SPSS Inc., Chicago, IL) was used for these analyses. For the data mining analysis, IBM-SPSS Modeler version 13.0 (IBM-SPSS Inc., Chicago, IL) software was utilized as reported previously [26]. The patients used for model building were divided into two groups at each step of the analysis based on split variables. Each value of each variable was considered as a potential split. The optimum variables and cut-off values were determined by a statistical search algorithm to generate the most significant division into two prognostic subgroups that were as homogeneous as possible for the probability of SVR. Thereafter, each subgroup was evaluated again and divided further into subgroups. This procedure was repeated until no additional significant variable was detected or the sample size was below 15. To avoid over-fitting, 10-fold cross validation was used in the tree building process. The reproducibility of the resulting model was tested with the data from the validation patients.

Results

Association between the IL28B (rs8099917) genotype and the PEG-IFN/RBV response

The rs8099917 allele frequency was 70% for TT ($n = 345$), 29% for TG ($n = 146$), and 1% for GG ($n = 5$). We defined the *IL28B* major allele as homozygous for the major sequence (TT) and the *IL28B* minor allele as homozygous (GG) or heterozygous (TG) for the minor sequence. The rate of NVR was significantly higher (72% vs. 12%, $p < 0.0001$) and the rate of SVR was significantly lower (14% vs. 50%, $p < 0.0001$) in patients with the *IL28B* minor allele compared to those with the major allele (Fig. 1).

Effect of the IL28B polymorphism, substitutions in the ISDR, Core70, and Core91 of HCV on time-dependent clearance of HCV

Patients were stratified according to their IL28B allele type, the number of mutations in the ISDR, the amino acid substitutions in Core70 and Core91, and the rate of undetectable HCV-RNA at 4, 8, 12, 24, and 48 weeks after the start of therapy was analyzed (Fig. 2A–D). The rate of undetectable HCV-RNA was significantly higher in patients with the IL28B major allele than the minor allele, in patients with two or more mutations in the ISDR compared to none or only one mutation, in patients with arginine (Arg) at Core70 rather than Gln/His, and in patients with leucine (Leu) at Core91 rather than Met. The difference was most significant when stratified by the *IL28B* allele type. The rate of RVR and cEVR was significantly more frequent in patients with the *IL28B* major allele compared with those with the *IL28B* minor allele: 9% vs. 3% for RVR ($p < 0.005$) and 57% vs. 11% for cEVR ($p < 0.0001$). These findings suggest that IL28B has the greatest impact on early virological response to therapy.

Association between substitutions in the ISDR and relapse after the completion of therapy

Patients were stratified according to the *IL28B* allele, number of mutations in the ISDR, and amino acid substitutions of Core70 and Core91, and the rate of relapse was analyzed (Fig. 3A and B). Among patients who achieved cEVR, the rate of relapse was significantly lower in patients with two or more mutations in the ISDR compared to those with only one or no mutations (15% vs. 31%, $p < 0.005$) (Fig. 3 B). On the other hand, the relapse rate was not different between the *IL28B* major and minor alleles within patients who achieved RVR (3% vs. 0%) or cEVR (28% vs. 29%) (Fig. 3A). Amino acid substitutions of Core70 and Core91 were not associated with the rate of relapse (data not shown).

Factors associated with response by multivariate logistic regression analysis

By univariate analysis, the minor allele of *IL28B* ($p < 0.0001$), one or no mutations in the ISDR ($p = 0.03$), high serum level of

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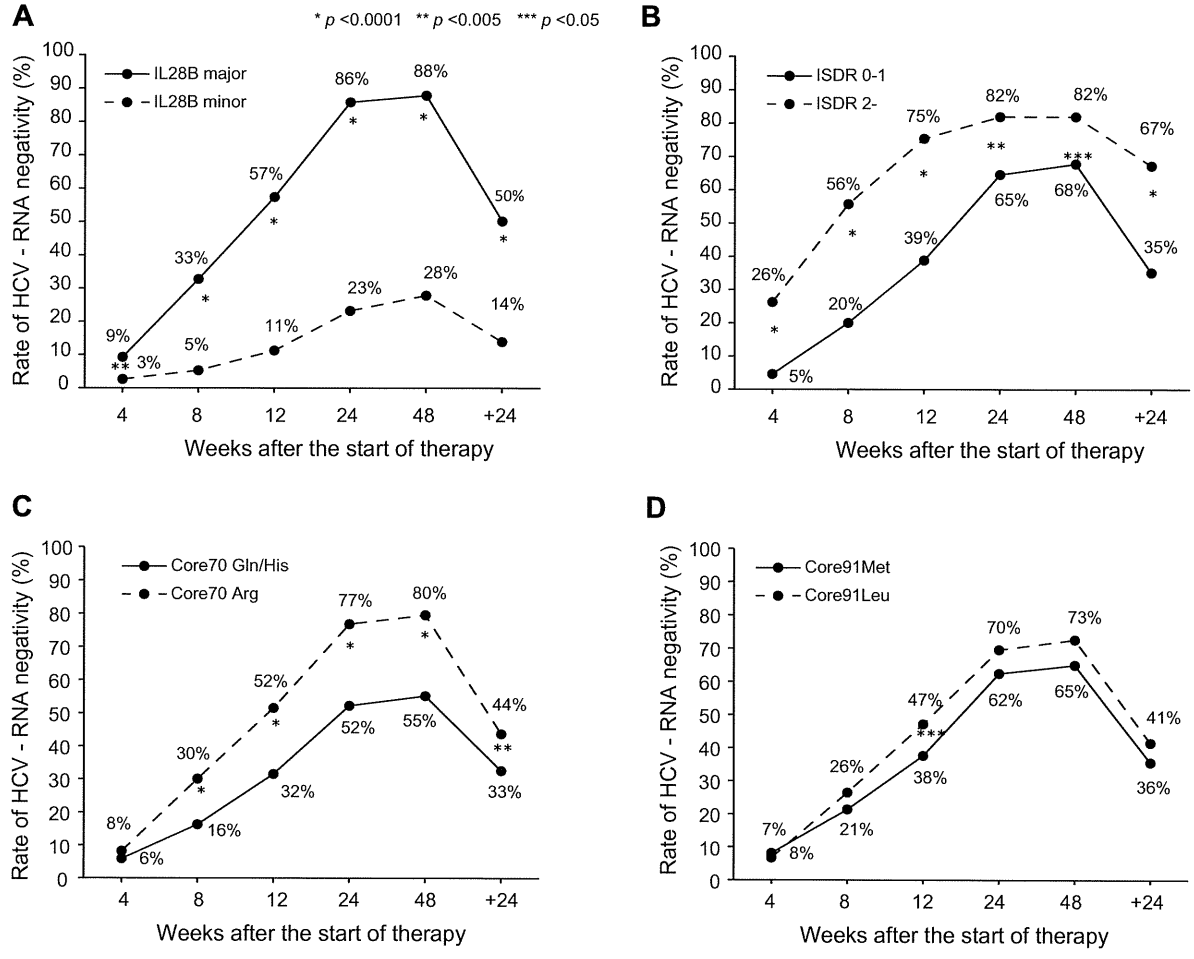

Fig. 2. Effect of *IL28B* mutations in the ISDR, Core70 and Core91 of HCV on time-dependent clearance of HCV. The rate of undetectable HCV-RNA was plotted for serial time points after the start of therapy (4, 8, 12, 24, and 48 weeks) and for 24 weeks after the completion of therapy. Patients were stratified according to (A) the *IL28B* allele (minor allele vs. major allele), (B) the number of mutations in the ISDR (0–1 mutation vs. 2 or more mutations), amino acid substitutions of (C) Core70 (Gln/His vs. Arg), and (D) Core91 (Met vs. Leu). The *p* values are from Fisher's exact test.

HCV-RNA ($p = 0.035$), Gln or His at Core70 ($p < 0.0001$), low platelet counts ($p = 0.009$), and advanced fibrosis ($p = 0.0002$) were associated with NVR. By multivariate analysis, the minor allele of *IL28B* (OR = 20.83, 95%CI = 11.63–37.04, $p < 0.0001$) was associated with NVR independent of other covariates (Table 2). Notably, mutations in the ISDR ($p = 0.707$) and at amino acid Core70 ($p = 0.207$) were not significant in multivariate analysis due to the positive correlation with the *IL28B* polymorphism ($p = 0.004$ for ISDR and $p < 0.0001$ for Core70, Fig. 4).

Genetic polymorphism of *IL28B* also was associated with SVR (OR = 7.41, 95% CI = 4.05–13.57, $p < 0.0001$) independent of other covariates, such as platelet counts, fibrosis, and serum levels of HCV-RNA. Mutation in the ISDR was an independent predictor of SVR (OR = 2.11, 95% CI = 1.06–4.18, $p = 0.033$) but the amino acid at Core70 was not (Table 3).

Factors associated with the IL28B polymorphism

Patients with the *IL28B* minor allele had significantly higher serum level of gamma-glutamyltransferase (GGT) and a higher frequency of hepatic steatosis (Table 4). When the association between the *IL28B* polymorphism and HCV sequences was analyzed, Gln or His at Core70, that is linked to resistance to PEG-IFN and RBV therapy [4,14,15], was significantly more frequent in patients with the minor *IL28B* allele than in those with the major allele (67% vs. 30%, $p < 0.0001$) (Fig. 4). Other HCV sequences with an IFN resistant phenotype also were more prevalent in patients with the minor *IL28B* allele than those with the major allele: Met at Core91 (46% vs. 37%, $p = 0.047$) and one or no mutations in the ISDR (94% vs. 85%, $p = 0.004$) (Fig. 4).

