Figure 1. Results of genome-wide association studies. a) HBV carriers and healthy controls, and b) HBV carriers and HBV-resolved individuals were compared. *P* values were calculated by chi-squared test for allele frequencies. Dots with arrows on chromosome 6 show strong associations with protective effects against persistent HB infection and with HBV clearance.
doi:10.1371/journal.pone.0039175.g001

Clearance of Hepatitis B virus in Japanese and Korean Individuals

We also conducted a GWAS to identify the host genetic factors related to clearance of HBV in the above 181 Japanese HBV carriers and 185 Japanese HBV-resolved individuals using a genome-wide SNP typing array (Affymetrix Genome-Wide Human SNP Array 6.0 for 900 K SNPs). The same two SNPs (rs3077 and rs9277542) showed strong associations in the allele frequency model ($P=9.24\times10^{-7}$ and $P=3.15\times10^{-5}$) with clearance of HBV (Figure 1b).

The above 32 SNPs, including the two associated SNPs (rs3077 and rs9277542), were selected for a replication study in two independent sets of HBV carriers and HBV resolved individuals (replication-1:256 Japanese HBV carriers and 150 Japanese HBV resolved individuals; and replication-2:344 Korean HBV carriers and 106 Korean HBV resolved individuals; Table 1). All 32 SNPs were genotyped using the DigiTag2 assay and 29 of 32 SNPs were successfully genotyped (Table S3). The associations of the original SNPs were replicated in both replication sets [replication-1 (Japanese): rs3077, $P=3.32\times10^{-2}$, OR $=0.72$ and rs9277542, $P=1.25\times10^{-2}$, OR $=0.68$; replication-2 (Korean): rs3077, $P=2.35\times10^{-7}$, OR $=0.41$ and rs9277542, $P=4.97\times10^{-6}$, OR $=0.46$; Table 3]. Meta-analysis using random effects model showed $P_{meta}=1.56\times10^{-4}$ for rs3077 (OR $=0.51$, 95% CI $=0.36$–0.72), and 5.91×10^{-7} for rs9277542 (OR $=0.55$, 95% CI $=0.43$–0.69). While there was evidence of heterogeneity between these studies for rs3077 ($P_{het}=0.03$) and no evidence for rs9277542 ($P_{het}=0.19$), significant associations with HBV clearance were observed with Mantel-Haenszel $P_{meta}=3.28\times10^{-12}$ for rs3077 and 1.42×10^{-10} for rs9277542, when using CMH fixed-effects model. Among the remaining 27 SNPs in the replication study, two SNPs (rs9276431 and rs7768538), located in a genetic region including *HLA-DQ* gene, were marginally replicated in the two sets of HBV carriers and HBV resolved individuals with Mantel-Haenszel *P* values of 2.10×10^{-5} (OR $=0.59$) and 1.10×10^{-5} (OR $=0.56$), respectively (Table S3), when using CMH fixed-effect model. Due to the existing heterogeneity among three groups (GWAS, Replication-1 and Replication-2) ($P_{het}=0.03$ for rs9276431 and 0.04 for rs7768538), weak associations were observed with $P_{meta}=0.03$ for rs9276431 and 0.02 for rs7768538 by the random effects model meta-analysis.

Meta-analysis across 6 independent studies, including 5 additional published data, showed $P_{meta}=1.48\times10^{-9}$, OR $=0.60$ for rs3077, $P_{meta}=1.08\times10^{-17}$, OR $=0.66$ for rs9277535 and $P_{meta}=5.14\times10^{-5}$, OR $=0.55$ for rs9277542 (Table S4). As shown in Table S4, the OR for the rs9277535 and rs9277542 were similar among the 6 independent studies, and heterogeneity was negligible ($P_{het}=0.03$ for rs9277535 and 0.14 for rs9277542). However, significant level of heterogeneity for rs3077 was observed with $P_{het}=9.57\times10^{-6}$ across 5 independent studies, including our study.

Table 1. Number of study samples.

		GWAS	Replication-1	Replication-2
population		**Japanese**	**Japanese**	**Korean**
HBV carriers	Total	**181**	**256**	**344**
	IC	20	94	–
	CH	67	101	177
	LC	3	10	–
	HCC	91	51	167
Healthy controls		**184**	**236**	**151**
Resolved individuals		**185**	**150**	**106**

Abbreviation: IC, Inactive Carrier; CH, Chronic Hepatitis; LC, Liver Cirrhosis; HCC, Hepatocellular Carcinoma.
doi:10.1371/journal.pone.0039175.t001

URLs

The results of the present GWAS are registered at a public database: https://gwas.lifesciencedb.jp/cgi-bin/gwasdb/gwas_top.cgi.

Discussion

The recent genome-wide association study showed that the SNPs located in a genetic region including *HLA-DPA1* and *HLA-DPB1* genes were associated with chronic HBV infection in the Japanese and Thai population [10,11]. In this study, we confirmed a significant association between SNPs (rs3077 and rs9277542) located in the same genetic region as *HLA-DPA1* and *HLA-DPB1* and protective effects against CHB in Korean and Japanese individuals. Mata-analysis using the random effects model across 6 independent studies including our study suggested that, widely in East Asian populations, variants in antigen binding sites of *HLA-DP* contribute to protective effects against persistent HBV infection (Table S2).

On GWAS and replication analysis with Japanese and Korean individuals, we identified associations between the same SNPs (rs3077 and rs9277542) in the *HLA-DPA1* and *HLA-DPB1* genes and HBV clearance; however, no new candidate SNPs from the GWAS were detected on replication analysis (Table S3). When the data of reference#18 was excluded from the meta-analysis across 6 independent studies, heterogeneity among 4 studies was estimated to be $P_{het}=0.15$ and significant association of rs3077 with HBV clearance was observed with $P_{meta}=5.88\times10^{-24}$, OR $=0.56$ (Table S4). In our study, a negligible level of heterogeneity for rs3077 was also observed ($P_{het}=0.03$) on meta-analysis by adding replication-1 (Table 3). Despite the heterogeneity in replication-1, a marginal association was observed for rs3077 with the same downward trend in the odds ratio ($P=3.32\times10^{-2}$, OR $=0.72$). Moreover, meta-analysis using GWAS and replication-2 showed significant association of $P_{meta}=1.89\times10^{-12}$, OR $=0.43$ for rs3077 with no evidence of heterogeneity ($P_{het}=0.75$). Although the reason why heterogeneity was observed in replication-1 is unclear, one possible reason is the clinical heterogeneity due to different kits being used for antibody testing. The associations of *HLA-DPA1/−DPB1* with CHB and HBV clearance showed the same level of significance in the comparison of HBV patients with HBV resolved individuals (OR $=0.43$ for rs3077 and 0.49 for rs9277542) as the one with healthy controls (OR $=0.46$ for rs3077 and 0.50 for rs9277542), when the replication-1 was excluded in the analysis (Table 2 and Table 3). The results of meta-analysis across 6 independent studies including our study also showed the same or slightly weaker associations in the

Table 2. Results of replication study for protective effects against CHB.

	Position			MAF[a]	Allele	Stage	HBV carriers			Healthy controls				OR[b]		
dbSNP rsID	Chr	Build 36.3	Nearest Gene	(allele)	(1/2)	(population)	11	12	22	11	12	22	HWEp	95% CI	*P*-value[c]	P_{het}[d]
rs3077	6	33141000	HLA-DPA1	0.44	T/C	GWAS	13	51	117	28	88	67	0.919	0.42	1.14×10^{-7}	
				(T)		(Japanese)	(7.2)	(28.2)	(64.6)	(15.3)	(48.1)	(36.6)		(0.30–0.58)		
						Replication-1	26	95	134	46	125	65	0.309	0.48	2.70×10^{-8}	
						(Japanese)	(10.2)	(37.3)	(52.5)	(19.5)	(53.0)	(27.5)		(0.37–0.62)		
						Replication-2	23	81	111	31	74	40	0.767	0.47	2.08×10^{-6}	
						(Korean)	(10.7)	(37.7)	(51.6)	(21.4)	(51.0)	(27.6)		(0.35–0.65)		
						Meta-analysis[e]								0.46	4.40×10^{-19}	0.80
														(0.39–0.54)		
rs9277542	6	33163225	HLA-DPB1	0.45	T/C	GWAS	18	53	110	29	102	52	0.073	0.42	5.32×10^{-8}	
				(T)		(Japanese)	(9.9)	(29.3)	(60.8)	(15.8)	(55.7)	(28.4)		(0.31–0.58)		
						Replication-1	30	106	118	54	114	67	0.681	0.54	3.33×10^{-6}	
						(Japanese)	(11.8)	(41.7)	(46.5)	(23.0)	(48.5)	(28.5)		(0.42–0.70)		
						Replication-2	30	87	94	35	72	36	0.933	0.54	8.29×10^{-5}	
						(Korean)	(14.2)	(41.2)	(44.5)	(24.5)	(50.3)	(25.2)		(0.40–0.74)		
						Meta-analysis[e]								0.50	1.28×10^{-15}	0.40
														(0.43–0.60)		

[a]Minor allele frequency and minor allele in 198 healthy Japanese (ref#19).
[b]Odds ratio of minor allele from two-by-two allele frequency table.
[c]P value of Pearson's chi-square test for allelic model.
[d]Heterogeneity was tested using general variance-based method.
[e]Meta-analysis was tested using the random effects model.
doi:10.1371/journal.pone.0039175.t002

comparison of HBV patients with HBV resolved individuals (OR = 0.56 for rs3077, 0.66 for rs9277535 and 0.55 for rs9277542) than in the one with healthy controls (OR = 0.55 for rs3077, 0.61 for rs9277535 and 0.51 for rs9277542), which was the opposite result as we expected (Table S2 and Table S4). These results may suggest that other unknown immune system(s) exist to eliminate the HBV in the HBV resolved individuals.

