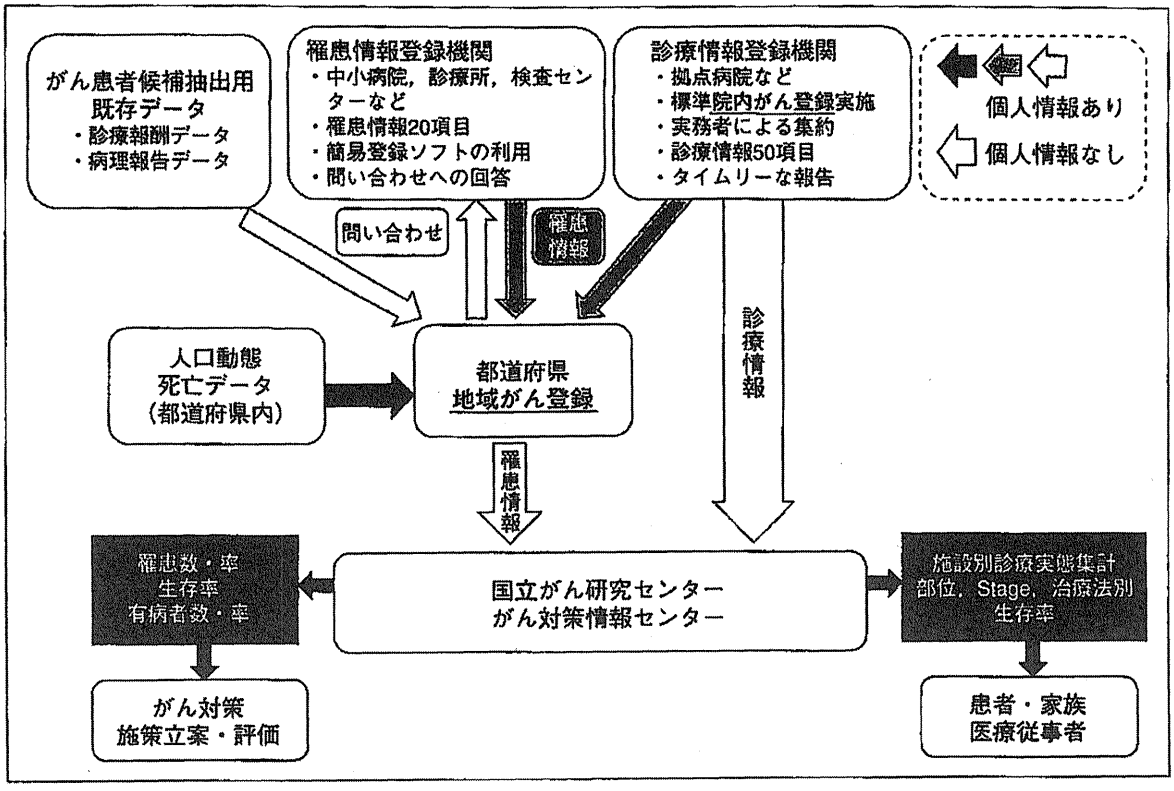

図5　がん登録のデータの流れ（将来案）

・国立がん研究センターがん対策情報センターがん情報サービス．がん診療連携拠点病院向け「院内がん登録」（http://ganjoho.jp/hospital/cancer_registration/index.html）

3．臓器がん登録

臓器がん登録は，学会・研究会が中心となって，会員医師が所属する比較的大きな病院から学会・研究会の中央事務局にデータを集約することにより，全国規模の登録を実施する仕組みである．専門的な医師のいる病院に限られるため，症例に偏りのある危険性があるが，詳細な臨床情報が収集されているため，より適切な進行度分類のあり方の検討，詳細な治療法別の生存率の計測などが可能である．臓器がん登録の横のつながりを保つ仕組みとして，厚生労働省がん研究助成金に臓器がん登録に関する研究班が組織されていたが，現在は，厚生労働省がん研究開発費「院内がん登録および臓器がん登録と連携した診療科データベースの構築と活用に関する研究」に，その役割が引き継がれている．同班が行った18臓器がん登録に対するアンケート調査によると，登録項目数は22～188項目，地域がん登録による全国推定罹患数を分母としたカバー率も6～78%と各登録によってさまざまであった．一方，多くの臓器がん登録でwebか電子媒体を利用し，連結可能匿名化した上でデータ収集を行っていた．生存率を計算するための予後調査の不明割合がいずれの臓器がん登録でも20%前後と高かった．

わが国におけるがん登録の今後の方向性

地域がん登録については，今後実施県の増加が見込まれるとともに，拠点病院からの届出数の増加により，実施県においても精度向上が予想され，全国推計に使用できるがん登録の数は，30道府県（総人口の60%）程度に増加することが期待できる．これまで，厚生労働省研究班を中心に行われてきた標準化，データ収集については，かなり定常化されてきているので，今後は，研究班活動から事業としての活動に移行していくことが考えられる．さらに，登録精度を向上させるためには，法制化（①国の事業，②届出義務，③個人情報を含む既存電子化資料の利用）が

必要と考える．特に，中小病院の届出漏れを確認するためには，レセプトなどの既存電子化資料を利用して，現在の死亡によるさかのぼり調査を前倒しで行うことで，悉皆性を担保し，データ固定の即時性を向上することができる(図5)．

院内がん登録については，拠点病院の登録項目について，必須22項目と標準49項目を整理した上で，地域がん登録との登録項目共通化が必須である．さらに，拠点病院全国集計について，施設別集計の公表を進めることが肝要である．さらに，診療の質評価のため，Quality Indicatorの測定への展開が考えられる．

地域がん登録，院内がん登録，臓器がん登録の3種類のがん登録は，それぞれ目的，実施主体，登録対象，登録項目，収集時期などが異なるため単純に統合することはできないが，共通する部分も多く，相互に連携を深めて，効率の良い登録体制を構築する必要がある．臓器がん登録に対する医療機関側の情報源は各診療科が管理する診療科データベースであることが多いが，患者の基本情報について，院内がん登録とともに病院情報システムから抽出することで省力化が可能である．こうした診療科データベースは，個人情報保護の観点からのシステム管理が徹底されていない場合が多く，院内がん登録や病院情報システムと同レベルのシステム管理の必要性が高まってきている．

一方，多くの地域がん登録は，人口動態統計死亡データおよび住民票照会や本籍地照会による予後調査を実施しているが，これらの情報について院内がん登録を通じて臓器がん登録へ還元することで，医療機関における予後調査の負担を大幅に軽減できる．既存統計資料の有効活用をすることで，予後調査の際のデータ収集を効率的に進めることができる環境を整えることが喫緊の課題である．さらに，がん医療の質の均てん化の程度を検証するためには，適切な対象に対して標準的な診断治療が実施されているかどうかのデータが必要であり，現在の地域・院内がん登録に含まれる項目だけでは，検証は難しく，サンプリング調査やデータベース間の照合などの追加的な調査が必要となる．

2006年10月に，国立がん研究センターにがん対策情報センターが設置され，がん統計・情報部に地域がん登録室と院内がん登録室が設置された．当面，種々の研究班と連携しながら，地域がん登録と院内がん登録の標準化と体制整備を支援するとともに，実務担当者の教育研修を行うことが想定されているが，今後は，種々のがん関連の統計を一元的に収集整理して，正確で役に立つがん統計情報の提供を進め，データ利用をより一層進めていく必要がある．

*　　*　　*

ciated with each health state, followed by micro-costing of the results. Costs were calculated according to Portuguese official databases. Only direct health costs were applied. The annual discount rate for costs and outcomes was considered to be 3%, according to Portuguese guidelines. A deterministic and probabilistic sensitive analysis was performed. **RESULTS:** Assuming a lifetime horizon, each patient gained 0.43, 0.55 and 0.63 life years and 0.17, 0.21 and 0.24 quality-adjusted life years with pegIFN alfa-2a plus RBV versus pegIFN alfa-2b plus RBV for all CHC genotypes, genotypes 1/4 and genotypes 2/3 respectively. The savings per patient treated with pegIFN alfa-2a plus RBV were 44€, 259€ and 1.647€ for all genotypes, genotypes 1/4 and genotypes 2/3, respectively. **CONCLUSIONS:** According to the present model, the treatment of patients with CHC with pegIFN alfa-2a plus RBV is a dominant strategy in comparison to pegIFN alfa-2b plus RBV for all genotypes, from the Portuguese NHS perspective.

PGI19

STRESS ULCER BLEEDING PROPHYLAXIS WITH PROTON PUMP INHIBITORS, H2 RECEPTOR ANTAGONISTS OR SUCRALFATE: A COST-EFFECTIVENESS ANALYSIS

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OBJECTIVES: Proton pump inhibitors (PPI), H2-receptor antagonists (H2RA) and sucralfate present varying pharmacological efficacy in preventing stress ulcer bleeding (SUB) in intensive care units. The literature also reports disparate rates of ventilator assisted pneumonia (VAP) as side-effects of these treatments. We compared the cost-effectiveness of these 3 pharmaco-prophylaxis options. **METHODS:** We constructed a decision tree for patients at high-risk for developing SUB (diagnoses of major trauma, hypovolemic shock, sepsis, septicaemia, acute respiratory failure, extensive burns, acute renal failure, shock, acute pancreatitis, coronary artery bypass graft surgery). Probabilities were obtained from a broad literature search. Costs were estimated using the Nationwide Inpatient Sample 2008, a representative US country-wide database and were expressed in 2010 US$. In each of the 3 treatment branches (PPI, H2RA and sucralfate), patients could be in one of three states of health: no complication (NC), SUB or VAP. A third-party payer perspective was adopted. Cost-effectiveness and sensitivity analyses were performed. A 60-day time horizon was adopted. **RESULTS:** PPI, H2RA and sucralfate treatments were associated with SUB and VAP probabilities of 5.9% and 17.2%, 5.1% and 17.7%, and 1.4% and 10.3%, respectively. Lengths of stay and per diem costs were 14 days and $2,993 for NC, 24 days and $2,764 for SUB, and 42 days and $3,310 for VAP. Average costs per no-rebleeding patient were $58,734 for PPI, $77,543 for H2RA, and $77,366 for sucralfate. H2RA and Sucralfate were dominated by PPI. These findings were robust on sensitivity and threshold analyses. Probability of complications would need to increase to 20% in the PPI group or drop to 1% in either of the other two treatment groups in order for PPI to cease being the dominant strategy. **CONCLUSIONS:** PPI prophylaxis is the dominant prophylactic strategy in patients at high-risk of developing SUB when compared to using H2RA or sucralfate.