Data mining analysis

Data mining analysis was performed to build a model for the prediction of SVR and the result is shown in Fig. 5. The analysis selected four predictive variables, resulting in six subgroups of patients. Genetic polymorphism of *IL28B* was selected as the best predictor of SVR. Patients with the minor *IL28B* allele had a lower probability of SVR and a higher probability of NVR than those with the major *IL28B* allele (SVR: 14% vs. 50%, NVR: 72% vs.

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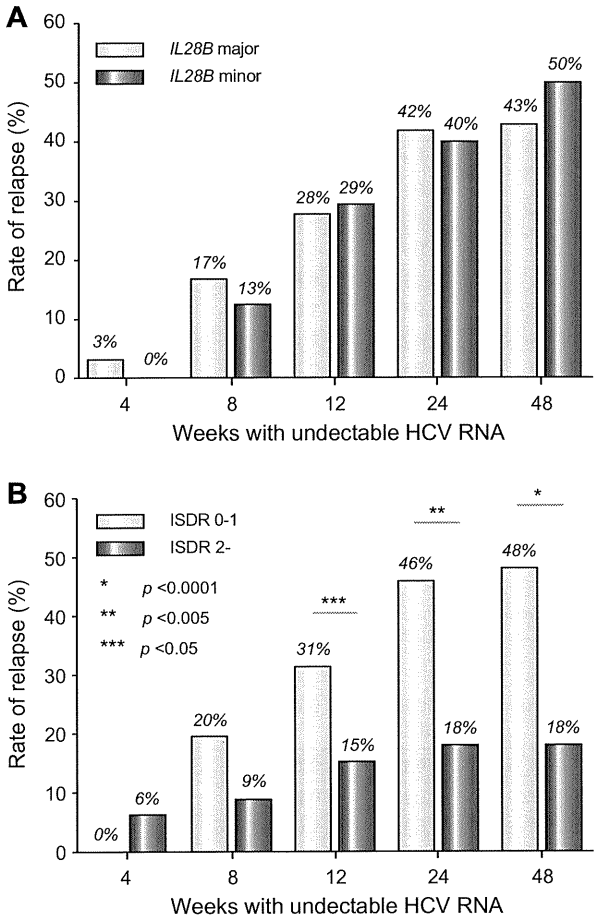

Fig. 3. Association between relapse and the *IL28B* allele or mutations in the ISDR. The rate of relapse was calculated for patients who had undetectable HCV-RNA at serial time points after the start of therapy (4, 8, 12, 24, and 48 weeks). Patients were stratified according to (A) the *IL28B* allele (minor allele vs. major allele) and (B) the number of mutations in the ISDR (0–1 mutation vs. 2 or more mutations). The *p* values are from Fisher's exact test. [This figure appears in colour on the web.]

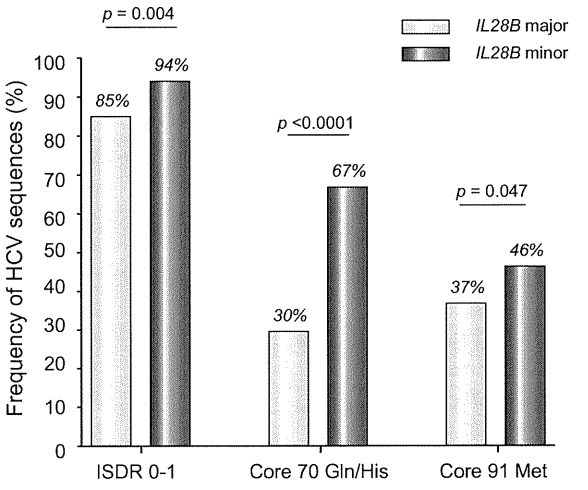

Fig. 4. Associations between the *IL28B* allele and HCV sequences. The prevalence of HCV sequences predicting a resistant phenotype to IFN was higher in patients with the minor *IL28B* allele than those with major allele. (A) 0 or 1 mutation in the ISDR of NS5A, (B) Gln or His at Core70, and (C) Met at Core91. *p* values are from Fisher's exact test. [This figure appears in colour on the web.]

12%). After stratification by the *IL28B* allele, patients with low platelet counts ($<140 \times 10^9$/L) had a lower probability of SVR and higher probability of NVR than those with high platelet counts ($\geqslant 140 \times 10^9$/L): for the minor *IL28B* allele, SVR was 7% vs. 19%, and NVR was 84% vs. 62%, and for the major *IL28B* allele, SVR was 32% vs. 66% and NVR was 16% vs. 8%. Among patients with the major *IL28B* allele and low platelet counts, those with two or more mutations in the ISDR had a higher probability of SVR and lower probability of relapse than those with one or no mutations in the ISDR (SVR: 75% vs. 27%, and relapse: 8% vs. 57%). Among patients with the major *IL28B* allele and high platelet counts, those with a low HCV-RNA titer (<600,000 IU/ml) had a higher probability of SVR and lower probability of NVR and relapse than those with a high HCV-RNA titer (SVR: 90% vs. 61%, NVR: 0% vs. 10%, and relapse: 10% vs. 29%). The sensitivity and specificity of the decision tree were 78% and 70%, respectively. The area under the receiver operating characteristic (ROC) curve of the model was 0.782 (data not shown). The proportion of patients with advanced fibrosis (F3-4) was 39% (84/217) in patients with low platelet counts ($<140 \times 10^9$/L) compared to 13% (37/279) in those with high platelet counts ($\geqslant 140 \times 10^9$/L).

Validation of the data mining analysis

The results of the data mining analysis were validated with 165 patients who differed from those used for model building. Each patient was allocated to one of the six subgroups for the validation using the flow-chart form of the decision tree. The rate of SVR and NVR in each subgroup was calculated. The rates of SVR and NVR for each subgroup of patients were closely correlated between the model building and the validation patients ($r^2 = 0.99$ and 0.98) (Fig. 6).

Discussion

The rate of NVR after 48 weeks of PEG-IFN/RBV therapy among patients infected with HCV of genotype 1 is around 20–30%. Previously, there have been no reliable baseline predictors of NVR or SVR. Because more potent therapies, such as protease and polymerase inhibitor of HCV [28,29] and nitazoxanide [30], are in clinical trials and may become available in the near future, a pre-treatment prediction of the likelihood of response may be helpful for patients and physicians, to support clinical decisions about whether to begin the current standard of care or whether to wait for emerging therapies. This study revealed that the *IL28B* polymorphism was the overwhelming predictor of NVR and is independent of host factors and viral sequences reported previously. The *IL28B* encodes a protein also known as IFN-lambda 3, which is thought to suppress the replication of various viruses including HCV [31,32]. The results of the current study and the findings of the GWAS studies [6–9] may provide the rationale for developing diagnostic testing or an IFN-lambda based therapy for chronic hepatitis C in the future.

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Table 2. Factors associated with NVR analyzed by univariate and multivariate logistic regression analysis.

	Univariate			Multivariate		
	Odds ratio	95%CI	*p* value	Odds ratio	95%CI	*p* value
Gender: female	0.98	0.67-1.45	0.938	1.29	0.75-2.23	0.363
Age	1.01	0.97-1.01	0.223	0.99	0.97-1.02	0.679
ALT	1.00	1.00-1.00	0.867	1.00	0.99-1.00	0.580
GGT	1.004	1.00-1.01	0.029	1.00	1.00-1.00	0.715
Platelets	0.95	0.91-0.99	0.009	0.92	0.87-0.98	0.006
Fibrosis: F3-4	2.23	1.46-3.42	0.0002	1.97	1.09-3.57	0.025
HCV-RNA: ≥600,000 IU/ml	1.83	1.05-3.19	0.035	2.49	1.17-5.29	0.018
ISDR mutation: ≤1	2.14	1.08-4.22	0.030	0.96	0.78-1.18	0.707
Core 70 (Gln/His)	3.23	2.16-4.78	<0.0001	1.41	0.83-2.42	0.207
Core 91 (Met)	1.39	0.95-2.06	0.093	1.21	0.72-2.04	0.462
IL28B: Minor allele	19.24	11.87-31.18	<0.0001	20.83	11.63-37.04	<0.0001

ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; ISDR, interferon sensitivity determining region; Gln, glutamine; His, histidine; Met, methionine; Minor allele, heterozygote or homozygote of minor allele.

Table 3. Factors associated with SVR analyzed by univariate and multivariate logistic regression analysis.

	Univariate			Multivariate		
	Odds ratio	95%CI	*p* value	Odds ratio	95%CI	*p* value
Gender: female	0.81	0.56-1.16	0.253	0.86	0.55-1.35	0.508
Age	0.97	0.95-0.99	0.0003	0.99	0.96-1.01	0.199
ALT	1.00	1.00-1.00	0.337	1.00	1.00-1.01	0.108
GGT	1.00	1.00-1.00	0.273	1.00	1.00-1.00	0.797
Platelets	1.12	1.01-116	<0.0001	1.13	1.08-1.19	<0.0001
Fibrosis: F0-2	2.64	1.65-4.22	<0.0001	1.87	1.07-3.28	0.029
HCV-RNA: <600,000 IU/ml	2.49	1.55-3.98	0.0001	2.75	1.55-4.90	0.001
ISDR mutation: 2≤	3.78	2.14-6.68	<0.0001	2.11	1.06-4.18	0.033
Core 70 (Arg)	1.61	1.11-2.28	0.012	0.84	0.52-1.35	0.470
Core 91 (Leu)	1.28	0.88-1.85	0.185	1.26	0.81-1.96	0.300
IL28B: Major allele	6.21	3.75-10.31	<0.0001	7.41	4.05-13.57	<0.0001

ALT, alanine aminotransferase; GGT, Gamma-glutamyltransferase; ISDR, interferon sensitivity determining region; Arg, arginine; Leu, leucine; Major allele, homozygote of major allele.