Among the HLA class II loci (*HLA-DPA1*, *HLA-DPB1* and *HLA-DQB2*), which were associated with CHB and HBV clearance, a weak linkage disequilibrium ($r^2<0.1$) was observed between *HLA-DQB2* locus and *HLA-DPA1/−DPB1* loci in Japanese and Korean populations (Figure S2). We also found that similar linkage disequilibrium blocks (r^2) were observed among three subgroups (HBV carriers, HBV resolved individuals and Healthy controls). Moreover, logistic regression analysis of *HLA-DP* (rs3077 and rs92775542) with use of *HLA-DQ* (rs9276431 and rs768538) as covariates showed that the same level of significant associations of *HLA-DP* with CHB and HBV clearance as shown in the single-point association analysis, while no associations of *HLA-DQ* with $P_{log}>0.05$ were detected both in Japanese and in Korean (Table S5). These results show that *HLA-DP* is the main genetic factor for susceptibility to CHB and HBV clearance, and the associations of *HLA-DQB2* would result from linkage disequilibrium of *HLA-DP*A1/−DPB1.

In this study, we confirmed the significant associations between *HLA-DPA1* and *HLA-DPB1*, and protective effects against CHB and HBV clearance in Japanese and Korean individuals. These results suggest that the associations between the *HLA-DP* locus, CHB and HBV clearance are widely replicated in East Asian populations, including Chinese, Thai, Japanese and Korean individuals; however, there have been no similar GWAS performed in Caucasian and African populations. Moreover, there were no significant SNPs associated with HCC development in this study, thus suggesting that it is necessary to increase the sample size. To clarify the pathogenesis of CHB or the mechanisms of HBV clearance, further studies are necessary, including a functional study of the *HLA-DP* molecule, identification of novel host genetic factors other than *HLA-DP*, and variation analysis of HBV.

Materials and Methods

Ethics Statement

All study protocols conform to the relevant ethical guidelines, as reflected in the *a priori* approval by the ethics committees of all participating universities and hospitals. The written informed consent was obtained from each patient who participated in this study and all samples were anonymized.

Genomic DNA Samples and Clinical Data

All of the 1,793 Japanese and Korean samples, including individuals with CHB, healthy controls and HBV-resolved individuals (HBsAg-negative and anti-HBc-positive), were collected at 20 multi-center hospitals (liver units with hepatologists) throughout Japan and Korea. The 19 hospitals in Japan were grouped into the following 8 areas: Hokkaido area (Hokkaido University Hospital, Teine Keijinkai Hospital), Tohoku area (Iwate Medical University Hospital), Kanto area (Musashino Red Cross Hospital, Saitama Medical University, Kitasato University Hospital, University of Tokyo), Koshin area (Shinshu University Hospital, Kanazawa University Hospital), Tokai area (Nagoya City University Hospital, Nagoya Daini Red Cross Hospital), Kinki area (Kyoto Prefectural University of Medicine Hospital, National Hospital Organization Osaka National Hospital, Osaka

Table 3. Results of replication study for clearance of hepatitis B virus.

	Position		MAF[a]	Allele	Stage	HBV carriers			Resolved individuals			OR[b]		
dbSNP rsID	Chr	Build 36.3 Nearest Gene	(allele)	(1/2)	(population)	11	12	22	11	12	22	95% CI	*P*-value[c]	P_{het}[d]
rs3077	6	33141000 HLA-DPA1	0.44	T/C	GWAS	13	51	117	29	82	74	0.44	9.24×10^{-7}	
			(T)		(Japanese)	(7.2)	(28.2)	(64.6)	(15.7)	(44.3)	(40.0)	(0.32–0.61)		
					Replication-1	26	95	134	20	64	60	0.72	3.32×10^{-2}	
					(Japanese)	(10.2)	(37.3)	(52.5)	(13.9)	(44.4)	(41.7)	(0.53–0.97)		
					Replication-2	23	81	111	29	48	28	0.41	2.35×10^{-7}	
					(Korean)	(10.7)	(37.7)	(51.6)	(27.6)	(45.7)	(26.7)	(0.29–0.58)		
					Meta-analysis[e]							0.51	1.56×10^{-4}	0.03
												(0.36–0.72)		
					Meta-analysis[e]							0.43	1.89×10^{-12}	0.75
					(GWAS+replication-2)							(0.34–0.54)		
rs9277542	6	33163225 HLA-DPB1	0.45	T/C	GWAS	18	53	110	28	88	69	0.51	3.15×10^{-5}	
			(T)		(Japanese)	(9.9)	(29.3)	(60.8)	(15.1)	(47.6)	(37.3)	(0.37–0.70)		
					Replication-1	30	106	118	28	62	52	0.68	1.25×10^{-2}	
					(Japanese)	(11.8)	(41.7)	(46.5)	(19.7)	(43.7)	(36.6)	(0.51–0.92)		
					Replication-2	30	87	94	30	53	22	0.46	4.97×10^{-6}	
					(Korean)	(14.2)	(41.2)	(44.5)	(28.6)	(50.5)	(21.0)	(0.33–0.64)		
					Meta-analysis[e]							0.55	5.91×10^{-7}	0.19
												(0.43–0.69)		
					Meta-analysis[e]							0.49	9.69×10^{-10}	0.65
					(GWAS+replication-2)							(0.39–0.61)		

[a]Minor allele frequency and minor allele in 198 healthy Japanese (ref#19).
[b]Odds ratio of minor allele from two-by-two allele frequency table.
[c]P value of Pearson's chi-square test for allelic model.
[d]Heterogeneity was tested using general variance-based method.
[e]Meta-analysis was tested using the random effects model.
doi:10.1371/journal.pone.0039175.t003

City University), Chugoku/Shikoku area (Tottori University Hospital, Ehime University Hospital, Yamaguchi University Hospital, Kawasaki Medical College Hospital) and Kyushu area (Kurume University Hospital). Korean samples were collected at Yonsei University College of Medicine.

HBV status was measured based on serological results for HBsAg and anti-HBc with a fully automated chemiluminescent enzyme immunoassay system (Abbott ARCHITECT; Abbott Japan, Tokyo, Japan, or LUMIPULSE f or G1200; Fujirebio, Inc., Tokyo, Japan). For clinical staging, inactive carrier (IC) state was defined by the presence of HBsAg with normal ALT levels over 1 year (examined at least four times at 3-month intervals) and without evidence of portal hypertension. Chronic hepatitis (CH) was defined by elevated ALT levels (>1.5 times the upper limit of normal [35 IU/L]) persisting over 6 months (at least by 3 bimonthly tests). Liver cirrhosis (LC) was diagnosed principally by ultrasonography (coarse liver architecture, nodular liver surface, blunt liver edges and hypersplenism), platelet counts <100,000/cm^3, or a combination thereof. Histological confirmation by fine-needle biopsy of the liver was performed as required. Hepatocellular carcinoma (HCC) was diagnosed by ultrasonography, computerized tomography, magnetic resonance imaging, angiography, tumor biopsy or a combination thereof.

The Japanese control samples from HBV-resolved subjects (HBsAg-negative and anti-HBc-positive) at Nagoya City University-affiliated healthcare center were used by comprehensive agreement (anonymization in an unlinkable manner) in this study. Some of the unrelated Japanese healthy controls were obtained from the Japan Health Science Research Resources Bank (Osaka, Japan). One microgram of purified genomic DNA was dissolved in 100 µl of TE buffer (pH 8.0) (Wako, Osaka, Japan), followed by storage at −20°C until use.

SNP Genotyping and Data Cleaning

For GWAS, we genotyped a total of 550 individuals, including 181 Japanese HBV carriers, 184 Japanese healthy controls and 185 spontaneously HBV-resolved Japanese individuals (HBsAg-negative and anti-HBc-positive), using the Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix, Inc., Santa Clara, CA), in accordance with the manufacturer's instructions. The average QC call rate for 550 samples reached 98.47% (95.00–99.92%), which had an average sample call rate of 98.91% (93.55–99.74%) by determining the genotype calls of over 900 K SNPs using the Genotyping Console v4.1 software (with Birdseed v1 algorithm) provided by the manufacturer [19]. We then applied the following thresholds for SNP quality control in data cleaning: SNP call rate ≥95% and MAF ≥1% for three groups (HBV carriers, healthy controls and HBV-resolved individuals), and HWE *P*-value ≥0.001 for healthy controls [20]. Here, SNP call rate is defined for each SNP as the number of successfully genotyped samples divided by the number of total samples genotyped. A total of 597,789 SNPs and 590,278 SNPs on autosomal chromosomes

passed the quality control filters in the genome-wide association analysis using HBV carriers and healthy controls, and using HBV carriers and HBV-resolved individuals, respectively (Figure 1). All cluster plots for the SNPs showing $P<0.0001$ on association analyses in the allele frequency model were confirmed by visual inspection, and SNPs with ambiguous cluster plots were excluded.