PGI20

PHARMACOECONOMIC STUDY OF GLUTAMINE DIPEPTIDE USAGE DURING TOTAL PARENTERAL NUTRITION (TPN)

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OBJECTIVES: To undertake a comparative analysis of 2 schemes of TPN: isolated standard scheme of TPN (2 types: "all in one bag" and "1+1+1") and scheme of TPN, which includes expenses for purchasing and usage of glutamine dipeptide. **METHODS:** Pharmacoeconomic analysis "cost-effectiveness" was provided. The study estimated direct costs, because appraisal from the stand point of the Russian healthcare system was chosen: expenses for drug therapy, hospitalization (intensive care unit and medical division) and late complications (pneumonia and sepsis) treatment. Effectiveness data was taken from clinical trial: Eandi M., Pradelli S., Lanazzo S.. Alanyl-glutamine Dipeptide (Dipeptiven) in Total Parenteral Nutrition (TPN) Therapy in Critically Ill Italian Patients: A Pharmacoeconomic Simulation Model. AdRes Health Economics and Outcomes Research - Torino (Italy), 2010. Survival rate of patients was the main effectiveness criterion. Three types of TPN were compared: "3 in 1" and "1+1+1" without glutamine dipeptide usage and "3 in 1" system with glutamine dipeptide. Two-factor sensitivity analysis was carried out, which showed that results of our pharmacoeconomic study were stable. **RESULTS:** In the course of analysis the following results were obtained: direct medical expenses for 1 patient treatment with TPN system "3 in 1" were 1561,92 €; "1+1+1" – 1651,25 €; "3 in 1" + glutamine dipeptide – 1652,66 €. Taking into account the value of effectiveness rate of 3 compared TPN systems ("3 in 1" and "1+1+1" – 0,6554 and "3 in 1" + glutamine dipeptide - 0,7624) the results of Cost-Effectiveness Ratio (CER) were the following: "3 in 1" – 2383,15 €; "1+1+1" - 2519,45 € and "3 in 1" + glutamine dipeptide – 2167,71 €. **CONCLUSIONS:** According to the results of our research TPN system "3 in 1" + glutamine dipeptide is a dominant alternative as at the greatest effectiveness rate, CER result is the least of all compared systems.

PGI21

COST-EFFECTIVENESS OF PEGINTERFERON AND RIBAVIRIN FOR ELDERLY PATIENTS WITH CHRONIC HEPATITIS C: RESULTS BASED ON THE NATIONWIDE HEPATITIS REGISTRATION IN JAPAN

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OBJECTIVES: The cost-effectiveness of peginterferon and ribavirin (PEG_IFN+RBV) for elderly patients with chronic hepatitis C (CHC) was investigated. A nationwide registration of interferon-treated hepatitis patients has been conducted in Japan since 2009. This study was based on individual patient data from the registration for investigation in a real-world setting. **METHODS:** PEG_IFN+RBV-treated CHC patients 65-years or older were analyzed. All registered patients received antiviral treatment, and were assumed to suffer if not treated. The incremental cost and effectiveness of treatment was estimated as the difference between actual events and the assumed longstanding disease status. The individual patient data regarding age, gender, and duration of and response to treatment was used to estimate cost of PEG_IFN+RBV, cost of following CHC, and quality-adjusted life-year (QALY). Incremental cost effectiveness ratio (ICER) and 95% bootstrap confidence interval (CI) were calculated, and probabilistic sensitivity analysis (PSA) was done for assumptions on the distribution of uncertain data. Conservative assumptions were used throughout the analysis. **RESULTS:** There were a total of 1378 patients (median age 68 y; range 65 – 80 y). 1005 patients had hepatitis C virus type 1 (72.9%), and 1269 had a high viral load (92.1%). A platelet count of <100,000/mm3 was found in 152 patients (11.0%), 100,000 – 150,000/mm3 in 541 patients (39.3%), and ³150,000/mm3 in 655 patients (47.5%). 1106 patients completed the planned treatment (80.3%). Sustained viral response was observed in 650 cases (47.2%), relapse in 404 cases (29.3%), and no response in 324 cases (23.5%). Incremental cost was calculated to be 1.885 million yen (approximately 16,390 euros) for a patient, and effectiveness was 0.657 QALY. ICER was 2.869 million yen (approximately 24,950 euros)/QALY (95% CI: 2.665 – 3.089 million yen /QALY). PSA showed that most trials had ICER of less than 4.00 million yen/QALY. **CONCLUSIONS:** The ICER of PEG_IFN+RBV for elderly patients with CHC seemed acceptable.

PGI22

THE EXTRA HEALTH CARE COSTS ASSOCIATED WITHANTIMICROBIAL PROPHYLAXIS IN COLORECTAL SURGICAL PATIENTS: AN EXPLORATION OF PROFILING DATA FROM A UNIVERSITY HOSPITAL IN JAPAN

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OBJECTIVES: Postoperative infections bring about an expansion of length of hospital stay (LOS) and extra medical costs. **METHODS:** We analyze the relationship between variations in antimicrobial prophylaxis (AMP) and extra medical costs in surgical patients with colorectal malignancies. Utilizing profiling administrative data, we analyzed 161 admitted patients between 2007 and 2009 to a university hospital. We classified the patients into two classes based on AMP duration: the control group (112) and the case group (49). Most patients from both groups were appropriately given AMP agents consistent with the guidelines of infection-related associations. **RESULTS:** The LOS of the control group (24.6 ± 12.1 days) was shorter than that of the case (49.4 ± 35.2) ($p<0.05$). Hospitalization charge of the control group (15130 ± 3930 USD) was lower than that of the case (23130 ± 1212) ($p<0.05$), but hospitalization charge per day of the control group (670 ± 160 USD) was higher than that of the case (530 ± 130) ($p<0.05$). Furthermore, 73 cases of the control group were given on the day of surgery till the first postoperative day, and 39 cases were given to the second and third postoperative days. LOS of the former (22.7 ± 9.5 days) was shorter than that of the latter (28.3 ± 15.5) ($p<0.05$). **CONCLUSIONS:** AMP agents in our hospital were found to generally given according to the recommended guidelines. It is important for the hospital administrators to quantify the additional costs on top of the primary diagnosis in order to properly deal with infection control and hospital management.

Gastrointestinal Disorders – Patient-Reported Outcomes & Preference-Based Studies

PGI23

ECONOMIC IMPACT OF MEDICATION ADHERENCE AND PERSISTENCE IN THE TREATMENT OF ULCERATIVE COLITIS IN CANADA: ANALYSES WITH THE RAMQ DATABASE

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OBJECTIVES: The aim of this study was to assess adherence and persistence to mesalamine treatment in ulcerative colitis (UC), and to evaluate the impact on health care resource utilization and cost from a Canadian health care system perspective. **METHODS:** A retrospective prescription and medical claims analysis was conducted using a random sample of UC patients with no diagnosis of Crohn's disease who were initiated on an oral mesalamine formulation from January 2005 to December 2009. Treatment adherence (medication possession ratio [MPR]) and persistence were calculated over a 1-year period after index prescription. To evaluate the economic impact of non-adherence and non-persistence, the number and all-cause costs of physician visits, emergency visits, and hospitalizations were estimated. Statistical comparisons, based on adherence and persistence, were made using the chi-square test for proportions and Student's t-test or the F-test from one-way ANOVA for means. Statistical significance was $p<0.05$. **RESULTS:** A sample of 1681 patients was obtained. The mean age of new oral mesalamine users was 55.3 years (SD=17.8), with a similar proportion of males and females. At month 12, 27.7% of patients had a MPR ≥80%, and 45.5% of patients were persistent on treatment. Over the 12-month period, there was a statistically significant difference in overall health care resource utilization and all-cause costs in non-persistent ($4973.57) versus persistent ($3256.23) patients to UC medications ($p<0.001$, unadjusted), with hospitalizations as the major cost driver. Similar numeric differences were observed for overall health care costs associated with non-adherence versus adherence ($4357.70 versus $3758.81, $p=0.277$, unadjusted). **CONCLUSIONS:** Adherence and persistence to oral mesalamine for the treatment of UC was relatively poor in this patient cohort. Furthermore, patients who were non-persistent

Research Article

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Predictive value of the *IL28B* polymorphism on the effect of interferon therapy in chronic hepatitis C patients with genotypes 2a and 2b

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3BcKHrouNE & 2JMR: Common *IL28B* locus polymorphisms (tnPs rs8099917 and rs129798600 have been reported to affect peg-interferon plus ribavirin combination therapy (PdG-saV0for hepatitis C virus (HCV0genotype 1b2but few reports have examined their effect on other two common genotypes22a and 2b.

MeSI oER: We analyzed predictive factors for sustained virological response (tVs0in a retrospective study of 719 patients with either genotype 2a (5300or 2b (1890 of these patients2160 were treated with PdG-saV and 559 were treated with interferon monotherapy. We evaluated predictive factors including HCV snA2 histological findings2 *IL28B* tnP genotypes (rs8099917 2 rs129798602and rs129802750 and the effect of treatment regimen and prior treatment history.

ReRulSR: HCV snA viral load2treatment regimen2and rs8099917 genotypes independently contributed to the effect of the therapy. eor patients treated with PdG-saV2 rs8099917 and viral load were independent predictive factors for tVs in genotype 2b but not in genotype 2a. Conversely2in patients treated with interferon monotherapy2viral load and rs8099917 were independent predictive factors for tVs in genotype 2a but not in genotype 2b. The favorable rs8099917 genotype is also associated with a steep decline in viral load by the second week of treatment.

4oNcluRJoNR: Initial viral load and rs8099917 genotype are significant independent predictors of tVs in genotype 2 patients.

Keywords: Interferon therapy; tingle nucleotide polymorphism; sibavirin; gepatitis b.

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Abbreviations: gbw2hepatitis b virus; len2interferon; pdf -len2pegylated interferon; saw2ribavirin; pdf -saw2pegylated interferon plus ribavirin combination therapy; tnp2single nucleotide polymorphism; tws2sustained viral responder; ns2non-responder.