Among baseline factors, IL28B was the most significant predictor of NVR and SVR. Moreover, the IL28B allele type was also correlated with early virological response: the rate of RVR and cEVR was significantly high for the IL28B major allele compared to the IL28B minor allele: 9% vs. 3% for RVR and 57% vs. 11% for cEVR (Fig. 2). On the other hand, the relapse rate was not different between the IL28B genotypes within patients who achieved RVR or cEVR (Fig. 3). We believe that optimal therapy should be based on baseline features and a response-guided approach. Our findings suggest that the IL28B genotype is a useful baseline predictor of virological response which should be used for selecting the treatment regimen: whether to treat patients with PEG-IFN and RBV or to wait for more effective future therapy including direct acting antiviral drugs. On the other hand, baseline IL28B genotype might not be suitable for determining the treatment duration in patients who started PEG-IFN/RBV therapy and whose virological response is determined because the IL28B genotype is not useful for the prediction of relapse. The duration of therapy should be personalized based on the virological response. Future studies need to explore whether the combination of baseline IL28B genotype and response-guided approach further improves the optimization of treatment duration.

The SVR rate in patients having the IL28B minor allele was 14% in the present study while it was 23% in Caucasians and 9% in African Americans in a study by McCarthy et al. [33]. On the other hand, the SVR rate in patients having the IL28B minor allele was 28% in genotypes 1/4 compared to 80% in genotypes 2/3 in a study by Rauch et al. [9]. These data imply that the impact of the IL28B polymorphism on response to therapy may be different in terms of race, geographical areas, or HCV genotypes, and that our data need to be validated in future studies including different populations and geographical areas before generalization.

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Table 4. Factors associated with IL28B genotype.

	IL28B major allele n = 345	*IL28B* minor allele n = 151	*p* value
Gender: male	166 (48%)	84 (56%)	0.143
Age (years)	57 ± 10	57 ± 10	0.585
ALT (IU/L)	79 ± 60	78 ± 62	0.842
Platelets (10^9/L)	153 ± 54	155 ± 52	0.761
GGT (IU/L)	51 ± 45	78 ± 91	0.001
Fibrosis: F3-4	76 (22%)	45 (30%)	0.063
Steatosis:			
>10%	16/88 (18%)	13/23 (57%)	0.024
>30%	6/88 (7%)	6/23 (26%)	0.017
HCV-RNA: >600,000 IU/ml	284 (82%)	125 (83%)	1.000

ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase.

Four GWAS studies have shown the association between a genetic polymorphism near the IL28B gene and response to PEG-IFN plus RBV therapy. The SNPs that showed significant association with response were rs12979860 [8] and rs8099917 [6,7,9]. There is a strong linkage-disequilibrium (LD) between these two SNPs as well as several other SNPs near the IL28B gene in Japanese patients [34] but the degree of LD was weaker in Caucasians and Hispanics [8]. Thus, the combination of SNPs is not useful for predicting response in Japanese patients but may improve the predictive value in patients other than Japanese who have weaker LD between SNPs.

Other significant predictors of response independent of IL28B genotype were platelet counts, stage of fibrosis, and HCV RVA load. A previous study reported that platelet count is a predictor of response to therapy [35], and the lower platelet count was related with advanced liver fibrosis in the present study. The association between response to therapy and advanced fibrosis independent of the IL28B polymorphism is consistent with a recent study by Rauch et al. [9].

There is agreement that the viral genotype is significantly associated with the treatment outcome. Moreover, viral factors such as substitutions in the ISDR of the NS5A region [10] or in the amino acid sequence of the HCV core [4] have been studied in relation to the response to IFN treatment. The amino acid Gln or His at Core70 and Met at Core91 are repeatedly reported to be associated with resistance to therapy [4,14,15] in Japanese patients but these data wait to be validated in different populations or other geographical areas. In this study, we confirmed that patients with two or more mutations in the ISDR had a higher rate of undetectable HCV-RNA at each time point during therapy. In addition, the rate of relapse among patients who achieved cEVR was significantly lower in patients with two or more mutations in ISDR compared to those with only one or no mutations (15% vs. 31%, $p < 0.05$). Thus, the ISDR sequence may be used to predict a relapse among patients who achieved virological response during therapy, while the IL28B polymorphism may be used to predict the virological response before therapy. A higher number of mutations in the ISDR are reported to have close association with SVR in Japanese [11–13,15,36] or Asian [37,38] populations but data from Western countries have been controversial [39–42]. A meta-analysis of 1230 patients including 525 patients from Europe has shown that there was a positive correlation between the SVR and the number of mutations in the ISDR in Japanese as well as in European patients [43] but this correlation was more pronounced in Japanese patients. Thus, geographical factors may account for the different impact of ISDR on treatment response, which may be a potential limitation of our study.

To our surprise, these HCV sequences were associated with the IL28B genotype: HCV sequences with an IFN resistant phenotype were more prevalent in patients with the minor *IL28B* allele than those with the major allele. This was an unexpected finding, as we initially thought that host genetics and viral sequences were completely independent. A recent study reported that the IL28B polymorphism (rs12979860) was significantly associated with HCV genotype: the IL28B minor allele was more frequent in HCV genotype 1-infected patients compared to patients infected with HCV genotype 2 or 3 [33]. Again, patients with the IL28B minor allele (IFN resistant genotype) were infected with HCV sequences that are linked to an IFN resistant phenotype. The mechanism for this association is unclear, but may be related to an interaction between the IL28B genotype and HCV sequences in the development of chronic HCV infection as discussed by McCarthy et al., since the *IL28B* polymorphism was associated with the natural clearance of HCV [44]. Alternatively, the HCV sequence within the patient may be selected during the course of chronic infection [45,46]. These hypotheses should be explored through prospective studies of spontaneous HCV clearance or by testing the time-dependent changes in the HCV sequence during the course of chronic infection.

How these host and viral factors can be integrated to predict the response to therapy in future clinical practice is an important question. Because various host and viral factors interact in the same patient, predictive analysis should consider these factors in combination. Using the data mining analysis, we constructed a simple decision tree model for the pre-treatment prediction of SVR and NVR to PEG-IFN/RBV therapy. The classification of patients based on the genetic polymorphism of *IL28B*, mutation in the ISDR, serum levels of HCV-RNA, and platelet counts, identified subgroups of patients who have the lowest probabilities of NVR (0%) with the highest probabilities of SVR (90%) as well as those who have the highest probabilities of NVR (84%) with the lowest probability of SVR (7%). The reproducibility of the model was confirmed by the independent validation based on a second

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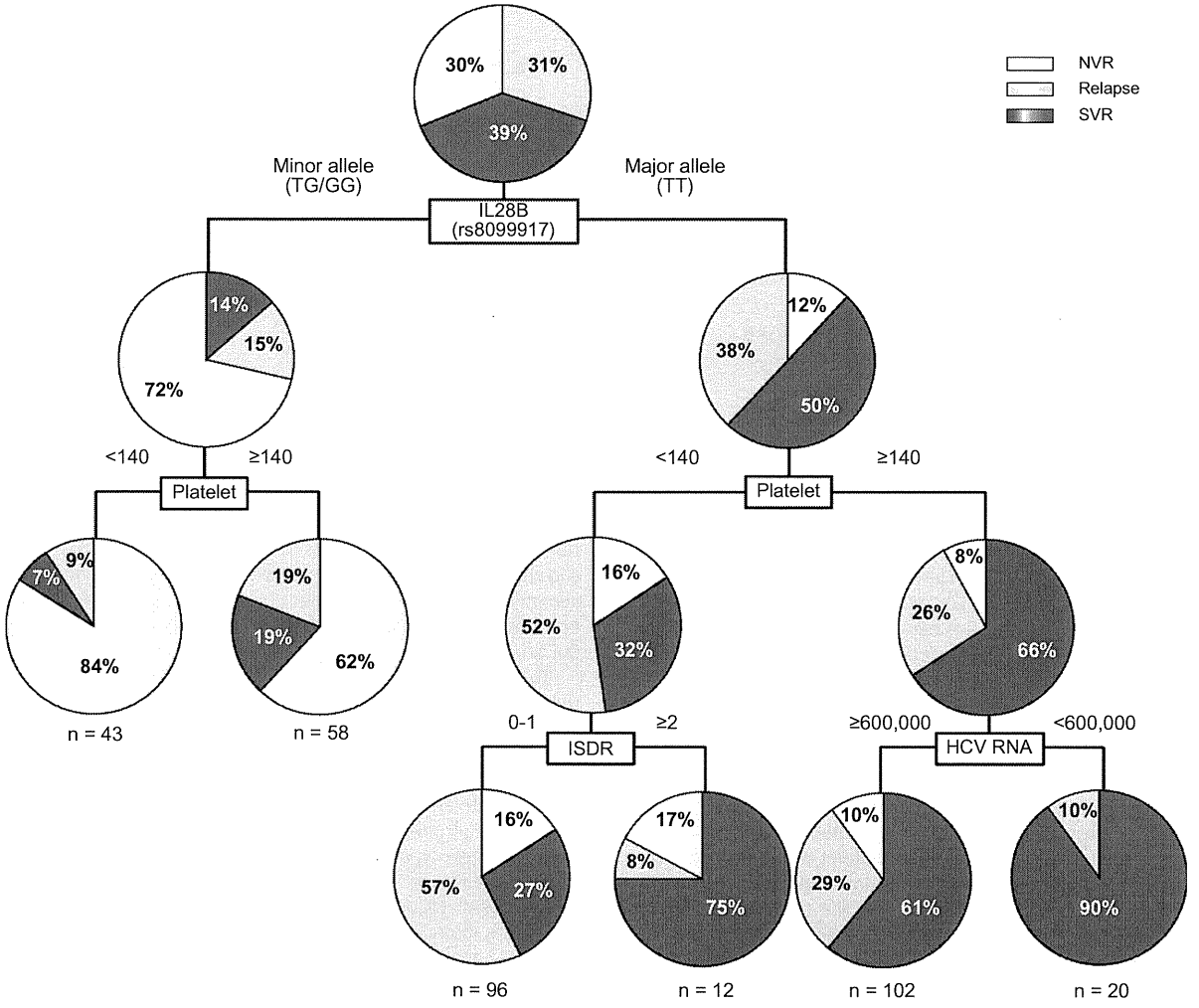