In the following replication stage, we selected a set of 32 SNPs with $P<0.0001$ in the GWAS using HBV carriers and HBV-resolved individuals. SNP genotyping in two independent sets of 256 Japanese HBV carriers, 236 Japanese healthy controls and 150 Japanese HBV-resolved individuals (Table 1, replication-1), and 344 Korean HBV carriers, 151 Korean healthy controls and 106 Korean HBV-resolved individuals (Table 1, replication-2) was completed for the selected 32 SNPs using the DigiTag2 assay [21,22] and custom TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA) on the LightCycler 480 Real-Time PCR System (Roche, Mannheim, Germany).

Statistical Analysis

The observed associations between SNPs and the protective effects on chronic hepatitis B or clearance of hepatitis virus B were assessed by chi-squared test with a two-by-two contingency table in allele frequency model. SNPs on chromosome X were removed because gender was not matched among HBV carriers, healthy controls and HBV-resolved individuals. A total of 597,789 SNPs and 590,278 SNPs passed the quality control filters in the GWAS stage; therefore, significance levels after Bonferroni correction for multiple testing were $P=8.36\times10^{-8}$ (0.05/597,789) and $P=8.47\times10^{-8}$ (0.05/590,278), respectively. For the replication study, 29 of 32 SNPs were successfully genotyped; therefore, we applied $P=0.0017$ (0.05/29) as a significance level, and none of the 29 markers genotyped in the replication stage showed deviations from the Hardy-Weinberg equilibrium in healthy controls ($P>0.01$).

The genetic inflation factor λ was estimated by applying the Cochrane-Armitage test on all SNPs and was found to be 1.056 and 1.030 in the GWAS using HBV carriers and healthy controls, and using HBV carriers and HBV-resolved individuals, respectively (Figure S3). These results suggest that the population substructure should not have any substantial effect on statistical analysis. In addition, the principal component analysis in a total of 550 individuals in the GWAS stage together with the HapMap samples also revealed that the effect of population stratification was negligible (Figure S4).

Based on the genotype data of a total of 1,793 samples including 1,192 Japanese samples and 601 Korean samples in both GWAS and replication stages, haplotype blocks were estimated using the Gabriel's algorithm using the Haploview software (v4.2) (Figure S2). In the logistic regression analysis, two SNPs (rs9276431 and rs7768538) within the HLA-DQ locus were individually involved as a covariate (Table S5). Statistical analyses were performed using the SNP & Variation Suite 7 software (Golden Helix, MT, USA).

Supporting Information

Figure S1 GWAS using samples from HBV carriers with LC or HCC, and HBV carriers without LC and HCC. *P* values were calculated using chi-squared test for allele frequencies.
(PPTX)

Figure S2 Estimation of linkage disequilibrium blocks in HBV patients, HBV resolved individuals and healthy controls in Japanese and Korean. The LD blocks (r^2) were analyzed using the Gabriel's algorithm.
(PPTX)

Figure S3 Quantile-quantile plot for test statistics (allele-based chi-squared tests) for GWAS results. Dots represent P values of each SNP that passed the quality control filters. Inflation factor λ was estimated to be: a) 1.056 in the analysis with HBV carriers and healthy controls; and b) 1.030 with HBV carriers and HBV-resolved individuals.
(PPTX)

Figure S4 Principal component analysis on a total of 550 individuals in GWAS, together with HapMap samples (CEU, YRI and JPT).
(PPTX)

Table S1 Results for 29 SNPs selected in replication study using samples of HBV carriers and healthy controls. [a]*P* values by chi-squared test for allelic model. [b]Odds ratio of minor allele from two-by-two allele frequency table. [c]Meta-analysis was tested using additive, two-tailed CMH fixed-effects model.
(XLSX)

Table S2 Results of meta-analysis for protective effects against persistent HB infection across 6 independent studies, including this study. [a]Minor allele frequency and minor allele in 198 healthy Japanese (ref#19). [b]Odds ratio of minor allele from two-by-two allele frequency table. [c]*P* value of Pearson's chi-squared test for allele model. [d]Heterogeneity was tested using general variance-based method. [e]Meta-analysis was tested using the random effects model.
(XLSX)

Table S3 Results for 29 SNPs selected in replication study using samples from HBV carriers and HBV-resolved individuals. [a]*P* values by chi-squared test for allelic model. [b]Odds ratio of minor allele from two-by-two allele frequency table. [c]Meta-analysis was tested using additive, two-tailed CMH fixed-effects model.
(XLSX)

Table S4 Results of meta-analysis for clearance of HBV across 6 independent studies, including this study. [a]Minor allele frequency and minor allele in 198 healthy Japanese (ref#19). [b]Odds ratio of minor allele from two-by-two allele frequency table. [c]*P* value of Pearson's chi-squared test for allele model. [d]Heterogeneity was tested using general variance-based method. [e]Meta-analysis was tested using the random effects model.
(XLSX)

Table S5 Logistic regression analysis of *HLA-DP* (rs3077 and rs9277542) and *HLA-DQ* (rs9276431 and rs7768538) with susceptibility to CHB and HBV clearance using the *HLA-DQ* genotypes individually as a covariate.
(XLSX)

Acknowledgments

We thank all the patients and families who contributed to the study and Ms. Yasuka Uehara-Shibata and Ms. Yoshimi Ishibashi for technical assistance.

Author Contributions

Conceived and designed the experiments: NN HS YT. Performed the experiments: HS Y. Mawatari M. Sageshima YO. Analyzed the data: NN MK AK. Contributed reagents/materials/analysis tools: KM M. Sugiyama SHA JYP SH JHK KS M. Kurosaki YA SM MW ET MH SK EO YI EM AT Y. Murawaki YH IS M. Korenaga KH TI NI KHH YT MM. Wrote the paper: NN M. Kawashima YT KT MM.

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Gut Online First, published on November 7, 2012 as 10.1136/gutjnl-2012-302553

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ORIGINAL ARTICLE

Hepatitis C virus kinetics by administration of pegylated interferon-α in human and chimeric mice carrying human hepatocytes with variants of the *IL28B* gene

Tsunamasa Watanabe,[1] Fuminaka Sugauchi,[2] Yasuhito Tanaka,[1] Kentaro Matsuura,[3] Hiroshi Yatsuhashi,[4] Shuko Murakami,[1] Sayuki Iijima,[1] Etsuko Iio,[3] Masaya Sugiyama,[5] Takashi Shimada,[6] Masakazu Kakuni,[6] Michinori Kohara,[7] Masashi Mizokami[5]

► Additional supplementary files are published online only. To view these files please visit the journal online (http://dx.doi.org/10.1136/gutjnl-2012-302553).

[1]Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan
[2]Department of Gastroenterology, Nagoya City Koseiin Medical Welfare Center, Nagoya, Japan
[3]Department of Gastroenterology and Metabolism, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan
[4]Department of Therapeutic Research, National Hospital Organization (NHO) Nagasaki Medical Center, Nagasaki, Japan
[5]The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa, Japan
[6]PhoenixBio Co. Ltd., Higashi-Hiroshima, Japan
[7]Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan

Correspondence to
Dr Masashi Mizokami, The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine 1–7–1, Kohnodai, Ichikawa 272–8516, Japan; mmizokami@hospk.ncgm.go.jp

Revised 4 October 2012
Accepted 9 October 2012

ABSTRACT

Objective Recent studies have demonstrated that genetic polymorphisms near the *IL28B* gene are associated with the clinical outcome of pegylated interferon α (peg-IFN-α) plus ribavirin therapy for patients with chronic hepatitis C virus (HCV). However, it is unclear whether genetic variations near the *IL28B* gene influence hepatic interferon (IFN)-stimulated gene (ISG) induction or cellular immune responses, lead to the viral reduction during IFN treatment.

Design Changes in HCV-RNA levels before therapy, at day 1 and weeks 1, 2, 4, 8 and 12 after administering peg-IFN-α plus ribavirin were measured in 54 patients infected with HCV genotype 1. Furthermore, we prepared four lines of chimeric mice having four different lots of human hepatocytes containing various single nucleotide polymorphisms (SNP) around the *IL28B* gene. HCV infecting chimeric mice were subcutaneously administered with peg-IFN-α for 2 weeks.

Results There were significant differences in the reduction of HCV-RNA levels after peg-IFN-α plus ribavirin therapy based on the *IL28B* SNP rs8099917 between TT (favourable) and TG/GG (unfavourable) genotypes in patients; the first-phase viral decline slope per day and second-phase slope per week in TT genotype were significantly higher than in TG/GG genotype. On peg-IFN-α administration to chimeric mice, however, no significant difference in the median reduction of HCV-RNA levels and the induction of antiviral ISG was observed between favourable and unfavourable human hepatocyte genotypes.