9NSroEucSJoN

Hepatitis C virus (HCV0infection is a major worldwide cause of chronic liver diseases2affecting an estimated 170 million people [1]. Chronic HCV infection may progress to hepatocellular carcinoma (HCC0 or liver cirrhosis (LC0 [2–6]2and in iapan260–70% of patients with HCC or LC are HCV carriers [7]. There are two major genotypes (1 and 20 and three sub-genotypes (1b2 2a2 and 2b0in iapan as well as in many other countries [8]. Although pathological features of these genotypes are similar [92 0]2interferon therapy is more effective against genotype 2 than genotype 1 [112 2]. Compared to the less than 50% of genotype 1 patients who respond to therapy [13–19]2more than 80% of genotype 2 patients who received 24-week peg-interferon and ribavirin (PdG-saV0 combination therapy achieved sustained virological response (tVs02defined as absence of HCV snA six months after the cessation of therapy. aecause of this otherwise high success rate2the small subset of genotype 2 patients who fail to respond to therapy should be examined more closely. Although treatment-resistant genotype 2 sub-populations have been reported [20–22]2the mechanism underlying variable response to treatment is unclear. multiple viral (e.g.2HCV genotype2amino acid substitutions in the nt5A and core region [22–26]0and host factors (e.g.2age [14]2body mass index [27]2and insulin resistance

Table 1. Baseline characteristics of patients with HCV genotypes 2a and 2b.

	All (n = 719)	2a (n = 530)	2b (n = 189)
Sex (M/F)	403/316	301/229	102/87
Age	57 (49-64)	56 (48-64)	59 (50-66)
Body weight (kg)	59.8 (51-71.4)	60.15 (53.75-71.65)	57.4 (48.5-70)
BMI (kg/m^2)	23.2 (20.3-25.7)	24.48 (21.43-26.4)	21.78 (19.89-24.79)
Fibrosis (F0-2/F3-4)	484/101	359/68	125/33
Treatment (IFN/PEG-RBV)	559/160	477/53	82/107
Treatment naïve (Y/N)	689/30	523/7	166/23
HCV RNA (log IU/ml)	5.3 (4.7-5.9)	5 (4.6-5.7)	5.9 (5.5-6.5)
rs8099917 (TT/GT/GG)	572/135/11	425/97/7	147/38/4
rs12979860 (CC/TC/TT)	565/137/11	422/98/7	143/39/4
rs12980275 (AA/GA/GG)	543/158/16	402/116/10	141/42/6
SVR/non-SVR	455/264	340/190	115/74

IFN, interferon monotherapy; PEG-RBV, peg-interferon plus ribavirin combination therapy; SVR, sustained viral responder.

[28]) have been reported to affect the outcome of interferon therapy in genotype 1-infected patients but such factors have not been closely examined in genotype 2 patients.

Single nucleotide polymorphisms (SNPs) and other genetic factors have been reported to be useful in predicting the outcome of interferon therapy. Polymorphisms in MxA [29,30], interferon alpha-receptor 1 [31], and osteopontin [32] have also been reported to be associated with interferon response. We also identified a MAPKAPK3 SNP [33] that is a predictive factor for interferon mono-therapy. Recently, several groups have reported an association between several SNPs in the *IL28* locus and the effect of PEG-RBV combination therapy for genotype 1b [34–38] but only a few studies have examined the role of these SNPs in the treatment of other genotypes. In this study, we analyzed predictive factors for SVR in genotype 2a and 2b patients treated with PEG-RBV. Because PEG-RBV was only approved for use in Japan in 2005, we also examined predictive factors in patients who were treated with interferon monotherapy, which is still used in the event of an adverse reaction to ribavirin.

Patients and methods

Patients and study design

We studied 719 Japanese patients with chronic hepatitis C (positive for HCV RNA for more than 6 months) who received interferon therapy with or without ribavirin between 2002 and 2008. Patients were treated at Toranomon Hospital in Tokyo, Hiroshima University Hospital, and hospitals belonging to the Hiroshima Liver Study Group (http://home.hiroshima-u.ac.jp/naika1/hepatology/english/study.html). All patients were negative for hepatitis B surface antigen, had no evidence of other liver diseases, such as auto-immune hepatitis or alcoholic liver disease, and had not received immunosuppressive therapy before enrollment in the study. All patients gave written informed consent to participate in the study in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and according to the process approved by the ethical committees of Hiroshima University and the SNP Research Center at the Institute of Physical and Chemical Research (RIKEN) in Yokohama.

PEG-RBV patients received weekly injections of peg-interferon-alpha-2b at 1.5 g/kg body weight for 24 weeks. Ribavirin was administered orally, and the dosage was determined based on the patient's body weight (600 mg for <60 kg, 800 mg for 60–80 kg, 1000 mg for >80 kg). Patients receiving interferon monotherapy were treated daily with 6 million units of IFN intramuscularly for 8 weeks, followed by the same dose three times a week for 16 weeks, for a total of 528 million units. Successful treatment was ascertained based on sustained virological response (SVR), defined as HCV RNA-negative six months after cessation of therapy. Fibrosis stage and activity were diagnosed by pathologists at each hospital according to the criteria of Desmet et al. [39]. Patients were classified as interferon treatment naïve or experienced based on prior interferon treatment but only parameters related to the most recent therapy were used in the analysis.

SNP Genotyping and quality control

We genotyped each patient for three *IL28B* SNPs previously reported to be associated with therapy outcome: rs8099917, rs12979860, and rs12980275. Samples were genotyped using the Illumina HumanHap610-Quad Genotyping BeadChip or the Invader assay, as described previously [40,41]. We were unable to determine genotypes for one of the 796 patients for rs8099917, six of the patients for rs12979860, and two for rs12980275.

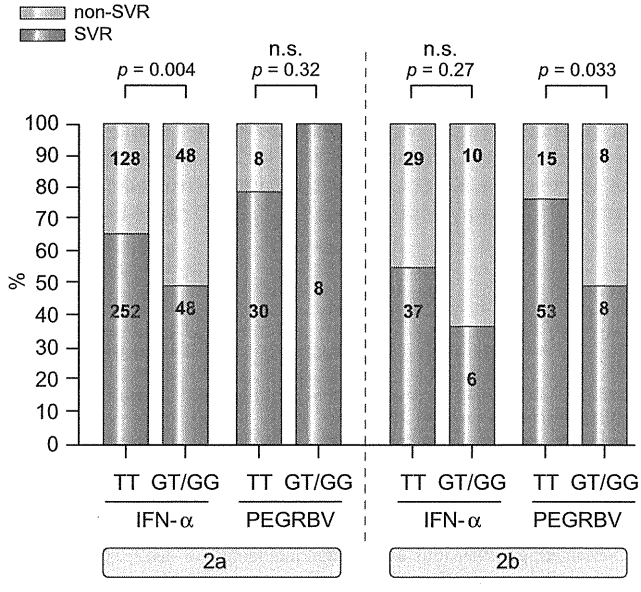

Fig. 1. Effect of interferon therapy on patients with genotype 2a and 2b infection. Sustained viral responders (SVR) and non-responders (non-SVR) were analyzed by IL28B SNP rs8099917 genotype, viral genotype, and treatment type. All patients were interferon-naïve.

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Table 2. Predictors for SVR in treatment-naïve patients treated with peg-interferon plus ribavirin combination therapy.

		Simple		Multiple			
Genotype	Variable	n	p	n	OR	(95% CI)	p
2a + 2b	Age	130	0.42				
	Sex	130	0.62				
	Genotype	130	0.21				
	Viral load	127	0.002 **	127	0.19	(0.06-0.55)	0.002 **
	Fibrosis	110	0.25				
	rs8099917	130	0.23				
	rs12980275	129	0.79				
2a	Age	46	0.77				
	Sex	46	0.62				
	Viral load	44	0.16				
	Fibrosis	39	0.75				
	rs8099917	46	0.8				
	rs12980275	45	0.77				
2b	Age	84	0.14				
	Sex	84	0.58				
	Viral load	83	0.01 *	83	0.13	(0.03-0.62)	0.01 *
	Fibrosis	71	0.08				
	rs8099917	84	0.03 *	83	0.23	(0.06-0.80)	0.02 *
	rs12980275	84	0.21				

1p <0.05; 11p <0.01; 111p <0.001.

HCV RNA levels

gbwsnV levels2corresponding to initial viral load2were measured using one of several sT-pbs-based methods .the original Vmplicor method2the high range method2or the TaqMan sT-pbs test). The measurement ranges of these assays were 0.5–H50 KIU/ml25–5000 KIU/ml2and 1.2–F.Hlog IU2respectively. taturated samples were diluted with pat and reanalyzed. Vll values were reported as log IU/ml.

Statistical analysis

f enotype-based associations were tested using the bochran–Vrmitage trend test. bombined analysis was performed using the Mantel–gaenszel method. timple and multiple regression analyses were used to examine the association between viral and clinical factors using p <0.05 as the criterion for inclusion in the multivariate model. gbwsnV was converted into a binary variable based on the median. Multivariate logistic regression analysis was performed using the cesign package in *R* .http://www.r-project.org) with fast backward elimination and validation based on Vlb score for model construction.

Results

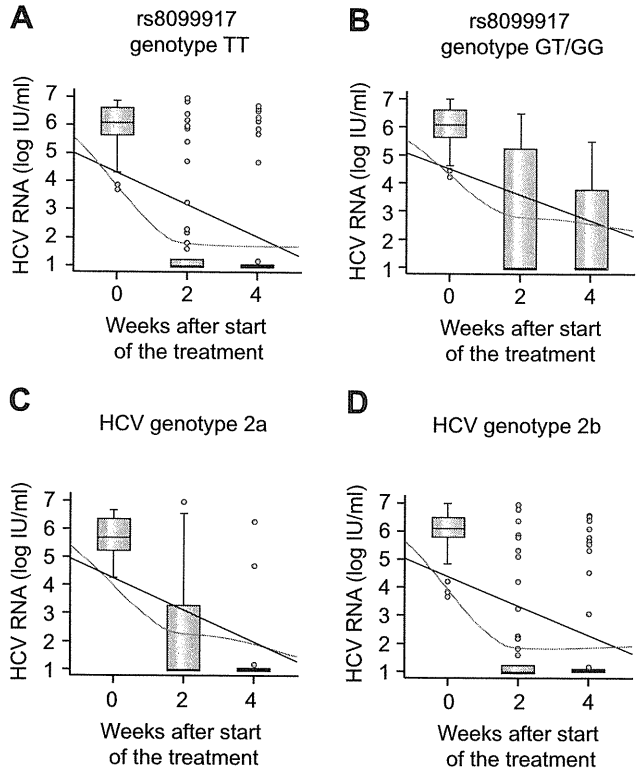
Clinical characteristics are summarized by genotype in Table 1. The tVs rate was slightly but not significantly higher among patients with genotype 2a (340 out of 530; 64%0 compared to genotype 2b patients (115 out of 189; 61%0 (p = 0.430 Patients who were treated with PdG-saV had a slightly but not significantly higher rate of tVs (111 out of 160; 69%0than patients treated with interferon monotherapy (344 out of 559261%0(p = 0.080 aecause the number of patients treated with interferon monotherapy (5590greatly exceeds the number of patients treated with PdG-saV (16002 patients were analyzed separately by treatment type. aecause 30 out of the 719 patients (4%0had received prior interferon treatment2only treatment-naïve patients were included in the analyses mentioned below2followed by a separate analysis of the effect of prior interferon treatment on tVs rate.