Fig. 5. Decision tree for the prediction of response to therapy. The boxes indicate the factors used for splitting. Pie charts indicate the rate of response for each group of patients after splitting. The rate of null virological response, relapse, and sustained virological response is shown. [This figure appears in colour on the web.]

group of patients. Using this model, we can rapidly develop an estimate of the response before treatment, by simply allocating patients to subgroups by following the flow-chart form, which may facilitate clinical decision making. This is in contrast to the calculating formula, which was constructed by the traditional logistic regression model. This was not widely used in clinical practice as it is abstruse and inconvenient. These results support the evidence based approach of selecting the optimum treatment strategy for individual patients, such as treating patients with a low probability of NVR with current PEG-IFN/RBV combination therapy or advising those with a high probability of NVR to wait for more effective future therapies. Patients with a high probability of relapse may be treated for a longer duration to avoid a relapse. Decisions may be based on the possibility of a response against a potential risk of adverse events and the cost of the therapy, or disease progression while waiting for future therapy.

We have previously reported the predictive model of early virological response to PEG-IFN and RBV in chronic hepatitis C [26]. The top factor selected as significant was the grade of steatosis, followed by serum level of LDL cholesterol, age, GGT, and blood sugar. The mechanism of association between these factors and treatment response was not clear at that time. To our interest, a recent study by Li et al. [47] has shown that high serum level of LDL cholesterol was linked to the IL28B major allele (CC in rs12979860). High serum level of LDL cholesterol was associated with SVR but it was no longer significant when analyzed together with the IL28B genotype in multivariate analysis. Thus, the association between treatment response and LDL cholesterol levels may reflect the underlining link of LDL cholesterol levels to IL28B genotype. Steatosis is reported to be correlated with low lipid levels [48] which suggest that IL28B genotypes may be also associated with steatosis. In fact, there were significant correlations between the IL28B genotype and the presence of steatosis in the present study (Table 4). In addition, the serum level of GGT, another predictive factor in our previous study, was significantly associated with IL28B genotype in the present study

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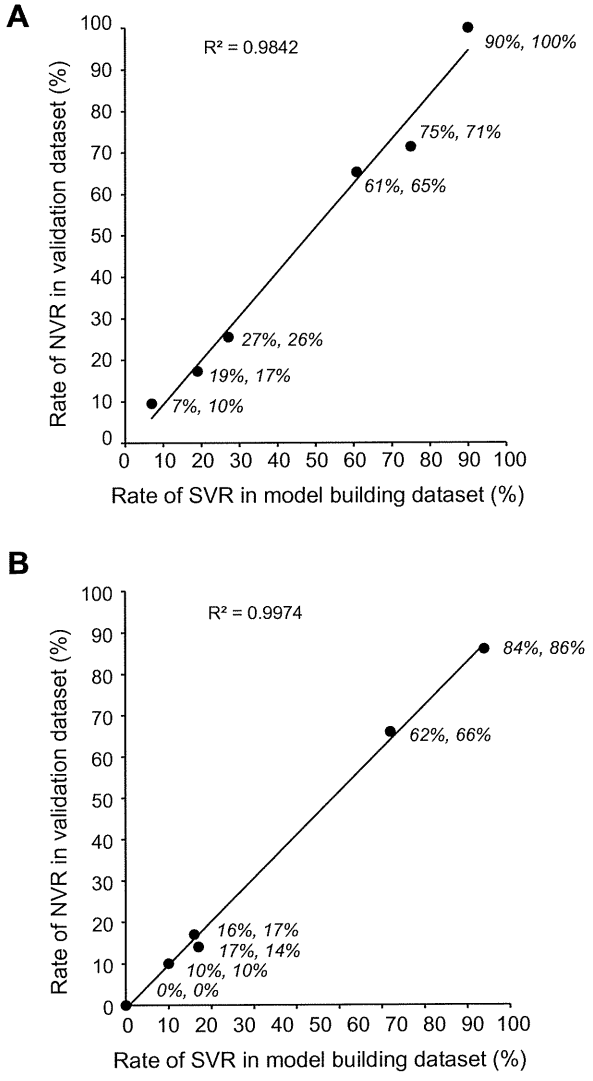

Fig. 6. Validation of the CART analysis. Each patient in the validation group was allocated to one of the six subgroups by following the flow-chart form of the decision tree. The rate of (A) sustained virological response (SVR) and (B) null virological response (NVR) in each subgroup was calculated and plotted. The *X*-axis represents the rate of SVR or NVR in the model building patients and the *Y*-axis represents those in the validation patients. The rate of SVR and NVR in each subgroup of patients is closely correlated between the model building and the validation patients (correlation coefficient: $r^2 = 0.98–0.99$).

(Table 4). The serum level of GGT was significantly associated with NVR when examined independently but was no longer significant when analyzed together with the IL28B genotype. These observations indicate that some of the factors that we have previously identified may be associated with virological response to therapy through the underlining link to the IL28B genotype.

In conclusion, the present study highlighted the impact of the *IL28B* polymorphism and mutation in the ISDR on the pre-treatment prediction of response to PEG-IFN/RBV therapy. A decision model including these host and viral factors has the potential to support selection of the optimum treatment strategy for individual patients, which may enable personalized treatment.

Conflicts of interest

The authors who have taken part in this study declare that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Financial support

This study was supported by a grant-in-aid from the Ministry of Health, Labor and Welfare, Japan, (H19-kannen-013), (H20-kannen-006).

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Please cite this article in press as: Kurosaki M et al. Pre-treatment prediction of response to pegylated-interferon plus ribavirin for chronic hepatitis C using genetic polymorphism in *IL28B* and viral factors. J Hepatol (2010), doi:10.1016/j.jhep.2010.07.037

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Please cite this article in press as: Kurosaki M et al. Pre-treatment prediction of response to pegylated-interferon plus ribavirin for chronic hepatitis C using genetic polymorphism in *IL28B* and viral factors. J Hepatol (2010), doi:10.1016/j.jhep.2010.07.037

Virus Research 158 (2011) 209–215

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Cross-species transmission of gibbon and orangutan hepatitis B virus to uPA/SCID mice with human hepatocytes

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ARTICLE INFO

Article history:
Received 10 February 2011
Received in revised form 31 March 2011
Accepted 1 April 2011
Available online 12 April 2011

Keywords:
Non-human primate
Cross-species transmission
Chimeric mouse
Phylogenetic analysis
HBV cccDNA
Immunofluorescence method

ABSTRACT

To investigate the potential of cross-species transmission of non-human primate HBV to humans, severe combined immunodeficiency mice transgenic for urokinase-type plasminogen activator, in which the mouse liver has been engrafted with human hepatocytes, were inoculated with non-human primate HBV. HBV-DNA positive serum samples from a gibbon or orangutan were inoculated into 6 chimeric mice. HBV-DNA, hepatitis B surface antigen (HBsAg), and HB core-related antigen in sera and HBV cccDNA in liver were detectable in 2 of 3 mice each from the gibbon and orangutan. Likewise, applying immunofluorescence HBV core protein was only found in human hepatocytes expressing human albumin. The HBV sequences from mouse sera were identical to those from orangutan and gibbon sera determined prior to inoculation. In conclusion, human hepatocytes have been infected with gibbon/orangutan HBV.

1. Introduction

Hepatitis B is caused by hepatitis B virus (HBV), a hepatotropic virus of the family *Hepadnaviridae*. This family comprises two genera, Avihepadnavirus and Orthohepadnavirus which can infect birds and mammals, respectively (Mason et al., 2005). As for humans, approximately 350 million chronic carriers have been infected by HBV worldwide (Lavanchy, 2004) and 15–40 percent have developed liver cirrhosis and hepatocellular carcinoma (Lee, 1997; McQuillan et al., 1989; Sharma et al., 2005). In addition to humans, HBV also infects higher non-human primates (apes) such as orangutans (*Pongo pygmaeus*), gibbons (*Hylobates* sp. and *Nomascus* sp.), gorillas (*Gorilla gorilla*), and chimpanzees (*Pan troglodytes*) (Grethe et al., 2000; MacDonald et al., 2000; Makuwa et al., 2003; Noppornpanth et al., 2003; Sall et al., 2005; Sa-nguanmoo et al., 2008; Starkman et al., 2003; Warren et al., 1998). In comparison with human HBV, non-human primate HBVs contain a 33 nucleotide deletion in the *PreS1* gene and all non-human primate HBVs cluster within their respective group separate from each human HBV genotype (Grethe et al., 2000; Kramvis et al., 2005; Robertson, 2001; Takahashi et al., 2000).