Conclusions As chimeric mice have the characteristic of immunodeficiency, the response to peg-IFN-α associated with the variation in *IL28B* alleles in chronic HCV patients would be composed of the intact immune system.

Significance of this study

What is already known on this subject?
- Genetic polymorphisms near the *IL28B* gene are associated with a chronic HCV treatment response.
- HCV-infected patients with the *IL28B* homozygous favourable allele had a more rapid decline in HCV kinetics in the first and second phases by peg-IFN-α-based therapy.
- During the acute phase of HCV infection, a strong immune response among patients with the *IL28B* favourable genotype could induce more frequent spontaneous clearance of HCV.

What are the new findings?
- In chronically HCV genotype 1b-infected chimeric mice that have the characteristic of immunodeficiency, no significant difference in the reduction in serum HCV-RNA levels and the induction of antiviral hepatic ISG by the administration of peg-IFN-α was observed between favourable and unfavourable human hepatocyte *IL28B* genotypes.
- By comparison of serum HCV kinetics between human and chimeric mice, the viral decline in both the first and second phases by peg-IFN-α treatment was affected by the variation in *IL28B* genotypes only in chronic hepatitis C patients.

How might it impact on clinical practice in the foreseeable future?
- The immune response according to *IL28B* genetic variants could contribute to the first and second phases of HCV-RNA decline and might be critical for HCV clearance by peg-IFN-α-based therapy.

INTRODUCTION

Hepatitis C is a global health problem that affects a significant portion of the world's population. The WHO estimated that, in 1999, 170 million hepatitis C virus (HCV)-infected patients were present worldwide, with 3–4 million new cases appearing per year.[1]

The standard therapy for hepatitis C still consists of pegylated interferon-α (peg-IFN-α), administered once weekly, plus daily oral ribavirin for 24–48 weeks in countries where protease inhibitors are not available.[2] This combination therapy is quite successful in patients with HCV genotype 2 or 3 infection, leading to a sustained virological response (SVR) in approximately 80–90% of patients treated; however, in patients infected with HCV genotype 1 or 4, only approximately half of all treated individuals achieved a SVR.[3 4]

Table 1 Characteristics of 54 patients infected HCV genotype 1

	IL28B SNP rs8099917		
	TT (n=34)	TG (n=19) + GG (n=1)	p Value
Age (years)	55.6±10.1	54.7±11.3	0.746
Gender (male %)	70	50	0.199
Body mass index (kg/m^2)	24.6±3.1	24.7±3.3	0.870
Viral load at therapy (log IU/ml)	6.0±0.7	5.8±0.8	0.357
SVR rate (%)	50	11	0.012
Serum ALT level (IU/l)	100.3±80.8	79.3±45.0	0.226
Platelet count (×10^4/μl)	17.1±9.0	16.5±5.8	0.771
Fibrosis (F3+4 %)	42	40	0.877

HCV, hepatitis C virus; SNP, single nucleotide polymorphism; SVR, sustained virological response.

Host factors were shown to be associated with the outcome of the therapy, including age, sex, race, liver fibrosis and obesity.[5] Genome-wide association studies have demonstrated that genetic variations in the region near the interleukin-28B (*IL28B*) gene, which encodes interferon (IFN)-λ3, are associated with a chronic HCV treatment response.[6–10] Furthermore, it was demonstrated that genetic variations in the *IL28B* gene region are also associated with spontaneous HCV clearance.[11–12]

Interestingly, a recent report showed the effect of genetic polymorphisms near the *IL28B* gene on the dynamics of HCV during peg-IFN-α plus ribavirin therapy in Caucasian, African American and Hispanic individuals;[13] HCV-infected patients with the *IL28B* homozygous favourable allele had a more rapid decline of HCV in the first phase, which is associated with the inhibition of viral replication as well as the second phase associated with immuno-destruction of viral-infected hepatocytes.[14] However, it is unknown how a direct effect by the *IL28B* genetic variation, such as the induction of IFN-stimulated genes (ISG) or cellular immune responses, would influence the viral kinetics during IFN treatment. Over recent periods, engineered severe combined immunodeficient (SCID) mice transgenic for urokinase-type plasminogen activator (uPA) received human hepatocyte transplants (hereafter referred to as chimeric mice)[15–17] and are suitable for experiments with hepatitis viruses in vivo.[18 19] We have also reported that these chimeric mice carrying human hepatocytes are a robust animal model to evaluate the efficacy of IFN and other anti-HCV agents.[20 21]

The purpose of this study was to reveal the association between genetic variations in the *IL28B* gene region and viral decline during peg-IFN-α treatment in patients with HCV, and to clarify the association between different *IL28B* alleles of human hepatocytes in chimeric mice and the response to peg-IFN-α without immune response. These studies will elucidate whether the immune response by the *IL28B* genetic variation affects the viral kinetics during peg-IFN-α treatment.

MATERIALS AND METHODS

Patients

Fifty-four Japanese patients with chronic HCV genotype 1 infection at Nagasaki Medical Center and Nagoya City University were enrolled in this study (table 1). Patients received peg-IFN-α2a (180 μg) or 2b (1.5 μg/kg) subcutaneously every week and were administered a weight-adjusted dose of ribavirin (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. Patients with other hepatitis virus infection or HIV coinfection were not included in the study. The study protocol conformed to the ethics guidelines of the 1975 Declaration of Helsinki as reflected by earlier approval by the institutions' human research committees.

Laboratory tests

Blood samples were obtained before therapy, as well as on day 1 and at weeks 1, 2, 4, 8 and 12 after the start of therapy and were analysed for the HCV-RNA level by the commercial Abbott Real-Time HCV test with a lower limit of detection of 12 IU/ml (Abbott Molecular Inc., Des Plains, Illinois, USA). Genetic polymorphism in the *IL28B* gene (rs8099917), a single nucleotide polymorphism (SNP) recently identified to be associated with treatment response,[6–8] was tested by the TaqMan SNP genotyping assay (Applied Biosystems, Foster City, California, USA).

HCV infection of chimeric mice with the liver repopulated for human hepatocytes

SCID mice carrying the uPA transgene controlled by an albumin promoter were injected with 5.0–7.5×10^5 viable hepatocytes through a small left-flank incision into the inferior splenic pole, thereafter chimeric mice were generated. The chimeric mice were purchased from PhoenixBio Co, Ltd (Hiroshima, Japan).[17] Human hepatocytes with the *IL28B* homozygous favourable allele, heterozygous allele or homozygous unfavourable allele were imported from BD Biosciences (San Jose, California, USA) (table 2). Murine serum levels of human albumin and the body weight were not significantly different among four chimeric mice groups, providing a reliable comparison for anti-HCV agents.[22] Three different serum samples were obtained from three chronic HCV patients (genotype 1b).[21 22] Each mouse was intravenously infected with serum sample containing 10^5 copies of HCV genotype 1b. Administration of peg-IFN-α2a (Pegasys; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) at the dose formulation (30 μg/kg) was consecutively applied to each mouse on days 0, 3, 7 and 10 (table 3).

HCV-RNA quantification

HCV-RNA in mice sera (days 0, 1, 3, 7 and 14) was quantified by an in-house real-time detection PCR assay with a lower quantitative limit of detection of 10 copies/assay, as previously reported.[21]

Quantification of IFN-stimulated gene-expression levels

For analysis of endogenous ISG levels, total RNA was isolated from the liver using the RNeasy RNA extraction kit (Qiagen, Valencia, California, USA) and complementary DNA synthesis

Table 2 Four lines of uPA/SCID mice from four different lots of human hepatocytes (donor) containing various SNP around the *IL28B* gene

uPA/SCID mice	Donor	Race	Age	Gender	rs8103142	rs12979860	rs8099917
PXB mice	A	African American	5 Years	Male	CC	TT	TG
	B	Caucasian	10 Years	Female	CC	TT	TG
	C	Hispanic	2 Years	Female	TT	CC	TT
	D	Caucasian	2 Years	Male	TT	CC	TT

PXB mice; urokinase-type plasminogen activator/severe combined immunodeficiency (uPA/SCID) mice repopulated with approximately 80% human hepatocytes.
SCID, severe combined immunodeficient; SNP, single nucleotide polymorphism.