IL28B polymorphisms

minor allele frequencies for rs80999172 rs129798602 and rs12980275 were 0.1092 0.1122 and 0.1322 respectively. The frequency of the rs8099917 risk allele was lower in tVs patients than non-tVs patients (0.089 vs. 0.14; p = 1.03e-050 The risk allele frequency among all patients was slightly higher than in the Hapmap-iPT population (0.109 vs. 0.093; p = 0.010but lower than in the Hapmap-CdU population (0.109 vs. 0.183; p = 1.6e-050 We compared rs8099917 allele and genotype frequencies with 900 healthy iapanese subjects but found no significant differences. 67% of patients (372 out of 5520with the favorable rs8099917 TT genotype achieved tVs 2compared to 51% (70 out of 1360of patients with GT or GG genotypes. eig. 1 shows the joint effects of treatment type2viral genotype2and rs8099917 genotype. In every case results for rs8099917 and rs12979860 are the same2but both factors cannot be included in a multivariate model simultaneously due to multicollinearity2so results for rs8099917 are presented due to the higher genotyping success rate.

Predictive factors for SVR in patients treated with PEG-RBV

Among treatment-naïve patients treated with PdG-saV278% (83 out of 1060of patients with rs8099917 TT achieved tVs compared



7JH(. (6GFDSoGQR0) 1111/ HFNoSWPF BNE 8 4A HFNoSWPF oN DI BNHF JN 8 4A R; 2 IFUFIR HCV RNA levels at 0, 2, and 4 weeks after the start of peg-interferon plus ribavirin combination therapy in treatment-naïve patients. (A and B) Change in viral load for patients with the protective TT genotype for rs8099917 (A) compared to patients with the GT or GG genotypes (B). (C and D) Change in viral load for patients with HCV genotype 2a (A) versus genotype 2b (B).

to 67© (16 out of 24) of patients with non-TT genotypes ($p = 0.29$). In univariate and multivariate analyses, only viral load was an independent predictive factor for SVR ($p = 0.002$; Table 2), but when we examined genotypes 2a and 2b separately, rs8099917 genotype ($p = 0.02$) and viral load ($p = 0.01$) were both significant independent predictors of SVR for patients with genotype 2b, whereas no significant univariate or multivariate predictors were found for patients with genotype 2a. Notably, however, all 8 patients with genotype 2a with rs8099917 GT/GG achieved SVR (Fig. 1). The same pattern held for patients with rs12979860 TC/TT (9 SVR, 0 non-SVR) and rs12980275 GA/GG (11 SVR, 0 non-SVR) genotypes. Moreover, none of these patients was homozygous for the risk allele at each SNP.

Change in HCV RNA levels for patients treated with PEG-RBV

HCV RNA levels at the start of PEG-RBV therapy and after 2 and 4 weeks of treatment are plotted by rs8099917 genotype and viral genotype in Fig. 2. Under multivariate analysis, rs8099917 genotype was an independent predictive factor for change in HCV RNA level by week 2 ($p = 0.036$) but viral genotype was not significant ($p = 0.15$). For changes in HCV RNA levels by week 4, neither the rs8099917 genotype nor the viral genotype was significant ($p = 0.17$ and $p = 0.22$, respectively).

Predictive factors for SVR in patients treated with interferon monotherapy

Among patients treated with interferon monotherapy, 65© of patients with rs8099917 TT achieved SVR, compared to only 48© of patients with GT or GG genotypes ($p = 0.002$). Viral load and the rs8099917 and rs12980275 genotypes were significant univariate predictors of SVR, and under multivariate analysis viral load and rs8099917 remained as independent predictors (Table 3). When genotypes 2a and 2b were analyzed separately, viral load ($p = 0.001$) and rs8099917 genotype ($p = 0.014$) were independent predictive factors for SVR in patients with genotype 2a but no significant univariate of multivariate terms were found for genotype 2b.

Effect of prior interferon treatment

Thirty out of the 719 patients (4©) had previously received treatment with interferon. Among these patients, only 40© achieved SVR, compared to the 64© SVR rate among treatment-naïve patients. Initial viral load was the only independent predictor of SVR in these patients, whereas in treatment-naïve patients, viral load, rs8099917 genotype, and treatment type (PEG-RBV vs interferon monotherapy) were independent predictors of SVR (Table 4).

Development of resistance to interferon therapy

Over the course of therapy five patients developed resistance to PEG-RBV treatment. In each case the patient showed an initial drop in viremia followed by viral breakthrough. Three out of the five patients were heterozygous (T/G) for the rs8099917 genotype and two out of the five were homozygous for the favorable allele (T/T).

5JRcuRRJoN

As the effect of *IL28B* polymorphism has not been reported separately for genotype 2 and its subtypes so far, we investigated whether the polymorphism influences treatment outcome in patients with HCV genotype 2a and 2b infections. In addition to previously reported effects for genotypes 1 and 4, our results demonstrate that polymorphisms in the *IL28B* locus are also predictive for SVR in genotype 2 (Table 2). We also showed that the favorable *IL28B* SNP genotype is associated with a rapid decrease in HCV RNA levels, which is itself a predictive factor for SVR [42]. Several studies have reported that polymorphisms at the *IL28B* locus affect the outcome of peg-interferon and ribavirin combination therapy in patients with HCV genotype 1b [34–36,38]. In particular, associations with therapy outcome have been reported for two SNPs in strong linkage disequilibrium, rs8099917 (T/G), and rs12979860 (C/T). Only a few studies have examined the effect of the SNP on the treatment outcome for other genotypes. Rallón et al. reported that the rs12979860 genotype is associated with treatment outcome for genotypes 1 and 4 but not genotype 3 in patients with HIV/HCV co-infection [43]. Similarly Rauch et al. reported an association between rs8099917 polymorphism and NVR for genotypes 1 and 4 (difficult-to-treat) but not for genotypes 2 and 3 (easier-to-treat) but the effect due to genotype 2 alone is unclear [38]. In a recent study, Mangia et al. also exam-

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Table 3. Predictors for SVR in treatment-naïve patients treated with IFN monotherapy.

		Simple			Multiple			
Genotype	Variable	n	p		n	OR	(95% CI)	p	
2a + 2b	Age	559	0.35						
	Sex	559	0.17						
	Genotype	559	0.068						
	Viral load	507	0.0002	***	506	0.59	(0.45-0.77)	0.0001	***
	Fibrosis	450	0.61						
	rs8099917	558	0.001	**	506	0.52	(0.33-0.82)	0.005	**
	rs12980275	558	0.009	**					
2a	Age	477	0.19						
	Sex	477	0.2						
	Viral load	425	0.001	**	424	0.6	(0.44-0.81)	0.001	***
	Fibrosis	382	0.37						
	rs8099917	476	0.003	**	424	0.53	(0.32-0.88)	0.014	*
	rs12980275	476	0.01	**					
2b	Age	82	0.67						
	Sex	82	0.56						
	Viral load	82	0.47						
	Fibrosis	68	0.53						
	rs8099917	82	0.19						
	rs12980275	82	0.44						

1p <0.05; 11p <0.01; 111p <0.001.

ined genotypes 2 and 3 and found a significant association between rs12979860 genotype and rapid virological response (sVs0 at week 4 for genotype 2 [44]. While rs12979860 was not directly associated with tVs in their study2rs12979860 genotype was significantly associated with tVs among those patients who failed to achieve sVs. In this study2we found a significant association between rs8099917 genotype and sVs in multivariate analysis for genotype 2b (p = 0.0282data not shown0but not for genotype 2a. When sVs was included as a factor in multivariate logistic regression analysis for genotype 2b2 sVs and rs8099917 genotype were both retained in the final model but only sVs was significant (sVs: p = 4.9e-05; rs8099917: p = 0.0850; data not shown0 When only non-sVs patients were included2no factors were significant; however2there were only six patients who achieved tVs without sVs and only one patient who achieved sVs but then failed to achieve tVs.

Although tVs rate was generally higher for genotype 2a2as reported previously [2021]2we found few differences between genotypes 2a and 2b. However2when analyzed separately2the results suggest an interesting interaction between the *IL28B* genotype2the viral genotype2and treatment type. In particular2we found that rs8099917 was a predictive factor for genotype 2a treated with len but not PdG-saV2and conversely for genotype 2b treated with PdG-saV but not len. This result is likely due to the relatively small sample sizes2but nonetheless all 8 (100%0of the genotype 2a PdG-saV patients lacking the favorable rs8099917 genotype achieved tVs 2compared to less than 50% for len therapy or either type of treatment with genotype 2b. In fact2each patient was heterozygous for each of the three *IL28B* tnPs examined. A further complication is that each of the five patients who developed resistance to interferon therapy was infected with genotype 2a2 and two of these patients had the favorable rs8099917 TT genotype while the others were heterozygous (GT0 more detailed analysis will be required to interpret these results.

aecause PdG-saV therapy was not covered by insurance in iapan until 20052we also present data comparing the effects of *IL28B* polymorphisms on treatment with the older len monotherapy versus the more recent PdG-saV combination therapy. Although the small sample sizes within each patient group likely underestimate the effect of tnP genotype2 we found that rs8099917 influences response to len monotherapy in patients with genotype 2a and also influences the response to PdG-saV therapy in patients with genotype 2b. Although PdG-saV is currently the standard treatment for chronic hepatitis C infection2 interferon monotherapy may still be used in the case of intolerance to ribavirin; therefore2it is important to understand the direct effects of interferon with and without ribavirin. moreover2 even with the advent of protease inhibitors and other antiviral drugs undergoing clinical trials2they are likely to be co-administered with interferon to prevent the otherwise rapid emergence of resistant quasispecies [45].

In summary2we showed that the *IL28B* tnP genotype is an important predictive factor for tVs and early viral dynamics in patients with HCV genotypes 2a and 2b.

Conflict 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.