Several experiments have been conducted to study cross-species transmission of human HBV to non-human primates. Human HBsAg positive sera were intravenously inoculated into chimpanzees. In all experiments, inoculated chimpanzees displayed HBsAg in their sera (Kim et al., 2008; Tabor et al., 1980). In 1977, Bancroft et al. inoculated pooled saliva collected from 5 human carriers into gibbons. Gibbons which received subcutaneous injections of the pooled saliva developed serological markers of HBV infection. In contrast, gibbons infected via either the nasal or oral route did not show evidence of HBV infection (Bancroft et al., 1977). However, the negative results in this study are probably attributable to the lack of a sufficiently sensitive test available at that time. Alter et al. transmitted semen and saliva of carrier patients to chimpanzees. Chimpanzees developed HBsAg and elevated ALT after inoculation (Alter et al., 1977). In 1980, Scott et al. inoculated semen donated by HBsAg and HBeAg positive patients

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doi:10.1016/j.virusres.2011.04.002

into gibbons via the subcutaneous and vaginal route. Moreover, saliva of carrier patients was pooled and inoculated into gibbons via the subcutaneous and oral route. The results showed that semen and saliva from carrier patients cause asymptomatic disease in gibbons when transmitted via the subcutaneous or vaginal route, yet not via the oral route (Scott et al., 1980).

In addition to these experiments, Mimms et al. performed studies by infecting a chimpanzee with gibbon HBV. The HBV-DNA sequence from this chimpanzee was similar to that of gibbon HBV (Mimms et al., 1993). In conclusion, human HBV can be transmitted to non-human primates and cross-species transmission of non-human primate HBV can occur among various non-human primate species. However, cross-species transmission of non-human primate HBV to humans has not yet been supported by scientific evidence. To avoid performing experiments in humans, severe combined immunodeficiency mice transgenic for urokinase-type plasminogen activator, with the liver replaced with human hepatocytes (chimeric mice) serve as a suitable model for studies on human liver-specific pathogens such as HCV and HBV, human hepatic metabolism of pharmaceutical agents, and human hepatic toxicity of candidate anti-proliferative agents (Kneteman and Mercer, 2005). The mice present evidence that more fully characterizes the repopulation of the mouse liver with human hepatocytes (Meuleman et al., 2005). Histological studies have revealed that chimeric mice show evidence of human hepatocyte replace integration with infiltration into mouse liver. Moreover, human albumin and 21 other human specific proteins can be detected in mouse sera (Dandri et al., 2001; Mercer et al., 2001). Subsequently, these mice were used to support woodchuck and human hepatocyte culture and were supported infection with woodchuck hepatitis virus (WHV) and HBV (Meuleman et al., 2005; Petersen et al., 1998; Tabuchi et al., 2008).

The aim of this study has been to demonstrate that non-human primate HBV can be replicated in human hepatocytes in order to consider preventive measures in case of potential HBV transmission from non-human primates to humans.

2. Materials and methods

The study was approved by the Faculty of Veterinary Science, Animal Care and Use Committee, Mahidol University. All experiments were performed in a biosafety level 2 laboratory.

2.1. Gibbon and orangutan HBsAg-positive serum

To study cross-species transmission of non-human primate HBV to humans, the HBsAg and HBV-DNA positive sera of white-cheeked gibbon (*Nomascus leucogenys*) and orangutan (*P. pygmaeus*) were collected from Dusit zoo, Bangkok and Khao Pratub Chang Wildlife Breeding Center, Ratchaburi, Thailand, respectively. These sera constitute the stored surplus sera from a previous study (Sa-nguanmoo et al., 2008).

2.2. Chimeric mice inoculation

Twelve-week-old SCID mice transgenic for urokinase-type plasminogen activator with human hepatocytes (PhoenixBio Co, Ltd., Hiroshima, Japan) were used in this study (Tateno et al., 2004). Real-time PCR was employed to detect non-human primate HBV DNA concentration in gibbon and orangutan serum. This detection method has been shown elsewhere (Abe et al., 1999).

The minimum infectious dose of pre-acute and late acute HBV for HBV transmission to chimeric mice with human hepatocyte repopulation is approximately 10^0 and 10^2 copies (Tabuchi et al., 2008). In this study, 10^4 gibbon or orangutan HBV genome equivalents were intravenously inoculated into 3 chimeric mice of each group. However, none of the chimeric mice showed evidence of HBV markers until week 4 after inoculation. Then, all chimeric mice were re-inoculated with 10^5 genome equivalents.

2.3. Serum collection and HBV DNA extraction

Twenty microliter serum samples were collected once a week after inoculation. HBV DNA was extracted from 5 μl mouse sera by using the QIAamp® DNA Mini kit (QIAGEN, QIAGEN Sciences Inc., MD) following the manufacturer's recommendation.

2.4. HBV DNA quantitative method

HBV DNA quantity was determined by real-time PCR (ABI 7500 Fast Real-time PCR, Applied Biosystems, Foster City, CA). To that end, the small S region was amplified as previously described (Abe et al., 1999). Briefly, 5 μl of DNA were subjected to quantitative HBV DNA analysis by ABI 7500 Fast Real-time PCR (Applied Biosystems, Foster City, CA). The reaction mixture comprised 12.5 μl TaqMan® Universal PCR MasterMix (Applied Biosystems, Foster City, CA), 0.5 μl of 10 μM forward primer (HBSF2: 5′-CTTCATCCTGCTGCTATGCCT-3′), 0.5 μl of 10 μM reverse primer (HBSR2: 5′-AAAGCCCAGGATGATGGGAT-3′), 0.5 μl of 10 μM probe (HBSP2G: FAM-ATGTTGCC CGTTTGTCCTCTAATTCCAG-TAMRA) and 6 μl distilled water. The real-time PCR was performed under the following conditions: 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 30 s, and 4 °C for the holding step. The HBV viral load in unknown samples was calculated by comparison with the standard curve. The detection limit in this study was 1000 copies/ml due to the small sample volume.

2.5. DNA extraction from mouse liver tissue and cccDNA detection in liver and sera of infected chimeric SCID mice

Mouse liver tissues from one HBV-DNA positive mouse each from the gibbon and orangutan HBV inoculation group were collected at week 15 after inoculation. To extract DNA from mouse liver tissue, 25 mg of liver tissue were extracted by using the DNeasy® Blood & Tissue kit (QIAGEN, QIAGEN Sciences Inc., MD) and eluted in 200 μl of elution buffer. HBV cccDNA was detected by conventional PCR (GeneAmp® PCR System 9700, Applied Biosystems, Foster City, CA). Primer sequences have been previously published (Suzuki et al., 2009). Partially double-stranded HBV DNA could not be amplified by these primers. The details have been previously described (Mason et al., 1998). Briefly, 5 μl of DNA were subjected to amplification by GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA). The reaction mixture comprised 1 U of Ampli Taq Gold® (Applied Biosystems, Foster City, CA), 2.5 μl of 10× PCR buffer containing 15 mM $MgCl_2$, 2 μl of GeneAmp® dNTP Mix (Applied Biosystems, Washington, UK), 1 μl of 10 μM forward primer (cccF2: 5′-CGTCTGTGCCTTCTCATCTGA-3′), 1 μl of 10 μM reverse primer (cccR4: 5′-GCACAGCTTGGAGGCTTGAA-3′), and 13.3 μl distilled water. The PCR was performed under the following conditions: 96 °C for 10 min, followed by 45 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s, and 4 °C for the holding step.

2.6. Entire genome sequencing and phylogenetic analysis

Mouse serum samples positive for HBV DNA were subjected to further studies by sequencing the entire genome sequences. To amplify the entire genome, 1 μl of DNA re-suspended solution was used as template for round I PCR. The entire genome was distinguished into two segments (fragment A and fragment B). Fragment A was amplified by 10 μM forward primer (HBV17F-SARU: 5′-CAAACTCTGCAAGATCCCAGAG-3′) and 10 μM reverse

primer (HBV1799R-SARU 5′-GACCAATTTATGCCTACAGCCTC-3′). Fragment B was amplified by 10 μM forward primer (HBV1595F-SARU: 5′-CTTCACCTCTGCACGTTGCATGG-3′) and 10 μM reverse primer (HBV262R-SARU: 5′-CCACCACGAGTCTAGACTCTGTGG-3′). Both fragment A and fragment B used the same reaction mixture as follows: 5 μl of 2.5 mM dNTP, 2 μl of 10 μM forward primer, 2 μl of 10 μM forward primer, 0.33 μl of LA-Taq (TaKaRa BIO INC, Shiga, Japan), and 29.67 μl distilled water. The amplification method was performed on GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA). The thermal cycle was continued as follows: 95 °C for 2 min (pre-denaturation) and followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min, and 72 °C for 15 min (final extension).