Table 3 Dosage and time schedule of pegIFN-α2a* treatment for HCV genotype 1b infected chimeric mice

Donor hepatocytes†	No of chimeric mice	Inoculum	Test compound	Dose			
				Level (μg/kg)	Concentrtion (μg/ml)	Volume (ml/kg)	Frequency
A	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
B	4	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
D	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
A	2	Serum B	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	2	Serum B	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
A	2	Serum C	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	2	Serum C	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10

*Pegasys; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan.
†The *IL28B* genetic variation of the donor hepatocytes was indicated in table 2.
HCV, hepatitis C virus; peg-IFN-α, pegylated interferon α.

was performed using 2.0 μg of total RNA (High Capacity RNA-to-cDNA kit; Applied Biosystems). Fluorescence real-time PCR analysis was performed using an ABI 7500 instrument (Applied Biosystems) and TaqMan Fast Advanced gene expression assay (Applied Biosystems). TaqMan Gene Expression Assay primer and probe sets (Applied Biosystems) are shown in the supplementary information (available online only). Relative amounts of messenger RNA, determined using a FAM-Labeled TaqMan probe, were normalised to the endogenous RNA levels of the housekeeping reference gene, glyceraldehyde-3-phosphate dehydrogenase. The delta Ct method ($2^{-(\text{delta Ct})}$) was used for quantitation of relative mRNA levels and fold induction.[23 24]

Statistical analyses

Statistical differences were evaluated by Fisher's exact test or the χ^2 test with the Yates correction. Mice serum HCV-RNA and intrahepatic ISG expression levels were compared using the Mann–Whitney U test. Differences were considered significant if p values were less than 0.05.

RESULTS

Characteristics of the study patients

Genotypes (rs8099917) TT, TG and GG were detected in 34, 19 and one patient infected with HCV genotype 1, respectively. SVR rates were significantly higher in HCV patients with genotype TT than in those with genotype TG/GG (50% vs 11%, p=0.012). The initial HCV serum load was comparable between genotypes TT and TG/GG (6.0±0.7 vs 5.8±0.8 log IU/ml). There were no significant differences in sex (male%, 70% vs 50%), age (55.6±10.1 vs 54.7±11.3 years), serum alanine aminotransferase level (100.3±80.8 vs 79.3±45.0 IU/L), platelet count (17.1±9.0 vs 16.5±5.8×10^4/μl) and fibrosis stages (F3/4%, 42% vs 40%) between HCV patients with the favourable (rs8099917 TT) and unfavourable (rs8099917 TG/GG) *IL28B* genotypes (table 1).

Changes in serum HCV-RNA levels in patients treated by peg-IFN-α plus ribavirin

Figure 1 shows the initial change in the serum HCV-RNA level for 14 days after peg-IFN-α plus ribavirin therapy in patients infected with HCV genotype 1 based on the genetic polymorphism near the *IL28B* gene. The immediate antiviral response (viral drop 24 h after the first IFN injection) was significantly higher in HCV patients with genotype TT than genotype TG/GG (−1.08 vs −0.39 log IU/ml, p<0.001). Figure 2 also shows the subsequent change in the serum HCV-RNA reduction after peg-IFN-α plus ribavirin therapy in patients infected with HCV genotype 1. Similarly, during peg-IFN-α plus ribavirin therapy, a statistically significant difference in the median reduction in serum HCV-RNA levels was noted according to the genotype (TT vs TG/GG). The median reduction in the serum HCV-RNA levels (log IU/ml) at 1, 2, 4, 8 and 12 weeks between genotypes TT and TG/GG was as follows: −1.58 vs −0.62, p<0.001; −2.35 vs −0.91, p<0.001;

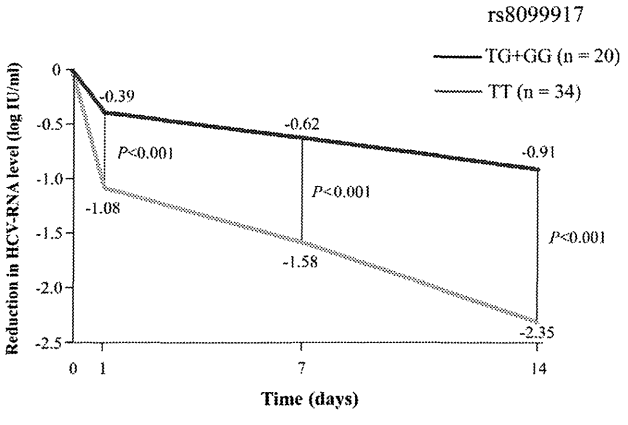

Figure 1 Rapid reduction of median hepatitis C virus (HCV)-RNA levels (log IU/ml) at 1, 7 and 14 days between *IL28B* single nucleotide polymorphisms rs8099917 genotype TT (n=34) and TG/GG (n=20) in HCV genotype 1-infected patients treated with peg-IFN-α plus ribavirin.

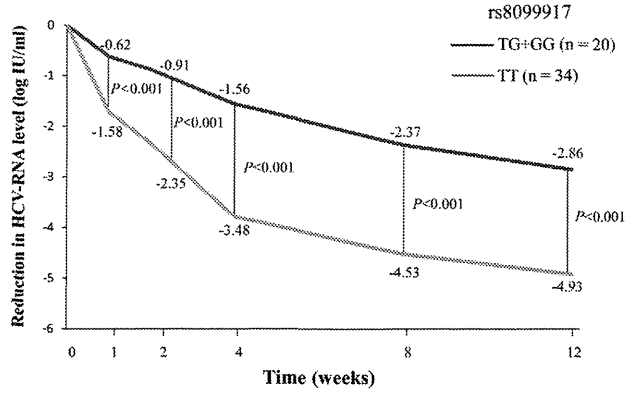

Figure 2 Weekly reduction of median hepatitis C virus (HCV)-RNA levels (log IU/ml) at 1, 2, 4, 8 and 12 weeks between *IL28B* single nucleotide polymorphisms rs8099917 genotype TT (n=34) and TG/GG (n=20) in HCV genotype 1-infected patients treated with pegylated interferon α plus ribavirin.

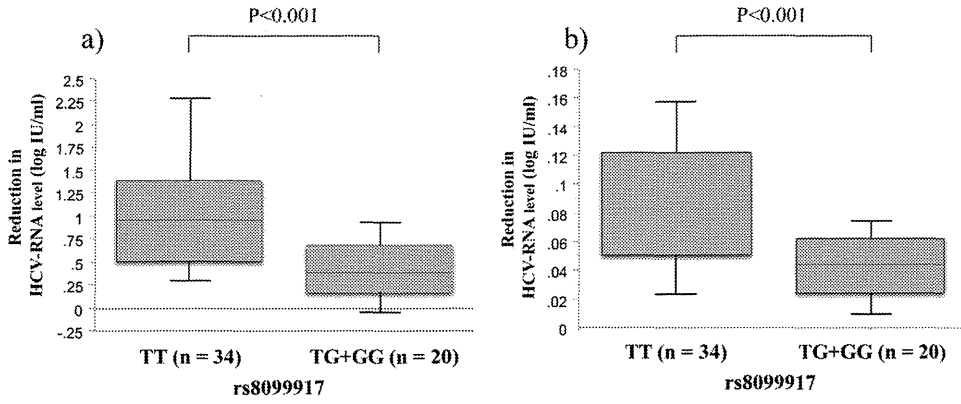

Figure 3 (A) The first-phase viral decline slope per day (Ph1/day) and (B) second-phase viral decline slope per week (Ph2/week) in hepatitis C virus (HCV) genotype 1-infected patients treated with pegylated interferon α plus ribavirin. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively.

−3.48 vs −1.56, p<0.001; −4.53 vs −2.37, p<0.01; −4.93 vs −2.86, p<0.001. Furthermore, the initial first-phase viral decline slope per day (Ph1/day) and subsequent second-phase viral decline slope per week (Ph2/week) in TT genotype were significantly higher than in genotype TG/GG (Ph1/day 0.94 ±0.83 vs 0.38±0.40 log IU/ml, p<0.001; Ph2/week 0.08±0.06 vs 0.04±0.03 log IU/ml, p<0.001) (figure 3).

Changes in serum HCV-RNA levels in chimeric mice treated by peg-IFN-α

In order to clarify the association between *IL28B* alleles of human hepatocytes and the response to peg-IFN-α, we prepared four lines of uPA/SCID mice and four different lots of human hepatocytes containing various rs8099917, rs8103142 and rs12979860 SNPs around the *IL28B* gene (table 2). The chimeric mice were inoculated with serum samples from each HCV-1b patient, and then HCV-RNA levels had increased and reached more than 10^6 copies/ml in all chimeric mice sera at 2 weeks after inoculation. After confirming the peak of HCV-RNA in all chimeric mice, they were subcutaneously administered with four times injections of the bolus dose of peg-IFN-α2a for 2 weeks (table 3). Figure 4 shows the change in the serum HCV-RNA levels for 14 days during IFN injection into chimeric mice transplanted with *IL28B* favourable or unfavourable human hepatocyte genotypes. On peg-IFN-α administration, no significant difference in the median reduction in HCV-RNA levels in the serum A-infected[22] chimeric mice sera was observed between favourable (n=7) and unfavourable