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		Simple			Multiple				
	Variable	n	p		n	OR	(95% CI)	p	
All	Age	719	0.70						
	Sex	719	0.28						
	Genotype	719	0.42						
	Viral load	663	6.00E-02	***	662	0.63	(0.51-0.79)	4.30E-05	***
	Fibrosis	585	0.83						
	rs8099917	718	0.002	**	662	0.57	(0.38-0.85)	0.0055	**
	rs12980275	717	0.03	*					
	Treatment	719	0.054						
Naïve	Age	689	0.58						
	Sex	689	0.18						
	Genotype	689	0.62						
	Viral load	634	0.0011	**	633	0.53	(0.41-0.69)	2.00E-06	***
	Fibrosis	560	0.95						
	rs8099917	688	0.00059	***	633	0.5	(0.33-0.77)	0.0015	**
	rs12980275	687	0.013	*					
	Treatment	689	0.0013	**	633	3.01	(1.82-4.99)	1.80E-05	***
Experienced	Age	30	0.91						
	Sex	30	0.75						
	Genotype	30	0.14						
	Viral load	29	0.032	*	29	0.21	(0.05-0.87)	0.032	*
	Fibrosis	25	0.53						
	rs8099917	30	0.12						
	rs12980275	30	0.1						
	Treatment	30	N/A						

*p <0.05; **p <0.01; ***p <0.001.

2cKNoV leEHMeNSR

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IL28 variation affects expression of interferon stimulated genes and peg-interferon and ribavirin therapy

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6FHOLS uRI © 5 Nn V: Common genetic variation within the IL28 locus has been found to influence the effect of peg-interferon and ribavirin combination therapy against chronic hepatitis C virus (HCV) infection. Expression of *IL28* in peripheral blood cells has been reported to be higher in patients with *IL28* SNP genotypes associated with favorable response.

MJ VWS I V: We analyzed 52 liver and 114 blood samples obtained from patients with HCV genotype 1b. We used reverse transcription-real time polymerase chain reaction to analyze expression levels of *IL28* and several interferon stimulated genes (ISGs), including *MxA*, double stranded RNA dependent protein kinase (*PKR*), 2′–5′ oligo-nucleotide synthetase (*OAS1*), *ISG15*, and *SOCS1*.

BJ VuPW: Interestingly, expression of *IL28* was significantly lower in patients with the response-favorable rs8099917 TT genotype compared to those with TG or GG genotypes (p <0.005). In hepatic cells, expression of *MxA*, *PKR*, *OAS1*, and *ISG15* were also significantly lower in rs8099917 TT patients (p <0.001, p = 0.005, p = 0.001, p <0.001, respectively), whereas in peripheral blood mononuclear cells ISG expression levels did not differ significantly. Among patients treated with peg-interferon plus ribavirin therapy, liver mRNA levels of *IL28*, *MxA*, *PKR*, *OAS1*, and *ISG15* were significantly or marginally lower in responders who became negative for HCV RNA (p = 0.001, 0.004, 0.014, 0.051, and 0.015, respectively).

7SRHuVS RV: Expression levels of ISGs are differentially regulated in the liver and peripheral blood. The mechanism underlying the expression levels of IL28 and ISGs and the correlation with the effect of the therapy should be further investigated.

Keywords: IL28; Liver biopsy; ISG15; MxA; Single nucleotide polymorphism.
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Abbreviations: HCV, hepatitis C virus; ISDR, interferon sensitivity determining region; IRRDR, interferon and ribavirin response determining region; SNP, single nucleotide polymorphism; SVR, sustained viral responder; NVR, non-viral responder; OAS1, 2′–5′ oligoadenylate synthetase 1 ; PKR, double stranded RNA dependent protein kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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Chronic hepatitis C virus infection often results in the development of chronic hepatitis, which leads to cirrhosis and hepatocellular carcinoma [1,2]. Currently, patients with chronic HCV infection are treated with a combination of pegylated interferon and ribavirin [3,4]. The eradication rate of the virus has been reported to be about 50% in patients treated with the standard 48 week therapy [4–6]. Although the eradication rate of the virus has been slightly improved by extending the treatment period to 72 weeks, there are many patients who fail to eradicate the virus [7]. Furthermore, many patients fail to complete the therapy because of severe side effects.

Many predictive factors have been reported so far that affect response to combination therapy. Viral factors, such as substitutions at core amino acids 70 and 91 [8,9], or within the interferon sensitivity determining region (ISDR) [10,11] or the interferon and ribavirin response determining region (IRRDR) [12] have been reported.

Among host factors, many single nucleotide polymorphisms (SNPs) associated with outcome of therapy have been identified. They include SNPs in interferon-alpha pathway genes [13] and interferon induced genes [14], within the promoters of the *MxA* [15] and osteopontin [16] genes, and within an intron of *MAPK APK3* [17].

Recently, Ge *et al.* [18] identified SNPs located 5′ to the *IL28B* gene that affect response to combination therapy. Furthermore, two other research groups also independently reported that these SNPs are associated with the effectiveness of combination therapy [19,20]. More recently, Thomas *et al.* reported that the SNP allele related to favorable therapy response is also associated with spontaneous clearance of HCV [21]. They reported that the allele related to HCV clearance is the major allele in the majority of Asian and European countries.

IL28A, *IL28B*, and *IL29* gene products belong to the interferon lambda family [22,23]. These cytokines are interferons functionally, but have been reported to be structurally related to the IL-10 family [24]. IL29 has been reported to reduce the replication levels of the HCV replicon [25] as well as hepatitis B virus [26]. IL29 has also been reported to reduce the replication of HCV cooperatively with interferon alpha and gamma [27]. These observations suggest that higher expression levels of interferon lambda should be observed in the liver and should correspond with a favorable response to therapy. However, no report has analyzed the expression levels of these cytokines and levels of ISG expression in the liver. In this study, we investigated mRNA expression levels of *IL28*, IL28 receptor, and several ISGs using biopsy samples obtained from patients with chronic hepatitis C and analyzed the relationship between the *IL28* genotype and the effect of combination therapy.

Materials and methods

Patients

We analyzed liver specimens from 52 patients who underwent liver biopsies at Hiroshima University Hospital between December 2002 and November 2008 and who were treated with a peg-interferon plus ribavirin combination for chronic hepatitis C genotype 1b at the same or other hospitals. Clinical characteristics of patients are shown in Table 1. Patients received weekly injections of peg-interferon-alpha-2b for 48 weeks with the dosage adjusted by body weight (60 μg for 35–45 kg, 80 μg for 46–60 kg, 100 μg for 61–75 kg, 120 μg for 76–90 kg, and 150 μg for 91–120 kg). Ribavirin was administered orally with the dosage based on body weight (600 mg for <60 kg, 800 mg for 60–80 kg, and 1000 mg for >80 kg). Ribavirin dosage was reduced when hemoglobin levels were reduced to 10.0 g/dl and stopped if hemoglobin levels reached 8.5 g/dl. The response to therapy categories are defined as follows: sustained viral responders (SVR) were negative for HCV RNA 24 weeks after cessation of therapy; relapsers were negative for HCV RNA only transiently during and after the therapy; and non-viral responders (NVR) never became negative for HCV RNA. Liver biopsy specimens, which were obtained in routine clinical practice in an amount beyond what was needed for pathological diagnosis, were kept frozen at −80 °C until analysis. Liver samples obtained by surgical operation from patients who received resection for hepatocellular carcinoma were also kept frozen. Fibrosis stage and activity were diagnosed according to the criteria of Desmet *et al.* [28].

Although we attempted to analyze blood samples from the same patients who provided liver specimens, more than half of these patients were not treated at Hiroshima University Hospital. Accordingly, we collected blood samples from 114 genotype 1b patients who visited Hiroshima University Hospital from November 2009 to March 2010 to analyze ISG mRNA levels. We excluded patients who were under treatment with therapies including interferon or immunosuppressants. Patients who had eliminated HCV with therapy were also excluded. Clinical characteristics of patients who contributed blood samples for ISG analysis are shown Table 1.

All patients provided written informed consent to participate in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved *a priori* by the ethical committee of Hiroshima University and RIKEN.

Genotyping

We genotyped SNPs rs8099917 and rs12979860 from 52 patients using either the Invader assay or the Taqman assay. In the Invader assay, allele-specific oligonucleotide pairs and invasive probes were designed and supplied by Third Wave Technologies (WI). FRET probes were labeled with FAM or VIC corresponding to alleles. The 10 μl reaction volume consisted of 0.5 μl of signal buffer, 0.5 μl of FRET probes, 0.5 μl of structure-specific cleavage enzyme, 1 μl of allele-specific probe mix, and 2 μl of PCR product diluted 1:10. Samples were incubated at 95 °C for 5 min and then at 63 °C for 15 min in an ABI PRISM 7700 (Applied Biosystems), and then fluorescence data were collected. Signal intensity was calculated as the ratio of FAM or VIC to ROX, an internal reference. Genotypes were determined visually in the dye components view of the SDS software.

In the TaqMan assay, we carried out PCR using TaqMan Universal PCR Master Mix (Applied Biosystems, CA), 1 ng DNA, 0.2 μM of each primer, and 40 nM of probe provided by Applied Biosystems in 3-μl reactions. Each 384-well plate contained 376 samples of an unknown genotype and 8 no-DNA control samples. Thermal cycle conditions were 50 °C for 2 min, 95 °C for 10 min, 50 cycles of 92 °C for 15 s, and 58 °C for 1 min. Thermal cycling was done on an ABI PRISM 7700 Sequence Detector System (Applied Biosystems), and then fluorescence data were collected and the genotypes were determined using the SDS software [29,30].

We calculated linkage disequilibrium using the LD method in the genetics library in the R 2.11 statistics package (http://www.r-project.org) and found high linkage disequilibrium between rs8099917 and rs12979860 ($r^2 = 0.99$ and $D' = 1$).