For the second round PCR, 2 μl of round I PCR was used as template. Round I PCR product of fragment A was nested by HBV47F-SARU forward primer (5′-CTGTATTTTCCTGC-TGGTGGCTCCAG-3′) and HBV1760R-SARU reverse primer (5′-TAACCTCGTCTCCGCCCCAAACTC-3′). The first round I PCR product of fragment B was nested by HBV1608F-SARU forward primer (5′-GCATGGAGACCACCGTGAACG-3′) and HBV201R-SARU reverse primer (5′-TGTAACACGAGCAGGGGTCCTAGG-3′). Both fragment A and fragment B used reaction mixtures as round I PCR except increasing in the first round PCR template to 2 μl and adjusting distilled water to 28.67 μl. The amplification program was performed as follows: 95 °C for 2 min (pre-denaturation) and followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min, and 72 °C for 20 min (final extension).

The second round PCR products were segregated by electrophoresis on 1% agarose gel stained with ethidium bromide. The bands of PCR products were purified using the QIAquick Gel Extraction kit (QIAGEN GmbH, Hilden, Germany). Purified products were further analyzed by ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

The genome was sequenced using the 8 primer sets previously published (Sugauchi et al., 2001). Cycle sequencing was performed using the BigDye Terminator 3.1V cycle sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations. The conditions for sequencing were programmed into the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA) as previously reported (Sugauchi et al., 2001). Nucleotide sequences were edited and assembled using SEQMAN 4.00 (LASERGENE program package, DNASTAR, DNASTAR Inc., Madison, WI). All complete HBV genomes isolated from mouse sera were compared to nucleotide sequences available at the GenBank database by using the Blast program (NCBI, Bethesda, MD). Moreover, the HBV sequences obtained from mouse sera were compared with gibbon and orangutan HBV strains determined prior to inoculation and also compared with other non-human primate HBVs and each human genotype from the GenBank database (NCBI, Bethesda, MD). Genetic comparison was performed by Clustal X program version 2.0.10 (European Bioinformatics Institute, Cambridge, UK). Subsequently, the phylogenetic tree was constructed using the Tamura – 3 parameter neighbor-joining method by Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (The Biodesign Institute, Tempe, AZ).

2.7. HBsAg, HBcrAg, and human albumin measurement in mouse sera

Mouse sera were diluted (1:10) and subjected to chemiluminescence enzyme immunoassay (CLEIA) (Fujirebio Diagnostic, Inc., Tokyo, Japan) to detect HBV surface antigen (HBsAg) and HBV core – related antigen: – the antigen which includes both the HBV precore/core proteins (HBcrAg) (Kimura et al., 2005; Shinkai et al., 2006). HBcrAg measurement by this assay implies detection of precore/core proteins, including core protein and HBeAg (Kimura et al., 2002, 2005; Rokuhara et al., 2003; Wong et al., 2007). HBcrAg also showed a good correlation with HBV DNA levels in Asian patients (Kimura et al., 2002; Rokuhara et al., 2003, 2005) and intrahepatic parameters, including fibrosis scores, intrahepatic HBV, cccDNA and nuclear HBcAg (Wong et al., 2007). To expose the core protein and HBeAg, the diluted serum was fist incubated with the solution that contains sodium dodecylsulfate. Subsequently, the lysate was added to the plate coated with primary antibody to HBcAg and HBeAg. After incubation, the plate was washed to discard excess primary antibody and the second antibody labeled with alkaline phosphatase was added. Upon addition of substrate solution, the incubated reaction was measured by chemiluminescent enzyme immunoassay (CLEIA). Fully automated analysis was performed using the Lumipulse® System (Fujirebio Diagnostic, Inc., Tokyo, Japan). Human serum albumin (h-Alb) levels were determined applying a commercial enzyme linked immunosorbent (ELISA) test kit (Bethyl Laboratories Inc., Montgomery, TX).

2.8. Immunohistofluorescence assay

To detect HBcAg and human albumin, thick mouse liver tissue was prepared by cutting the frozen mouse liver with a Leica CM1900 Cryostat-microtome (Meyer Instruments, Inc., Houston, TX) and mounting the slices on glass slides. Histological analysis was performed by immunofluorescence assay as previously reported (Sugiyama et al., 2006). Briefly, mouse liver tissue was blocked by DakoCytomation antibody diluent (Dako North America, Inc., Carpinteria, CA) for 10 min at room temperature. After drying by air, the tissue was incubated in the dark with 50 μg/ml of polyclonal rabbit anti-hepatitis B virus core antigen (HBcAg) (Dako North America, Inc., Carpinteria, CA) for 1 h at 37 °C. After washing 5 times with 1× phosphate buffered saline (PBS) (GIBCO, Invitrogen Corporation, Carlsbad, CA) the tissue was incubated with 50 μg/ml of Cy3® goat anti-rabbit IgG (H + L) (Invitrogen Molecular Probes, Eugene, OR) or 5 μg/ml of goat anti-human albumin FITC (Bethyl Laboratories, Inc., Montgomery, TX) in the dark at 37 °C for 1 h. After washing 5 times with 1× PBS, the tissue was mounted by VECTASHIELD mounting medium with DAPI (Vector Laboratories, Inc., Birmingham, CA). The stained mouse tissue was examined under a Nikon Microscope ECLIPSE E800 (Nikon Instruments, Inc., Melville, NY).

3. Results

3.1. Serum HBV DNA, HBsAg, HBcrAg and human albumin level quantitation

Upon first inoculation with serum containing 10^4 copies of gibbon or orangutan HBV, none of the mice could be infected. Then, chimeric mice were re-inoculated with 10^5 copies. One mouse died before re-inoculation. After re-inoculation, mouse sera were collected once a week. Samples were subjected to quantitative HBV DNA analysis by real-time PCR while HBsAg and HBcrAg were quantitatively determined by CLIEA. Four of 5 mice could be infected with gibbon or orangutan HBV. Two mice each from the gibbon and orangutan groups showed levels of HBV DNA, HBsAg, and HBcrAg with the remaining mouse not displaying any of these markers. In detail, HBV DNA and HBcrAg could be detected in serum samples from two mice of the gibbon group (code 101 and 103) and two mice of the orangutan group (code 201 and 202) 4 weeks after inoculation. HBsAg was present in the orangutan group 4 weeks and in the gibbon groups 6 weeks after inoculation, respectively.

In this experiment, the expected HBV markers HBV DNA, HBsAg and HBcrAg could be detected in mouse serum around 4–5 weeks after inoculation. This finding matched previous studies that had

inoculated human HBV genotypes A2, C2, B1 and J into chimeric SCID mice (Sugiyama et al., 2009; Tatematsu et al., 2009). The time appearance and progression of non-human primate HBV markers presented as same as with human HBV markers (Ganem and Prince, 2004). Human albumin (h-Alb) was measured by ELISA as a quality control. Serum h-Alb levels prior to inoculation of all mice in this study exceeded 7 mg/ml indicating a human hepatocyte replacement index (RI) of over 70 percent (PhoenixBio Co, Ltd., Hiroshima, Japan) and were stable during the experiment (Fig. 1). Mean alanine aminotransferase (ALT) levels were around 200 IU/L in the uPA/SCID mouse sera. After non-human primate HBV inoculation, ALT levels slightly increased in this study (data not shown).

3.2. Intrahepatic cccDNA detection in liver tissue and mouse sera

Using the specific primers that amplify only cccDNA (Suzuki et al., 2009), HBV cccDNA was detected in mouse liver tissue from those mice that had been infected with gibbon and orangutan HBV (Fig. 2A). Moreover, cccDNA was found in the sera of mice infected with gibbon HBV (Fig. 2B).

3.3. Phylogenetic analysis of the entire HBV genome from mouse sera

HBV-DNA from all four mice was amplified and subjected to sequencing of the entire genome. The sequences from mouse sera were identical to HBV from gibbon or orangutan serum determined prior to inoculation (gibbon code GD14, GenBank ID: HQ603061; orangutan code OS25, GenBank ID: EU155824) (Fig. 3). Comparison between the complete HBV sequences from mouse sera and gibbon or orangutan sera prior to inoculation showed 99.9% and 100% similarity, respectively.

3.4. HBcAg and human albumin detection in mouse liver tissue

The mouse liver was also tested for HBcAg by staining with polyclonal rabbit anti-HBcAg and goat anti-rabbit IgG labeled with Cy3 (Fig. 4A). To locate the human hepatocyte area in chimeric mouse liver, the tissue was examined for human albumin. The same mouse liver tissue was stained with goat anti-human albumin conjugated with FITC (Fig. 4B). The study confirmed that HBcAg was found in the same area of human hepatocytes (Fig. 4C).

4. Discussion

In a previous study, Hu et al. (2000) constructed a phylogenetic tree and found that the *S* gene sequence from two chimpanzees clustered with human HBV genotypes A and C which could suggest possible virus transmission from human to chimpanzee. Currently, there is no evidence indicating natural infection of humans with non-human primate HBV (Noppornpanth et al., 2003). However, non-human primate HBV would be transmitted to humans because the respective HBV genomes are largely similar.

In this study, cross-species transmission was performed using chimeric mice containing human hepatocytes. The results showed that HBV-DNA, HBsAg and HBcrAg can be detected in sera of mice inoculated with HBV-DNA positive sera from orangutan or gibbon carriers. Detection of HBV cccDNA in liver as well as immune staining data have provided the evidence that gibbon and orangutan HBV can be replicated in human hepatocytes of the chimeric mice sero-positive for HBV DNA. HBsAg and HBV DNA concentrations could increase over time following inoculation. Interestingly, based on phylogenetic analysis, all strains of HBV sequences obtained from mouse sera inoculated with gibbon or orangutan HBV carrier sera grouped with HBV from gibbon and orangutan sera determined prior to inoculation. Nucleotide comparison between HBV in mouse sera and the HBV strain used for inoculation showed 100% identity.