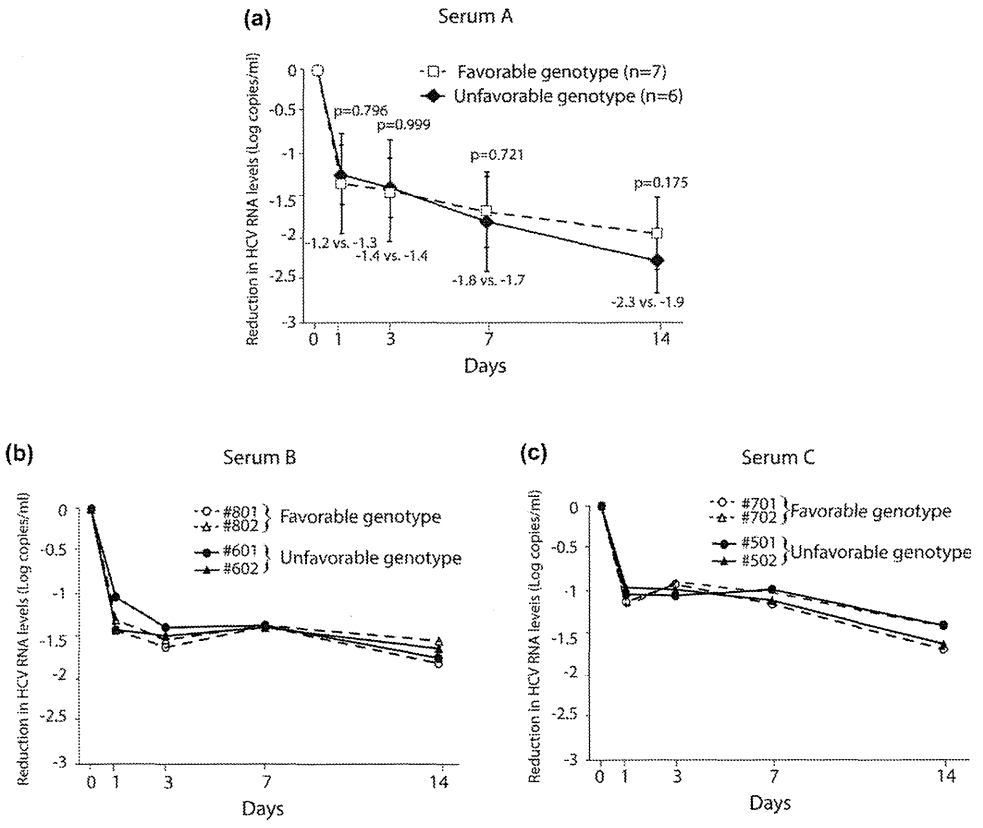

Figure 4 Median reduction of hepatitis C virus (HCV)-RNA levels (log copies/ml) after administering pegylated interferon α to chimeric mice having human hepatocytes containing various single nucleotide polymorphisms around the *IL28B* gene as favourable (rs8099917 TT) and unfavourable (rs8099917 TG) genotypes. Data are represented as mean+SD. Chimeric mice infected with a) serum A (n=7; favourable genotype, n=6; unfavourable genotype), (B) serum B (n=2, each genotype), and (C) serum C (n=2, each genotype). All serum samples were obtained from HCV-1b patients.

(n=6) *IL28B* genotypes on days 1, 3, 7 and 14 (−1.2 vs −1.3, −1.4 vs −1.4, −1.8 vs −1.7, and −2.3 vs −1.9 log copies/ml) (figure 4A). Moreover, we prepared two additional serum samples from the other HCV-1b patients (serum B and C)[21] to confirm the influence of *IL28B* genotype in early viral kinetics during IFN treatment. After establishing persistent infection with new HCV-1b strains in all chimeric mice, they were also administered four times injections of the bolus dose of peg-IFN-α2a for 2 weeks (figure 4B,C). In a similar fashion, no significant difference in HCV-RNA reduction in chimeric mice sera was observed between favourable and unfavourable *IL28B* genotypes.

Expression levels of ISG in chimeric mice livers

Because chimeric mice have the characteristic of severe combined immunodeficiency, the viral kinetics in chimeric mice sera during IFN treatment could be contributed by the innate immune response of HCV-infected human hepatocytes. Therefore, ISG expression levels in mice livers transplanted with human hepatocytes were compared between favourable and unfavourable *IL28B* genotypes (figure 5).

As shown in figure 5A, ISG expression levels in mice livers were measured at 8 h and 24 h after IFN treatment. The levels of representative antiviral ISG (eg, myxovirus resistance protein A, oligoadenylate synthetase 1, RNA-dependent protein kinase) and other ISG for promoting antiviral signalling (eg, Toll-like receptor 3, retinoic acid-inducible gene 1) were significantly induced at least 8 h after treatment, and prolonged at 24 h. No significant difference in ISG expression levels in HCV-infected livers was observed between favourable and unfavourable *IL28B* genotypes. The other inoculum for persistent infection of HCV-1b also demonstrated no significant difference in ISG

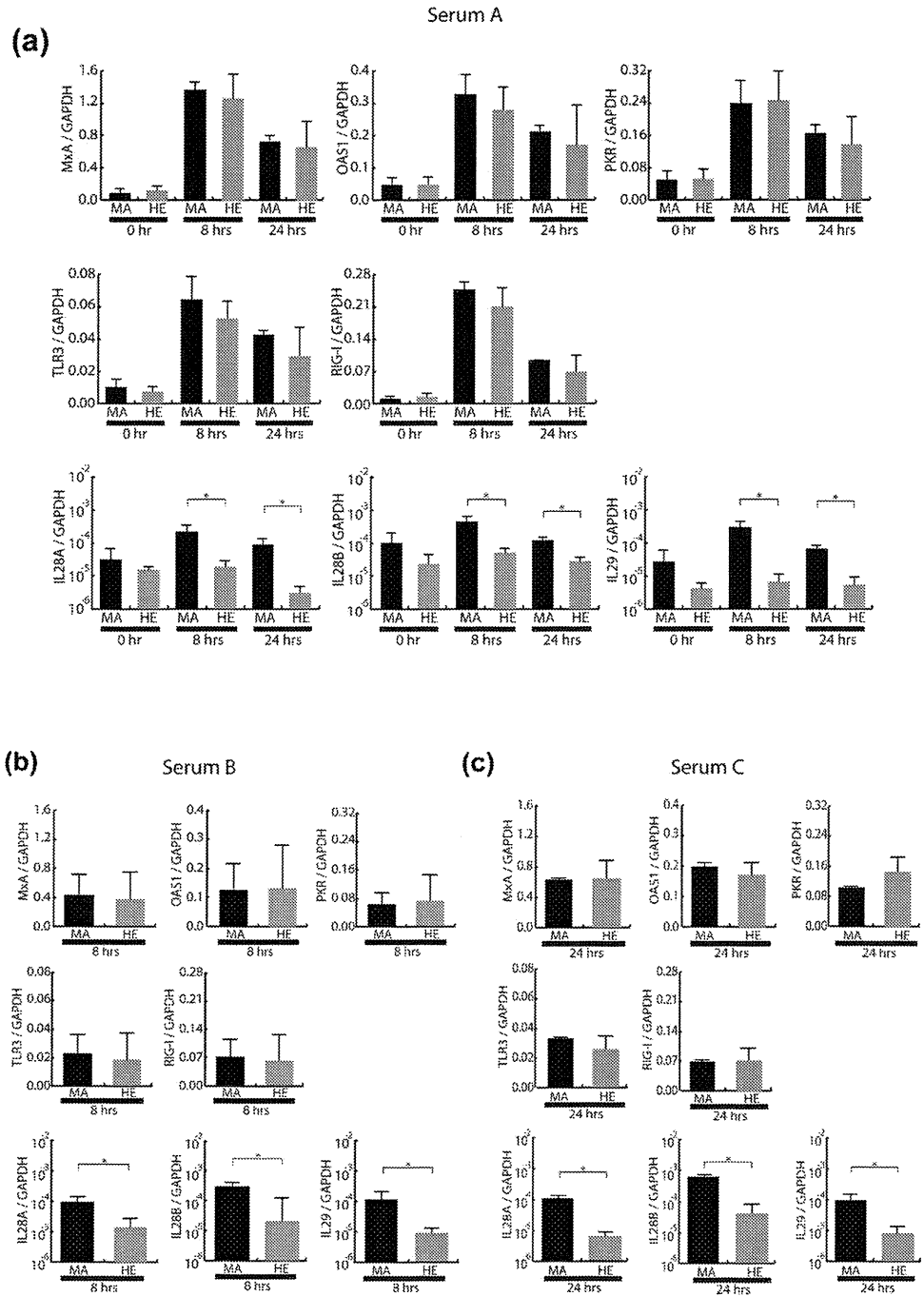

Figure 5 Intrahepatic interferon (IFN)-stimulated gene (ISG) expression levels in the pegylated interferon α (peg-IFN-α)-treated chimeric mice having human hepatocytes containing homozygous favourable allele (rs8099917 TT; MA) and heterozygous unfavourable allele (rs8099917 TG; HE) were measured and expressed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA. Data are represented as mean+SD. (A) Time kinetics of ISG after administration of the peg-IFN-α in serum A-infected chimeric mice (n=3, each genotype). Comparison of ISG expression levels at (B) 8 h in serum B-infected mice and (C) 24 h in serum C-infected mice after administering peg-IFN-α (n=3, each genotype). Predesigned real-time PCR assay of IL28B transcript purchased from Applied Biosystems can be cross-reactive to IL28A transcript. *p<0.05. MxA, myxovirus resistance protein A; OAS1, oligoadenylate synthetase 1; PKR, RNA-dependent protein kinase; RIG-1, retinoic acid-inducible gene 1; TLR3, Toll-like receptor 3.

expression levels between favourable and unfavourable *IL28B* genotypes (figure 5B,C). Interestingly, IFN-λ expression levels by treatment of peg-IFN-α were significantly induced in HCV-infected human hepatocytes harbouring the favourable *IL28B* genotype (figure 5 A–C).