Quantitative analysis of mRNA of ISGs

Total RNA was extracted from cell lines using the RNeasy Mini Kit (Qiagen, Valencia, CA). One microgram of each RNA sample was reverse transcribed with ReverseTra Ace (TOYOBO Co. Ltd., Japan) and Random Primer (Takara Bio, Kyoto, Japan). We quantified the mRNA for *IL28*, *MxA*, 2′–5′ oligoadenylate synthetase1 (*OAS1*), double stranded RNA dependent protein kinase (*PKR*), *ISG15*, and *SOCS1* with the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). As it was difficult to measure *IL28A* and *IL28B* mRNA separately, we measured *IL28A* plus *IL28B* mRNA and expressed *IL28* mRNA. Amplification and detection were performed using an ABI PRISM 7300 (Applied Biosystems). Results were normalized to the transcript levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Measurement of MxA protein in peripheral mononuclear cells

The MxA protein level in whole blood sample was measured using an ELISA system (MxA ELISA Kit, Kyowa Medex, Tokyo, Japan). Briefly, lysing solution was added to blood samples and the lysate was applied to ELISA plates coated with

	Treated patients	WBC patients
Characteristic	(n = 52)	(n = 114)
Age [median (range)]	56 (31-77)	63 (30-88)
Sex (Male/Female)	29/23	63/51
ALT [median (range)] IU/L	47 (13-246)	62 (15-259)
γ-GTP [median (range)] IU/L	47 (15-708)	53 (10-469)
Fibrosis (F1/F2/F3/F4)	20/18/4/10	23/28/17/11
Activity (A1/A2/A3)	13/30/9	14/48/14
Virus titer [median (range)] kIU/L	850 (15-6500)	850 (0.5-8200)
Core 70[a] (Wild/Mutant/ND)	27/19/6	40/21/53
Core 91[a] (Wild/Mutant/ND)	24/22/6	34/27/53
ISDR[b] substitutions (0/1/>2/ND)	14/16/12/10	37/12/9/56
rs8099917 allele (TT/TG/GG)	30/17/5	88/33/1
Outcome of therapy (SVR/relapser/NVR)[c]	25/19/8	not applicable

Outcome of therapy	TT	TG	GG
SVR	20	5	0
Relapser	4	8	2
NVR	1	4	3
Total	42	24	6

[a]Hepatitis C virus core amino acid (aa) 70R and 91L are considered wild type, while substituted amino acids are considered mutants. ND, not determined.
[b]Interferon sensitivity determining region: the number of substitutions relative to the ISDR of the reference sequence [31].
[c]SVR, sustained viral responder; NVR, non-viral responder.

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a MAb (KM1135, Kyowa Medex, Tokyo, Japan). After 2 h of incubation, the plates were washed, and a different peroxidase-labeled MAb (KM1124, Kyowa Medex) was added. After 1 h of incubation and washing, substrate was added. Chemiluminescence was detected using Multiskan MS (Labsystems Version 8.0, Helsinki, Finland). The sensitivity of MxA in this ELISA system was 3.2 ng/ml.

Analysis of amino acid sequences in the core and ISDR region

PCR amplification and nucleotide and amino acid sequence analysis of core and ISDR were performed as reported previously [31] with a slight modification. Briefly, HCV RNA was extracted from 100 μl serum samples by SepaGene RV-R (Sanko Junyaku Co., Tokyo, Japan) and dissolved in 20 μl of H_2O. The RNA was then reverse-transcribed with random primers and MMLV reverse transcriptase (Takara Shuzo, Tokyo, Japan). The resultant cDNA was then amplified by nested PCR. PCR was performed in 25 μl of the reaction mixture containing 2.5 mM $MgCl_2$, 0.4 mM of each dNTP, 20 pmol of each primer, and 1.25 U of LA Taq (Takara Bio Inc., Otsu, Japan) with a buffer supplied by the manufacturer. One microliter of 10 × -diluted products from the first PCR was used as a template for the second PCR. The PCR primer sequences are listed in Table 2. The PCR protocol involved initial denaturation at 95 °C for 5 min, 35 cycles of denaturation for 30 s at 94 °C, annealing of primers for 1 min at 57 °C and extension for 1 min at 72 °C, followed by a final extension at 72 °C for 7 min. The amplified DNA fragments were separated onto a 2% agarose gel and purified with the QIAquick gel extraction Kit (Qiagen, Hilden, Germany). Nucleotide sequences were determined using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc., CA).

The obtained nucleotide and amino acid sequences were compared with the prototype sequences of genotype 1b HCV-J (GenBank Accession No.: D90208) [32]. Amino acids at positions 70 and 91 of the core region identical to the reference sequence (arginine and leucine, respectively), were considered as wild type. The number of amino acid substitutions in the ISDR was determined as in Enomoto *et al.* [11,12].

Statistical analysis

Statistical analysis was performed using R version 2.11 or PASW Statistics 18 (SPSS Inc., IL). Categorical data were analyzed using Fisher exact tests, and continuous data were analyzed using the non-parametric Mann–Whitney *U* test. Given the large number of possible predictors, we used multiple logistic regression with variable selection to identify a model with the most important predictors for virological response. To identify independent predictive factors, variables that were significant at the 0.05 level in univariate non-parametric tests were considered as candidate factors for multiple logistic regression analysis. Multicollinearity among predictor variables were examined using hierarchical clustering based on Spearman rank. The model was reduced using forward/backward stepwise selection using the stepAIC function in R, and then boostrap validation was performed using the rms library (formerly called the Design library). Partial residual plot and leverage plots were examined to identify outliers and assess model assumptions. The rms calibrate function was used to calculate R^2 shrinkage, and log odds were corrected for over-optimism using penalized maximum likelihood [33].

Table 2. Primers used in this study.

HCV core protein		
	outer forward	5'-GCC ATA GTG GTC TGC GGA AC-3'
	outer reverse	5'-GGA GCA GTC CTT CGT GAC ATG-3'
	inner forward	5'-GCT AGC CGA GTA GTG TT-3'
	inner reverse	5'-GGA GCA GTC CTT CGT GAC ATG-3'
HCV NS5A ISDR[a]		
	outer forward	5'-TTC CAC TAC GTG ACG GGC AT-3'
	outer reverse	5'-CCC GTC CAT GTG TAG GAC AT-3'
	inner forward	5'-GGG TCA CAG CTC CCA TGT GAG CC-3'
	inner reverse	5'-GAG GGT TGT AAT CCG GGC GTG C-3'

[a]Interferon sensitivity determining region.

Results

IL28B SNP genotype and mRNA expression levels of ISGs in liver samples

We genotyped two SNPs (rs8099917 and rs12979860) in the *IL28B* locus, which have been reported to affect the outcome of the therapy, and compared them with mRNA expression levels in ISGs. Because of linkage disequilibrium, the results are the same for both SNPs, and thus only results for rs8099917 are presented. Other SNPs in this locus for the association with therapy outcome were several orders of magnitude less significant (data not shown). Expression levels of *IL28* mRNA in blood cells have been reported to be significantly higher in the patients homozygous for the response favorable allele (rs8099917 TT or rs12979860 CC) in peripheral blood [19,20]. However, our results showed that expression levels of *IL28* mRNA in the liver were significantly lower in rs8099917 TT patients (Table 3). Furthermore, hepatic mRNA levels of each of the major anti-viral ISGs, i.e., *MxA*, *PKR*, *OAS1*, and *ISG15* were significantly lower in rs8099917 TT patients (Table 3). In contrast, expression levels of *SOCS1*, which functions as a repressor of interferon signaling, did not differ significantly between the two groups of patients (Table 3).

IL28B SNP genotype and mRNA expression levels of ISGs in peripheral blood

We examined mRNA expression levels in blood cells. In contrast to liver expression levels, mRNA expression levels of *IL28* and other ISGs were not statistically different between the two groups of patients (Table 3). *IL28B* mRNA levels, as well as four of the five ISGs, were only slightly higher in rs8099917 TT patients (Table 3).

MxA protein levels in peripheral mononuclear cells

We examined the levels of MxA protein in the peripheral mononuclear cells of 43 patients with genotype 1b chronic hepatitis C who were treated with combination therapy. In this case, consistent with previous reports [19,20], the protein levels of MxA were marginally higher in patients homozygous for the major allele (Fig. 3). Furthermore, MxA protein levels in these patients were significantly higher two days after the beginning of therapy (Fig. 1).

IL28 locus genotypes and the effect of combination therapy

Fifty-two patients with chronic hepatitis C genotype 1b were treated with combination therapy. Numbers of SVR, relapser, and NVR patients were 25 (48%), 19 (37%) and 8 (15%), respectively. Responses to therapy by rs8099917 genotype are noted in Table 1. SVR was most frequent in rs8099917 TT patients.

Effect of the combination therapy and mRNA expression levels

As shown in Fig. 2, when patients were divided into VR (SVR + relapser) and NVR categories, expression levels of *IL28*, *MxA*, *PKR*, *OAS1*, and *ISG15* were significantly higher in NVR patients (Fig. 2). There was no significant difference in *SOCS1* mRNA expression between the two groups of patients. Similarly, when patients were classified as SVR and non-SVR (relapser and

Table 3. Effect of rs8099917 genotype on ISG expression in hepatic and peripheral blood cells.

ISG	TT		GT/GG		p
Liver					
IL28	0.00044	(0.00012-0.005)	0.012	(3E-04-0.023)	0.00493
IL28RA	0.0013	(0.00084-0.0019)	0.0015	(0.0011-0.0019)	0.39
MxA	0.0034	(0.0011-0.0094)	0.02	(0.0084-0.06)	8.04E-05
PKR	0.25	(0.022-0.45)	0.77	(0.26-1.1)	0.00493
OAS1	0.18	(0.10-0.31)	0.54	(0.22-1.1)	0.00106
ISG15	0.29	(0.14-0.59)	2	(0.87-3.9)	8.65E-07
SOCS1	0.0016	(0.0011-0.0024)	0.0017	(0.0012-0.0030)	0.707
Peripheral blood					
IL28	0.00078	(0.00045-0.0010)	0.00062	(0.00032-0.001)	0.31
IL28RA	0.016	(0.011-0.023)	0.015	(0.011-0.02)	0.34
MxA	0.011	(0.0043-0.029)	0.011	(0.0036-0.053)	0.9
PKR	0.18	(0.12-0.3)	0.18	(0.10-0.27)	0.386
OAS1	1.9	(0.75-3.4)	1.3	(0.85-2.3)	0.242
ISG15	3	(1.2-7.7)	2.7	(1.7-4.9)	0.59
SOCS1	0.022	(0.014-0.032)	0.019	(0.014-0.027)	0.292

The median and interquartile range are shown for the TT and GT/TT genotypes for SNP rs8099917 in the hepatic cells (upper) and in peripheral blood mononuclear cells (lower). Results of Mann–Whitney *U* test for effect of genotype on ISG expression levels are shown.

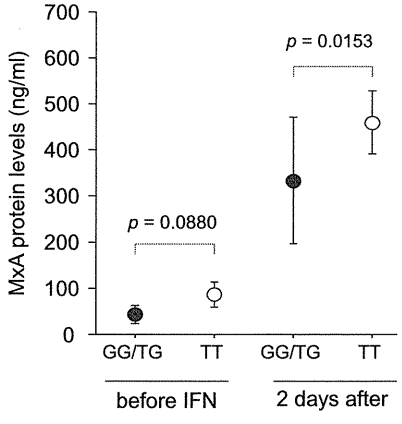

Fig. 1. MxA protein levels in peripheral white blood cells before and two days after the beginning of therapy. Points are classified by rs8099917 genotype (GG/TG vs. TT).