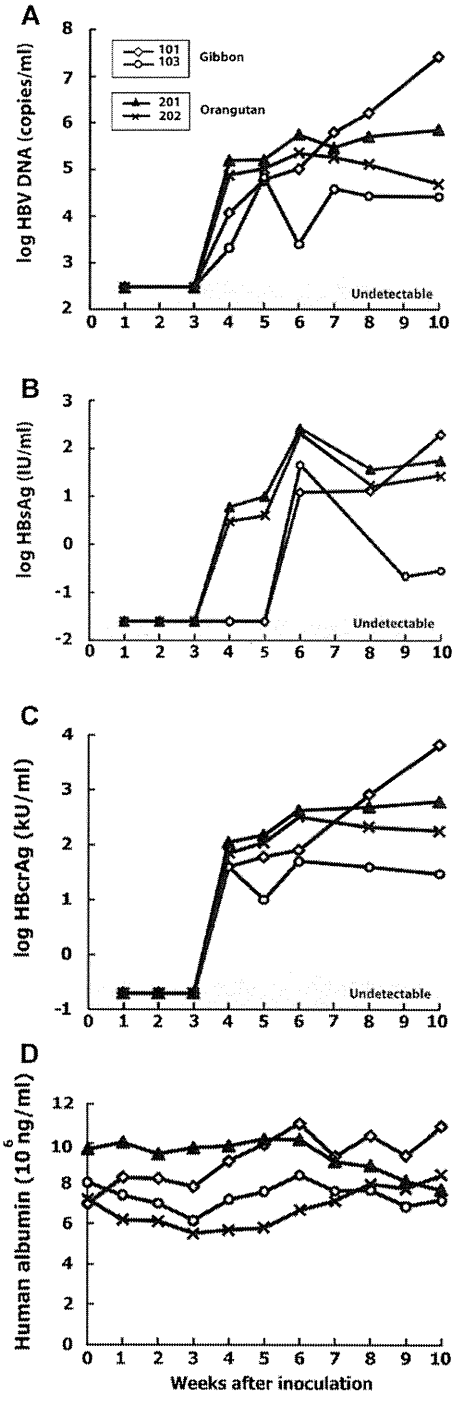

Fig. 1. HBV DNA, HBsAg, HBcrAg, and human albumin concentration in inoculated mouse sera on secondary inoculation. (A) Serum HBV DNA level. Gray zone indicates an area below the minimum sensitivity of real-time PCR (<10^3 copies/ml) (B) HBsAg concentration. The limitation of the test is 0.05 IU/ml. (C) HBcrAg level with the limited sensitivity at 1 kU/ml and (D) h-Alb concentration.

HBV infection depends on the infectious doses of HBV inoculums and host factors. In our experiment, one SCID mouse with human hapatocytes could not be infected with non-human primate HBV. This mouse lacks T- and B-lymphocytes as a protection from viral infection but still, it remains clear from viral infection. Some

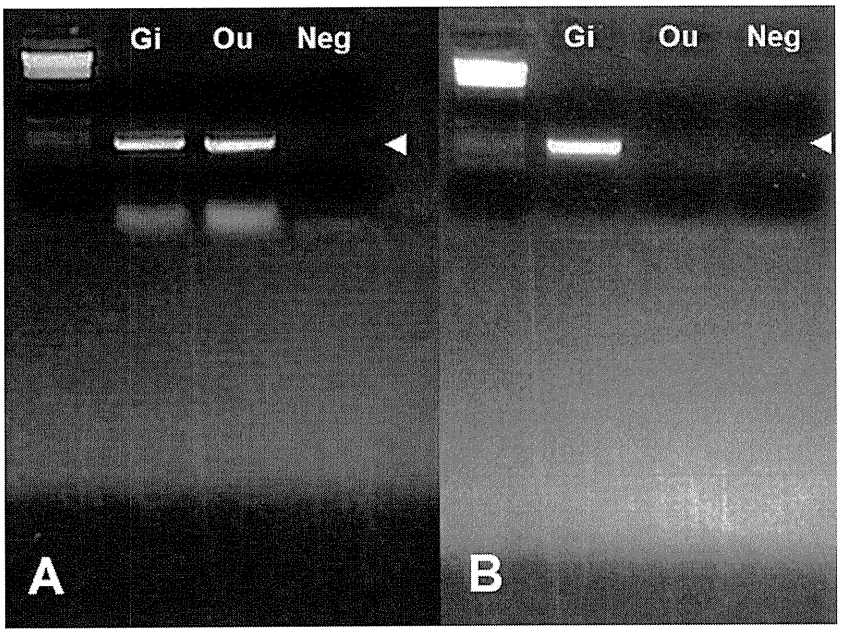

Fig. 2. CccDNA detection in liver (A) and sera (B) of mice that infected with gibbon HBV (Gi) or orangutan HBV (Ou). Neg represents negative PCR control (lacking DNA template). Arrow represents the target cccDNA PCR product.

researchers have attributed this to innate immunity of SCID mice (Lin et al., 1998). SCID mice have a normal innate immune system such as monocytes and macrophages (Ansell and Bancroft, 1989) which probably plays an important role in these mice. Moreover, infection of human hepatocytes with non-human primate HBV may be difficult due to the higher infectious dose required. Moreover, research on the early step of non-human primate HBV attachment to human hepatocytes has not been performed and the pathway of non-human primate HBV infection is still unclear. In comparison with human HBV, it might not be easy for non-human primate HBV to infect human hepatocytes.

Notably, a previous study has reported a new human HBV genotype (HBV-J) isolated from a Japanese patient with hepatocellular carcinoma (Tatematsu et al., 2009). The first HBV strain of interspecies HBV genotype J was closely related to gibbon and orangutan HBV strains and had a deletion of 33 nucleotides at the *preS1* region identical to non-human primate strains. Interestingly, this patient used to live in Borneo—a gibbon and orangutan habitat and hence, an endemic area (Tatematsu et al., 2009). He may have been infected with non-human primate HBV either by close contact or by eating raw meat of non-human primate HBV carriers (personal communication). However, infection of humans with non-human

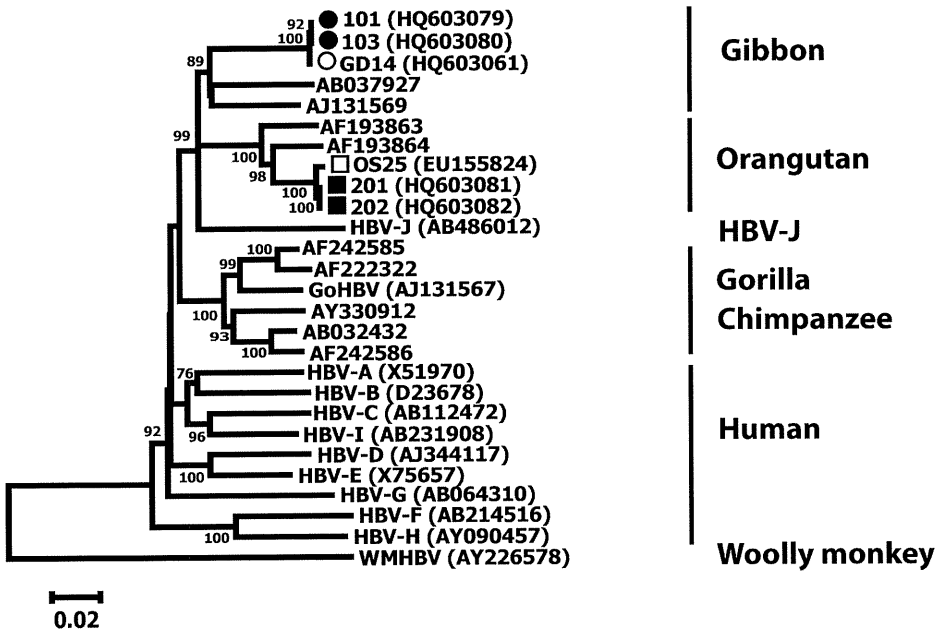

Fig. 3. Phylogenetic analysis of the entire HBV sequence obtained from mouse sera and available sequences of non-human primate HBV strains from GenBank database. Support of each branch as determined from 1000 bootstrap samples. Only 75% bootstrap values are indicated at each node. The scale bar at the bottom represents the genetic distance. Non-human primate HBV sequences obtained from our study are indicated by symbol (gibbon, ○; orangutan, □). HBV sequences obtained from mouse sera were ● and ■ for mice inoculated with gibbon and orangutan sera, respectively.