DISCUSSION

Several recent studies have demonstrated a marked association between the chronic hepatitis C treatment response[6–9] and SNP (rs8099917, rs8103142 and rs12979860) near or within the region of the *IL28B* gene, which affected the viral dynamics during peg-IFN-α plus ribavirin therapy in Caucasian, African American and Hispanic individuals.[13]

It has been reported that when patients with chronic hepatitis C are treated by IFN-α or peg-IFN-α plus ribavirin, HCV-RNA generally declines after a 7–10 h delay.[25] The typical decline is biphasic and consists of a rapid first phase lasting for approximately 1–2 days during which HCV-RNA may fall 1–2 logs in patients infected with genotype 1, and subsequently a slower second phase of HCV-RNA decline.[26] The viral kinetics had a predictive value in evaluating antiviral efficacy.[14] In this study, biphasic decline of the HCV-RNA level during peg-IFN-α treatment was observed in both patients and chimeric mice infected with HCV genotype 1; however, in the first and second phases of viral kinetics, a difference between *IL28B* genotypes was observed only in HCV-infected patients; a more rapid decline in serum HCV-RNA levels after administering peg-IFN-α plus ribavirin was confirmed in patients with the TT genotype of rs8099917 compared to those with the TG/GG genotype.

On the other hand, in-vivo data using the chimeric mouse model showed no significant difference in the reduction of HCV-RNA titers in mouse serum among four different lots of human hepatocytes containing *IL28B* favourable (rs8099917 TT) or unfavourable (rs8099917 TG) genotypes, which was confirmed by the inoculation of two additional HCV strains. These results indicated that variants of the *IL28B* gene in donor hepatocytes had no influence on the response to peg-IFN-α under immunosuppressive conditions, suggesting that the immune response according to *IL28B* genetic variants could contribute to the first and second phases of HCV-RNA decline and might be critical for HCV clearance by peg-IFN-α-based therapy.

Two recent studies indeed revealed an association between the *IL28B* genotype and the expression level of hepatic ISG in human studies.[27 28] Quiescent hepatic ISG before treatment among patients with the *IL28B* favourable genotype have been associated with sensitivity to exogenous IFN treatment and viral eradication; however, it is difficult to establish whether the hepatic ISG expression level contributes to viral clearance independently or appears as a direct consequence of the *IL28B* genotype. Another recent study addressed this question and the results suggested that there is no absolute correlation with the *IL28B* genotype and hepatic expression of ISG.[29] Our results on the hepatic ISG expression level in immunodeficient chimeric mice also suggested that no significant difference in ISG expression levels was observed between favourable and unfavourable *IL28B* genotypes. However, these results were not consistent with a previous report using chimeric mice that the favourable *IL28B* genotype was associated with an early reduction in HCV-RNA by ISG induction.[30] The reasons for the discrepancy might depend on the dose and type of IFN treatment, as well as the time point when ISG expression was examined in the liver. In addition, although IFN-λ transcript levels measured in peripheral blood mononuclear cells or liver revealed inconsistent results in the context of an association with the *IL28B* genotype,[7 8] our preliminary assay on the IL28A, IL28B and IL29 transcripts in the liver first indicated that the induction of IFN-λ on peg-IFN-α administration could be associated with the *IL28B* genotype. Therefore, the induction of IFN-λ followed by immune response might contribute to different viral kinetics and treatment outcomes in HCV-infected patients, because no difference was found in chimeric mice without immune response.

It has also been reported that the mechanism of the association of genetic variations in the *IL28B* gene and spontaneous clearance of HCV may be related to the host innate immune response.[11] Interestingly, participants with seroconversion illness with jaundice were more frequently rs8099917 homozygous favourable allele (TT) than other genotypes (32% vs 5%, p=0.047). This suggests that a stronger immune response during the acute phase of HCV infection among patients with the *IL28B* favourable genotype would induce more frequent spontaneous clearance of HCV.

Taking into account both the above results in acute HCV infection and our results conducted on chimeric mice that have the characteristic of immunodeficiency, it is suggested that the response to peg-IFN-α associated with the variation in *IL28B* alleles in chronic hepatitis C patients would be composed of the intact immune system.

Acknowledgements The authors would like to thank Kyoko Ito of Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan for doing the quantification of gene-expression assays.

Contributors YT and MM conceived the study. TW and FS and YT conducted the study equally. TW and FS coordinated the analysis and manuscript preparation. All the authors had input into the study design, patient recruitment and management or mouse management and critical revision of the manuscript for intellectual content. TW, FS and YT contributed equally.

Funding This study was supported by a grant-in-aid from the Ministry of Health, Labour, and Welfare of Japan (H22-kannen-005) and the Ministry of Education, Culture, Sports, Science and Technology, Japan, and grant-in-aid for research in Nagoya City University.

Competing interests None.

Patient consent Obtained.

Ethics approval This study was conducted with the approval of each ethics committee at the Nagoya City University and Nagasaki Medical Center (see supplementary information, available online only).

Provenance and peer review Not commissioned; externally peer reviewed.

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Hepatology Research

Hepatology Research 2012; 42: 1089–1099 doi: 10.1111/j.1872-034X.2012.01032.x

Original Article

Development of specific and quantitative real-time detection PCR and immunoassays for λ3-interferon

Masaya Sugiyama,[1] Tatsuji Kimura,[2] Shigeko Naito,[2] Motokazu Mukaide,[1,3] Takanori Shinauchi,[2] Masakatsu Ueno,[2] Kiyoaki Ito,[1] Kazumoto Murata[1] and Masashi Mizokami[1]

[1]*The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa,* [2]*Institute of Immunology, and* [3]*SRL, Tokyo, Japan*

Aim: Single nucleotide polymorphisms (SNP) around interferon (IFN)-λ3 have been associated with the response to pegylated IFN-α treatment for chronic hepatitis C. Specific quantification methods for IFN-λ3 are required to facilitate clinical and basic study.

Methods: Gene-specific primers and probes for IFN-λ1, 2 and 3 were designed for real-time detection PCR (RTD–PCR). Dynamic range and specificity were examined using specific cDNA clones. Total RNA from hematopoietic and hepatocellular carcinoma cell lines was prepared for RTD–PCR. Monoclonal antibodies were developed for the IFN-λ3-specific immunoassays. The immunoassays were assessed by measuring IFN-λ3 in serum and plasma.

Results: The RTD–PCR had a broad detection range (10–10^7 copies/assay) with high specificity (~10^7-fold specificity). Distinct expression profiles were observed in several cell lines. Hematopoietic cell lines expressed high levels of IFN-λ compared with hepatocellular carcinoma cells, and Sendai virus infection induced strong expression of IFN-λ. The developed chemiluminescence enzyme immunoassays (CLEIA) detected 0.1 pg/mL of IFN-λ3 and showed a wide detection range of 0.1–10 000 pg/mL with little or no cross-reactivity to IFN-λ1 or IFN-λ2. IFN-λ3 could be detected in all the serum and plasma samples by CLEIA, with median concentrations of 0.92 and 0.86 pg/mL, respectively.

Conclusion: Our newly developed RTD–PCR and CLEIA assays will be valuable tools for investigating the distribution and functions of IFN-λ3, which is predicted to be a marker for predicting outcome of therapy for hepatitis C or other virus diseases.

Key words: chemiluminescence enzyme immunoassay, enzyme-linked immunoassay, interleukin-28B, λ-interferon

INTRODUCTION

TYPE III INTERFERONS (IFN), designated -λ1, -λ2 and -λ3 (or interleukins [IL]-29, -28A and -28B, respectively) were recently discovered and shown to have antiviral activity.[1,2] A recent report also confirmed that while IFN-λ exhibit IFN-like activity,[3] they are structurally related to members of the IL-10 cytokine family, particularly IL-22.[4] The expression of types I and III IFN is induced in response to stimulation of pattern recognition receptors (PRR), including Toll-like receptors (TLR) and RIG-like receptors (RLR).[5–7] IFN-λ also induce several IFN-stimulated genes (ISG), including dsRNA-activated serine/threonine protein kinase, 2′,5′-OAS, and MxA (also known as MX1) proteins, all of which mediate antiviral protection.[8,9] IFN-λ treatment for tumors or viral infections could potentially be associated with fewer side-effects than type I IFN because IFN-λ receptor 1 has a more limited organ distribution than the type I IFN receptor.[1,2]

A recent genome-wide association study using a single nucleotide polymorphism (SNP) microarray revealed associations between SNP surrounding the IFN-λ3 gene and both the response to pegylated IFN-α therapy in hepatitis C patients[10–13] and spontaneous clearance of acute hepatitis C virus (HCV) infection.[11,14] In patients with these risk alleles, low expression of IFN-λ2 and -λ3 mRNA was observed in peripheral blood mononuclear cells and was associated with the treatment response to

Correspondence: Professor Masashi Mizokami, The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, 1-7-1 Kohnodai, Ichikawa 272-8516, Japan. Email: mmizokami@hospk.ncgm.go.jp

Received 30 January 2012; revision 24 March 2012; accepted 10 April 2012.

pegylated IFN-α therapy.[12,13] However, the mechanistic link between these SNP and IFN-λ3 expression is unclear because specific detection of the gene or protein for IFN-λ2 or -λ3 has been difficult.