NVR), expression levels were also higher in non-SVR patients than SVR for all ISGs except *SOCS1*, although statistical significance was seen only for *Mx1* and *ISG15* ($p = 0.033$ and 0.031, respectively) (data not shown).

IL28 locus genotypes and amino acid sequences of core and ISDR

As amino acid mutations in the core protein and ISDR region have been reported to be associated with the effect of combination therapy, we examined the relationship between *IL28* genotype and amino acid substitutions within the ISDR and at core amino acids 70 and 91. mRNA expression of the genes examined tended to be higher in patients with core amino acid 70 and 91 mutants and ISDR mutants, and expression levels of *IL28* ($p = 0.035$), *MxA* ($p = 0.031$), and *SOCS1* ($p = 0.018$) were significantly higher in patients with amino acid 91 substitutions (Fig. 3).

Factors associated with the effect of combination therapy

We examined combinations of factors associated with the effect of combination therapy for patients with genotype 1b. Gene expression levels among ISGs were correlated (Fig. 4). To identify factors that contribute independently to virological response, we performed multiple logistic regression analysis using ISG expression levels as well as *IL28B* genotypes and the number of viral substitutions for patients with HCV genotype 1b (Table 4). Following forward/backward stepwise selection based on AIC score, only *ISG15*, *MxA*, *IL28*, and *OAS1* remained in the model, and only *MxA* was significant at the 0.05 level. Age, sex, and other patient and viral factors were not significant.

Discussion

The association of *IL28* locus polymorphisms and response to peg-interferon and ribavirin combination therapy has been reported independently by three groups of researchers [18–20]. Two of the three studies have reported that expression of IL28 in peripheral leukocytes was higher in patients homozygous for the favorable allele [19,20]. It seems reasonable that higher levels of IL28 combined with administration of peg-interferon and ribavirin is related to better response to the therapy. In fact, an additive effect of lambda interferon and alpha interferon has been reported [27]. Accordingly, we assumed that expression levels should be also higher in the liver in such patients.

Interestingly, however, the expression levels of *IL28* were significantly lower in rs8099917 TT patients (Table 3). Expression

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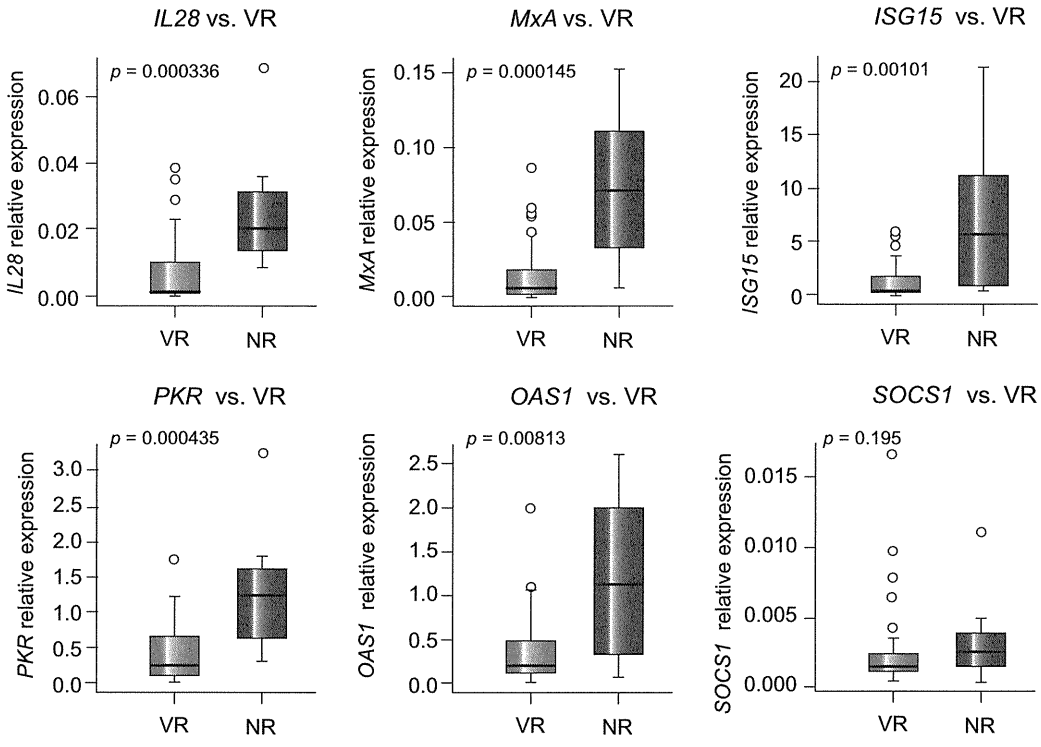

Fig. 2. Intrahepatic expression of *IL28* and interferon stimulated genes by response to therapy. aigures under each panel show the classification of patients with HCs genotype 1b by response to therapy: **s n**, sustained viral responder and relapser; **i n**, nonviral responder.

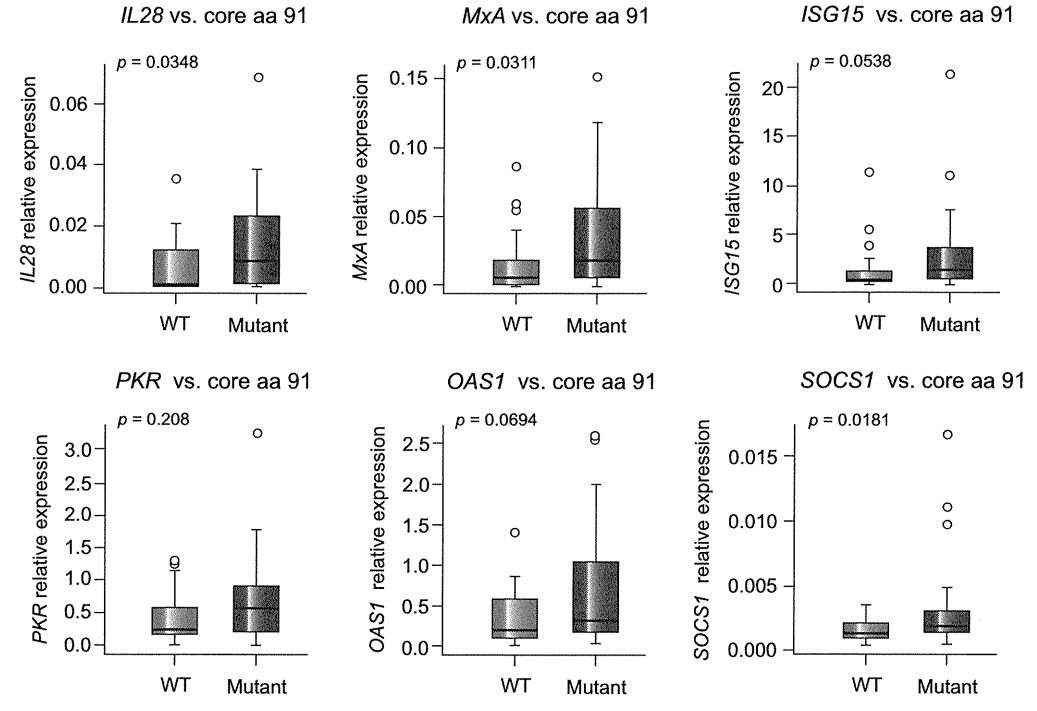

Fig. 3. Association between viral factors and ISG expression. I atients with a substitution at HCs core protein amino acid (aa) 91, which is associated with poorer response to treatment, showed significantly or marginally significantly increased expression of several **dbs** involved in establishment of the antiviral state as well as decreased expression of one **dsb** involved in the suppression of interferon signaling.

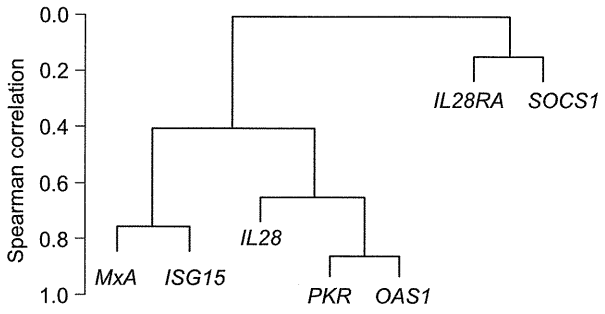

Fig. 4. Spearman correlation among predictor variables. Hierarchical clustering identified groups of genes with similar expression patterns.

levels of each of the ISGs involved in the establishment of antiviral defense (*MxA*, *PKR*, *OAS* and *ISG15*) were also lower in rs8099917 TT patients. We infer from these results that expression levels of *IL28* and other ISGs are regulated differently in the liver compared to peripheral blood cells. The finding that ISG expression levels were lower in patients homozygous for the major allele associated with a favorable response is consistent with Sarasin-Filipowicz *et al.* [34], who showed that lower ISG expression levels in the liver are associated with positive response to therapy, and vice versa. Feedback mechanisms that down-regulate the response to interferon administered during therapy might negatively affect the response to therapy in HCV infected liver cells. Our result showed that only the expression level of *SOCS1* did not differ among patients with rs8099917 genotype, which implies that the expression level of *SOCS1* relative to ISGs is higher in rs8099917 TT patients. Such relatively higher expression levels of inhibitory genes may contribute to the poor response to the therapy.

The relationship between *IL28B* polymorphisms and *IL28B* expression level remains unknown. Another SNP in strong linkage disequilibrium with the SNPs analyzed in this study resides in a possible promoter region of the *IL28B* gene [18], which might, therefore, affect mRNA expression levels, but different expression levels of ISG mRNA between liver and peripheral mononuclear cells cannot be explained simply by a single SNP in the promoter region. Further study is necessary to address this issue.

As it has been reported that amino acid substitutions in the core protein and the ISDR are associated with different responses to therapy [8–11], we attempted to uncover a relationship between core aa70 and 91 substitutions and expression levels of ISGs. Previously we found that core aa70 wild type viruses accumulated in rs8099917 TT patients, and several studies have reported poor response to therapy in the case of aa70 and aa91 substitutions [8]. Consistent with these results, in this study we found an association between elevated ISG expression and core aa91 substitutions, both of which are associated with poor response to therapy.

Multivariate analysis in this study reflected the tiered relationships among the predictors. The *IL28* rs8099917 genotype term was highly significant when analyzed alone, but it was

Table 4. Factors associated with virological response in patients with HCV genotype 1b (sustained viral response or transient/relapse response).