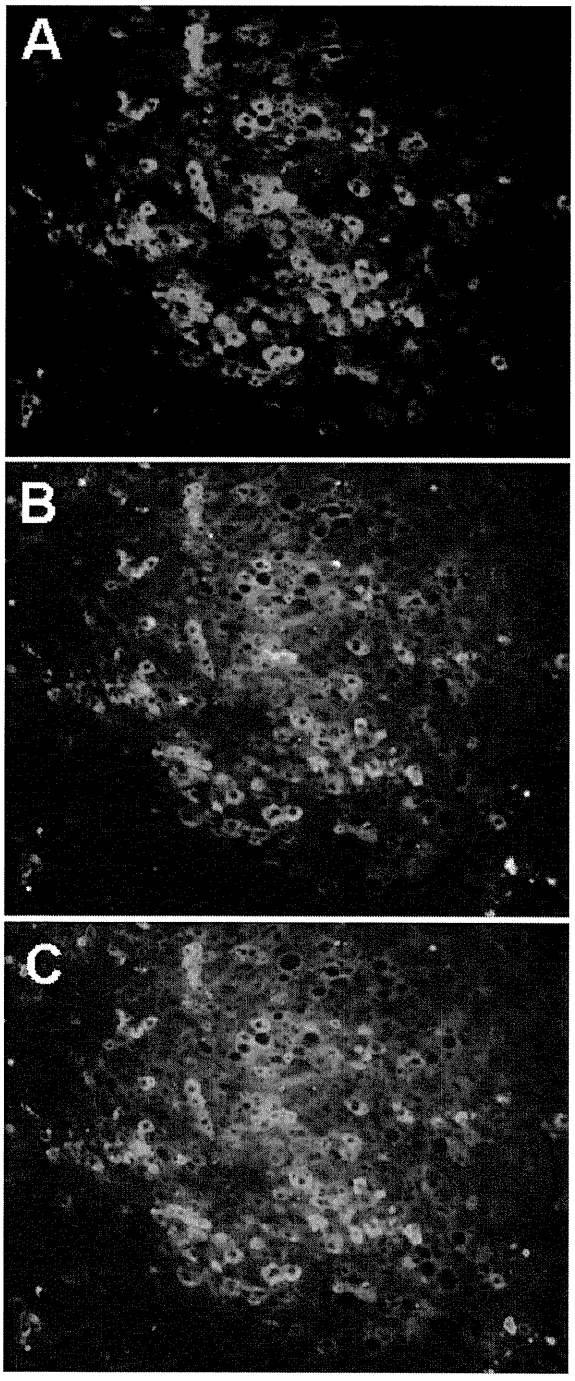

Fig. 4. Immunohistofluorescence of SCID mice infected with gibbon HBsAg-positive serum. Mouse liver tissue incubated for HBcAg (A), human albumin (B), and co-localization of HBcAg and human albumin (C).

primate HBV by eating raw meat or close contact with non-human primate HBV carriers would be hypothesis.

Yet, it has been reported that chimeric SCID mice with human hepatocytes can be infected by inoculation with HBV positive chimpanzee sera (Tabuchi et al., 2008) similar to what has been found in this study. In that previous study, human hepatocyte transplanted chimeric mice were used to study the HBV infectious titer in sera of pre-acute and late acute phase patients. These mice were inoculated with HBV infected chimpanzee sera. The chimeric mice also displayed HBV infection markers such as HBsAg, anti-HBc and anti-HBs as has been shown in this research. But the HBV in chimpanzee sera used to inoculate chimeric mice was human HBV, in contrast to the non-human primate HBV used in this study. Thus, this study is the first scientific evidence to prove and confirm that non-human primate such as gibbon and orangutan HBV can infect and replicate in human hepatocytes. Moreover, this finding can support the discovery of the HBV-J genotype which was found in the human and the assumption that humans can be infected with non-human primate HBV strains is still hypothesis.

Even though uPA-SCID mice with human hepatocytes constitute a useful animal model to study cross-species transmission, this model does not mirror the humoral and cellular immune response of the natural host. In real life, humans may be infected with non-human primate HBV and may clear this virus by their immune system. However, the results of this study indicated that human hepatocytes of chimeric mice have been infected with HBV from gibbon, orangutan and also with human HBV from infected chimpanzee sera as previously reported (Tabuchi et al., 2008). Previous studies have demonstrated cross-species transmission of human HBV to non-human primates, of non-human primate HBV to other species of non-human primates, and this study has demonstrated that non-human primate HBV can replicate in human hepatocytes. As non-human primates represent various virus reservoirs, not only of HBV but also lymphocryptovirus (LCV), Epstein-Barr virus (EBV), or simian foamy virus (SFV), people in close contact with animal HBV carriers should be aware and protect themselves from animal bites or exposure to infected blood or body fluids of non-human primates.

Acknowledgements

This research was supported by a grant-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology and a grant-in-aid from the Ministry of Health, Labour, and Welfare of Japan, Takeda Science Foundation, the Department of Virology & liver Unit, Nagoya City University Graduate School of Medical Sciences, Japan, the exchange program fellowship under the program "Strategic Scholarship for Frontier Research Network" of Thailand's Commission on Higher Education, the Center of Excellence in Clinical Virology, Chulalongkorn University, the Chulalongkorn University Graduate Scholarship to Commemorate the 72nd Anniversary of His Majesty King Bhumibol Adulyadej, the Postdoctoral Fellowship, the National Research University Project of CHE (HR1155A) and Ratchadaphiseksomphot Endowment Fund.

We would like to thank the veterinary doctors and staff of the Dusit zoo, Krabok Koo and Khao Pratubchang Wildlife Breeding Center for sera and taking care of all those non-human primates. We also would like to thank Ms. Kanako Tatematsu, Ms. Hatsue Naganuma, and the entire staff of the Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University, Thailand and the Department of Virology & Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya City University, Japan for their assistance. Finally, we would like to thank Ms. P. Hirsch for reviewing the manuscript.

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Plasma Free Amino Acid Profiling of Five Types of Cancer Patients and Its Application for Early Detection

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Abstract

Background: Recently, rapid advances have been made in metabolomics-based, easy-to-use early cancer detection methods using blood samples. Among metabolites, profiling of plasma free amino acids (PFAAs) is a promising approach because PFAAs link all organ systems and have important roles in metabolism. Furthermore, PFAA profiles are known to be influenced by specific diseases, including cancers. Therefore, the purpose of the present study was to determine the characteristics of the PFAA profiles in cancer patients and the possibility of using this information for early detection.

Methods and Findings: Plasma samples were collected from approximately 200 patients from multiple institutes, each diagnosed with one of the following five types of cancer: lung, gastric, colorectal, breast, or prostate cancer. Patients were compared to gender- and age- matched controls also used in this study. The PFAA levels were measured using high-performance liquid chromatography (HPLC)–electrospray ionization (ESI)–mass spectrometry (MS). Univariate analysis revealed significant differences in the PFAA profiles between the controls and the patients with any of the five types of cancer listed above, even those with asymptomatic early-stage disease. Furthermore, multivariate analysis clearly discriminated the cancer patients from the controls in terms of the area under the receiver-operator characteristics curve (AUC of ROC >0.75 for each cancer), regardless of cancer stage. Because this study was designed as case-control study, further investigations, including model construction and validation using cohorts with larger sample sizes, are necessary to determine the usefulness of PFAA profiling.

Conclusions: These findings suggest that PFAA profiling has great potential for improving cancer screening and diagnosis and understanding disease pathogenesis. PFAA profiles can also be used to determine various disease diagnoses from a single blood sample, which involves a relatively simple plasma assay and imposes a lower physical burden on subjects when compared to existing screening methods.

Citation: Miyagi Y, Higashiyama M, Gochi A, Akaike M, Ishikawa T, et al. (2011) Plasma Free Amino Acid Profiling of Five Types of Cancer Patients and Its Application for Early Detection. PLoS ONE 6(9): e24143. doi:10.1371/journal.pone.0024143

Editor: Libing Song, Sun Yat-sen University Cancer Center, China

Received April 8, 2011; **Accepted** August 1, 2011; **Published** September 7, 2011

Funding: This work has been supported by Grant-in-Aid for Scientific Research on Basic Research B (No. 17390195) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Ajinomoto, Co., Inc. did have a role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have read the journal's policy and have the following conflicts: Dr. Horimoto, Dr. Tochikubo, Dr. Yamakado, and Dr. Okamoto have been consultants for Ajinomoto, Co., Inc. and receive consultancy fees from Ajinomoto, Co., Inc. Dr. Imaizumi, Dr. Yamamoto, and Dr. Miyano are employees of Ajinomoto, Co., Inc. Dr. Miyagi, Dr. Higashiyama, Dr. Gochi, Dr. Akaike, Dr. Ishikawa, Dr. Miura, Dr. Saruki, Dr. Bando, Dr. Kimura, Dr. Imamura, Dr. Moriyama, Dr. Ikeda, Dr. Chiba, Dr. Oshita, Dr. Tochikubo, Dr. Mitsushima, Dr. Yamakado, and Dr. Okamoto received research grants from Ajinomoto, Co., Inc. Dr. Higashiyama, Dr. Imamura, Dr. Imaizumi, and Dr. Okamoto have applied for patents for plasma amino-acid profiling using multivariate analysis as a diagnostic tool for lung cancer and cancers (WO2008/016111 and WO2009/110517), Dr. Gochi, Dr. Imaizumi, and Dr. Yamamoto have applied for patents for plasma amino-acid profiling using multivariate analysis as a diagnostic tool for gastric cancer (WO2009/099005), Dr. Imaizumi and Dr. Okamoto have applied for patents for plasma amino-acid profiling using multivariate analysis as a diagnostic tool for colorectal cancer (WO2008/075663), Dr. Imaizumi and Dr. Okamoto have applied for patents for plasma amino-acid profiling using multivariate analysis as a diagnostic tool for breast cancer (WO2008/075662), Dr. Miyagi, Dr. Miura, Dr. Imaizumi, Dr. Yamamoto, and Dr. Okamoto have applied for patents for plasma amino-acid profiling using multivariate analysis as a diagnostic tool for prostate cancer (WO2009/154297), and Dr. Miyano has applied for patents for plasma amino acid measurement systems(WO2003/069328 and WO2005/116629). This does not alter the authors' adherence to all the PLoS One policies on sharing data and materials.

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