Variable	Univariate tests			Multiple logistic regression			
	n	OR	*p*	n	OR	(95% CI)	*p*
Age	52	1.13	0.4573				
Sex	52	0.604	0.2777				
rs8099917 (TT vs TG/GG)	52	3.68	0.0072				
Fibrosis stage	52	1.67	0.5672				
Activity	52	0.495	0.5788				
ALT	47	0.597	0.1845				
Gamma-GTP	47	0.539	0.0881				
Core aa70 (WT vs mutant)	46	1.24	0.7002				
Core aa91 (WT vs mutant)	46	1.43	0.4513				
ISDR (0 vs ≥1)	42	1.12	1.0000				
Titer	44	1.2	0.6377	52	0.297	(0.0794-1.11)	0.0706
IL28	52	0.273	0.0003				
IL28RA	51	0.792	0.3381	52	0.186	(0.047-0.736)	0.0165
MxA	52	0.255	0.0001	52	0.38	(0.124-1.16)	0.0892
ISG15	52	0.44	0.0010				
PKR	52	0.186	0.0004				
OAS1	52	0.372	0.0081	52	9.14	(0.974-85.7)	0.0528
SOCS1	52	0.87	0.1954	-	-	-	-

Univariate tests (Fisher exact and Mann–Whitney *U* tests) and multiple logistic regression analysis were used to examine the association between viral response and *IL28B* rs8099917 genotype, ISG gene expression, age and sex. Following multiple logistic regression *IL28*, *MxA*, and *OAS1* expression remained significant at the 0.05 level. Odds ratios for multiple logistic regression were adjusted using penalized maximum likelihood.

not significant when *IL28* and ISG mRNA expression levels were included in the model, suggesting that whatever the mechanism of action reflected by this polymorphism, it may directly or indirectly affect expression of the *IL28B* gene and downstream ISGs. Similarly, *MxA* and *ISG15* clustered together by Spearman rank correlation (Fig. 4), making it unlikely that both would remain significant in a multivariate model, and in this case the ISG with the stronger univariate effect (*MxA*) was selected.

Conclusions

In summary we found that the expression levels of ISGs in hepatic cells are inversely related with *IL28* SNP genotype relative to peripheral mononuclear cells. Analysis of the mechanism underlying different expression levels among *IL28* genotypes, especially differential regulation of anti-viral ISGs and *SOCS1*, should be important in understanding the mechanism behind variations in response to therapy and give us an insight into ways to develop more effective therapeutic regimens.

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Conflict of interest

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

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Prediction of Response to Peginterferon-Alfa-2b Plus Ribavirin Therapy in Japanese Patients Infected With Hepatitis C Virus Genotype 1b

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Variation at the IL-28B locus was recently reported to be a significant predictive factor of viral response to pegylated-interferon plus ribavirin combination therapy against chronic hepatitis C. Predictive factors for the effect of therapy, including IL-28B polymorphism rs8099917 and viral and clinical factors were investigated. A total of 288 patients were enrolled who were chronically infected with hepatitis C virus (HCV) genotype 1b and treated with combination therapy. Among them, 87 patients completed 48 weeks of therapy without dose reduction or discontinuation. In multivariate regression analysis, the rs8099917 TT genotype was the only independent factor significantly associated with sustained viral response ($P = 0.016$, OR 61.5), whereas substitutions at amino acid 70 (aa 70) of the HCV core protein ($P = 0.038$, OR 5.9) and non-TT genotypes ($P = 0.002$, OR 17.2) were associated with nonvirological response. Both factors were also associated with viral dynamics during the initial stage of the therapy. Correlation analysis revealed that rs8099917 genotype was correlated with γ-glutamyl transpeptidase, hyaluronic acid, and HCV core aa 70. In conclusion, host (IL-28B polymorphism) and viral (aa 70) factors independently affect response to combination therapy. ***J. Med. Virol. 83:981–988, 2011.*** © 2011 Wiley-Liss, Inc.

KEY WORDS: HCV; IL-28B; pegylated-interferon; ribavirin; rs8099917; core protein

INTRODUCTION

Hepatitis C virus (HCV) is a major cause of liver disease, including liver cirrhosis and hepatocellular carcinoma [Alter, 2006]. HCV eradication therapy is recommended for persons at risk for liver disease progression. Consensus guidelines recommend the use of pegylated-interferon (PEG-IFN) alfa-2b or PEG-IFN alfa-2a in combination with ribavirin (RBV) for the treatment of chronic hepatitis C [McHutchison et al., 2009]. Large-scale studies on 48-week PEG-IFN alfa and RBV treatment in the United States and Europe showed that 42–52% of patients with HCV genotype 1 achieved sustained viral response (SVR) [Manns et al., 2001; Fried et al., 2002]. In Japan, about 70% of patients with chronic hepatitis C are infected with HCV genotype 1b. Various factors have been reported to be associated with viral response to the treatment. In general, both viral (e.g., HCV genotype, amino acid substitutions in the core and nonstructural 5A (NS5A) regions)

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[Enomoto et al., 1996; Pascu et al., 2004; Akuta et al., 2007; Shirakawa et al., 2008] and host factors (age, body mass index, and insulin resistance) [Gao et al., 2004; Walsh et al., 2006] influence the outcome of IFN therapy against genotype 1. Viral load and stage of liver fibrosis vary among patients even when they have been infected from the same source. These two factors have also been reported to influence the outcome of IFN therapy [Tsubota et al., 1994]. The different disease outcomes, as well as the effect of IFN therapy may also partly depend on host genetic factors, and different responses among ethnic groups have been reported [Conjeevaram et al., 2006; Welzel et al., 2009]. To date, polymorphisms in myxovirus resistance protein A [Hijikata et al., 2000; Knapp et al., 2003], interferon alpha-receptor 1 [Matsuyama et al., 2003], osteopontin [Naito et al., 2005], and mitogen-activated protein kinase-activated protein kinase 3 [Tsukada et al., 2009] have been reported to be associated with IFN response by candidate gene approach. However, most of these studies were limited by their relatively small sample sizes and lack of validation. Thus, the host genetic factors that influence the IFN responsiveness in HCV-infected patients have not been fully explored. Recently, there were reports of association between variation at the IL-28B locus and the outcome of IFN therapy among HCV-infected patients [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Thomas et al., 2009].

In this report, the influence of potentially important prognostic factors (IL-28B variants rs8099917 and rs12979860, mutations at amino acid 70 (aa 70) of HCV core protein, and other clinical factors) on treatment response and viral kinetics were examined. Since these two SNPs were found to be in strong linkage disequilibrium ($r^2 \approx 1$) based on Plink/Haploview analysis and represent a common haplotype in the present dataset, only the results for rs8099917 are shown in this paper to avoid redundancy. Furthermore, correlations among the predictive factors were analyzed because most of the predictive factors identified in univariate analysis were not identified as independent predictive factors in multivariate analysis, suggesting these factors are correlated with each other.

PATIENTS AND METHODS

A total of 288 patients with chronic HCV genotype 1b infection who were treated with PEG-IFN plus RBV therapy between 2004 and 2008 at Hiroshima University Hospital and hospitals belonging to the Hiroshima Liver Study Group (Fig. 1, Group A) were enrolled. All patients were positive for both anti-HCV antibody and serum HCV RNA for more than 6 months. All subjects gave written informed consent to participate in the study according to the process approved by the Ethical Committee at the SNP Research Center, the Institute of Physical and Chemical Research (RIKEN), Yokohama. All patients were negative for hepatitis B surface antigen, had no evidence of other liver diseases, and had not received immunosuppressive therapy before enrollment

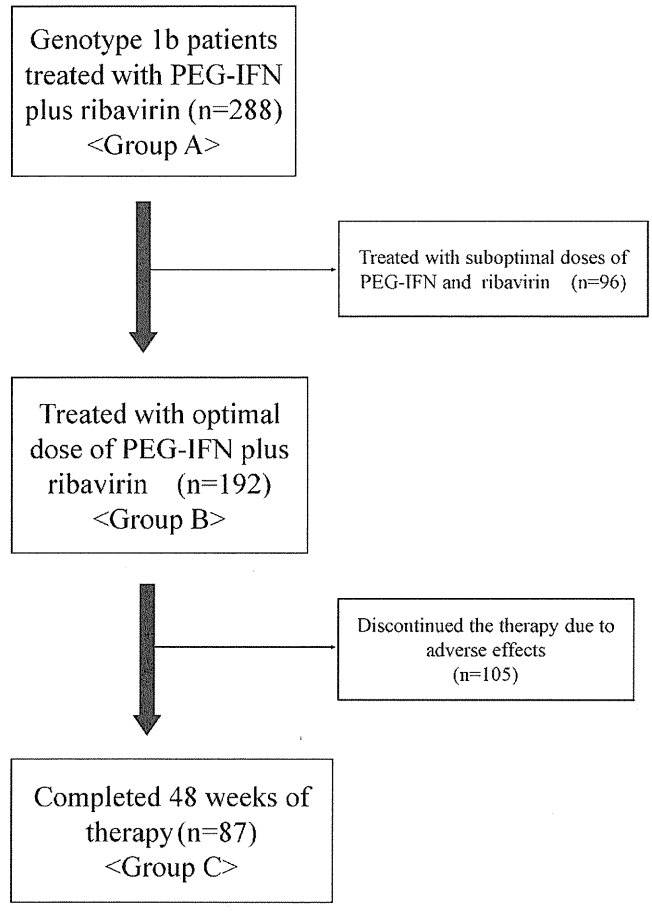

Fig. 1. Flow diagram of patients selected for the analysis. Among 288 patients with chronic HCV genotype1b infection who were treated with PEG-IFN plus RBV (Group A), 192 patients were treated with the standard dose of both peg-interferon and ribavirin (Group B). Eighty-seven patients completed 48 weeks of treatment without dose reduction or discontinuation (Group C).

in the study. Among them, 192 patients were treated with weekly injections of PEG-IFN-alfa-2b at 1.5 μg/kg plus oral administration of RBV for 48 weeks (Fig. 1, Group B). The amount of RBV was adjusted based on the subject's body weight (600 mg for <60 kg, 800 mg for 60–80 kg, 1,000 mg for >80 kg). The remaining patients were treated with suboptimal doses of the drugs because of conditions such as advanced age, low body weight, anemia, and low platelet counts. From Group B, 87 patients who completed 48 weeks of therapy without dose reduction or discontinuation were selected (Fig. 1, Group C). Group C patients were classified into the following three groups based on treatment outcome: SVR (sustained viral responder), TVR (transient viral responder), and NVR (nonresponder). SVR patients had normal alanine transaminase levels and no evidence of viremia at 24 weeks after completion of IFN therapy. TVR patients became nonviremic during therapy, but relapsed following cessation of the therapy. NVR patients remained viremic throughout therapy. The clinical characteristics of the patients are summarized in Table I.