Immunoassays were previously constructed to measure IFN-λ2 in culture medium and clinical samples.[15–17] However, cross-reactivity with IFN-λ3 is a possibility with these immunoassays because IFN-λ2 and -λ3 share 97.5% amino acid identity (193 of 200 amino acids).[1,2] Therefore, conflicting results were obtained using these immunoassays to measure the IFN-λ2/-λ3 concentration in sera – 40 pg/mL,[16] 24 pg/mL[15] or 3.6 ng/mL[17] – in three different studies.

Specific measurement IFN-λ3 levels will be necessary to understand the pathogenesis of disease in HCV-infected and other IFN-λ-related diseases, and for this, gene-specific quantification of each IFN-λ will be required. In the present study, we developed real-time detection polymerase chain reaction (RTD–PCR) specific for each IFN-λ mRNA with approximately 10^7-fold specificity among IFN-λ genes. We also constructed highly specific immunoassays for IFN-λ3, which had little or no cross-reactivity with IFN-λ2.

METHODS

Gene-specific RTD–PCR for IFN-λ1, -λ2 or -λ3

TOTAL RNA WERE isolated from cell lines using RNeasy Mini Kit with an RNase-free DNase Set to avoid DNA contamination, according to the manufacturer's instructions (Qiagen, Hilden, Germany). First-strand cDNA was synthesized using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science, Basel, Switzerland). The kit primer was added to 3 μg of total RNA solution and incubated at 65°C for 10 min. Reaction mixtures were sequentially incubated at 50°C for 60 min and at 85°C for 5 min. To test for DNA contamination, samples without reverse transcriptase were also prepared. Specific primer sets to detect IFN-λ1, -λ2 or -λ3 cDNA were designed within the region containing the gene-specific substitution and the primer sequences are shown in Table 1. The final concentration of the forward and reverse primers was adjusted to 900 nM, and that of the probe was 250 nM. For IFN-λ2 detection, dimethylsulfoxide was added at a final concentration of 10% to improve the specificity of the IFN-λ2 probe. cDNA aliquots (1 μL) were amplified in triplicate using RTD–PCR LightCycler 480 probe Master Mix (Roche Applied Science, Basel, Switzerland) or TaqMan Gene Expression Master Mix (Life Technologies, Carlsbad, CA, USA) together with a LightCycler 480II (Roche Applied Science), according to the manufacturer's instructions. The PCR conditions for IFN-λ2 or -λ3 were: stage 1, 95°C for 5 min; and stage 2, 95°C for 10 s followed by amplification at 65°C for 90 s. Stage 2 was repeated for 45 cycles. The conditions for IFN-λ1 quantification were the same but with an amplification temperature of 67°C. Specificity of amplification was confirmed by examination of dissociation reaction plots, with a distinct single peak indicating amplification of a single DNA sequence. The PCR products were analyzed on 2% agarose gels and by sequencing to confirm molecular sizes and specific amplification. Data were analyzed by absolute quantification using LightCycler 480 software and were normalized using human glyceraldehyde-3-phosphate dehydrogenase. A standard curve was prepared using serial 10-fold dilutions of plasmids containing IFN-λ1, -λ2 or -λ3 cDNA. The standard curve was linear over 7 log with a 0.998 correlation coefficient.

Table 1 Primers and probes for gene-specific real-time polymerase chain reaction

	ID	Sequence
Primer	IFN-λ1/F	5′-ggccagggacgccttggaagagtc-3′
	IFN-λ1/R	5′-tgggctgaggctggatacag-3′
	IFN-λ2/3/F	5′-gccaaagatgccttagaagagtc-3′
	IFN-λ2/3/R	5′-tgggctgaggctggatacag-3′
Probe	IFN-λ1	FAM-ctaggacgtcctccagggctg-MGB
	IFN-λ2	FAM-ccaagacgtccaccagggctg-MGB
	IFN-λ3	VIC-ccaagacatcccccagggctg-MGB

F, forward; IFN, interferon; R, reverse.

Cell lines

Human hepatocellular carcinoma cell lines HepG2 and HuH7, and the human cervical cancer cell line HeLa, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin. The Burkitt lymphoma cell line Raji, the human T-cell leukemia cell line Jurkat and the human promyelocytic leukemia cell line HL-60 (kindly provided by Dr Ryuzo Ueda, Nagoya City University) were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin. All incubations were performed at 37°C in an incubator with 5% CO_2. Natural human IFN-α was purchased from Hayashibara (Okayama, Japan). Lipopolysaccharide (LPS, 0127:B8; Sigma-Aldrich, St Louis, MO, USA), polyinosinic–polycytidylic acid (poly

I:C; Sigma-Aldrich) and Sendai virus (SeV; Hayashibara) were used for the induction of IFN-λ.

Serum/plasma samples and patients

Serum and plasma paired samples were obtained from 20 healthy volunteers (13 males and seven females). Sera containing high levels of IFN-λ3 were obtained from hepatitis C patients. Informed consent was obtained from all subjects and the study was conducted in accordance with the Declaration of Helsinki of 1983. All samples were stored at −80°C until tested.

Recombinant IFN-λs and peptides

Recombinant human IFN-λ1, -λ2 and -λ3 were purchased from R&D Systems (Minneapolis, MN, USA). Full-length recombinant IFN-λ3 (rIFN-λ3) was expressed in HeLa cells as previously reported.[18] Briefly, the rIFN-λ3 with 6× tagged His at the C-terminal was secreted into culture media and purified by Ni^{2+}-affinity chromatography. The N-terminal of the purified protein started from Val-26, indicating that the signal peptide was ablated naturally.[18] The concentration of rIFN-λ3 was determined using the Bradford protein assay (Bio-Rad, Hercules, CA, USA). Mutant IFN-λ3 with a K74R substitution was also expressed in HeLa cells.

Monoclonal antibodies specific for IFN-λ3

BALB/c mice were immunized i.p. with rIFN-λ3 mixed with Freund's adjuvant (Wako Pure Chemical Industries, Osaka, Japan). Splenocytes from the immunized mice were fused with NS-1 myeloma cells. The fused hybridoma cells were selected in RPMI-1640 medium supplemented with 10% fetal calf serum, hypoxanthine, aminopterin and thymidine. Hybridomas producing anti-IFN-λ3 monoclonal antibody (mAb) were selected by enzyme-linked immunoassay (ELISA) using rIFN-λ3 and then cloned by limiting dilution. mAb subclasses were determined by the micro-Ouchterlony method. All hybridoma cell lines were transplanted into the mouse abdominal cavity. From the mouse ascites, mAb were purified by protein-G column chromatography (GE Healthcare Japan, Tokyo, Japan). All antibodies were mapped by ELISA using 20-residue-long synthetic peptides with 10 overlapping amino acids (Sigma-Aldrich Japan, Tokyo, Japan).[19]

ELISA

Microtiter wells were coated with 100 μL of anti-IFN-λ2 mAb (Clone 248512; R&D Systems) or anti-IFN-λ3 mAb (Clone TA2602). The wells were washed, blocked with Casein-Na solution and air-dried. Samples (50 μL) were then added to each well together with 50 μL of assay buffer containing 1% mouse serum, 5 mM ethylenediamine tetraacetic acid, 0.05% Tween-20, 150 mM NaCl, 20 mM HEPES, 0.1% Casein-Na and 1% bovine serum albumin (pH 7.4). The wells were incubated for 1 h at 25°C and then washed threefold. A 100-μL aliquot of biotinylated anti-IFN-λ2 polyclonal antibody (R&D Systems) or biotin-conjugated TA2664 mAb was added to each well and incubated for 1 h at 25°C, followed by three washes. A 100-μL aliquot of avidin-conjugated horseradish peroxidase (Vector Laboratories, Burlingame, CA, USA) was added to each well and incubated for 1 h at 25°C. The wells were washed fivefold and 50 μL of substrate solution (TMB, BioFX; SurModics, Eden Prairie, MN, USA) was added. The absorbance at 450 nm was measured using a microplate reader (MTP-120; Corona Electric, Ibaraki, Japan).

Chemiluminescence enzyme immunoassay (CLEIA)

TA2664 mAb was digested with pepsin (Worthington Biochemical, Freehold, NJ, USA) in 100 mM acetate buffer (pH 3.8). The $F(ab)''_2$ fragments were isolated by gel filtration on Superdex 200HR (GE Healthcare Japan). The $F(ab)'_2$ fragments were reduced to Fab″ and conjugated to alkaline phosphatase (ALP; Roche Diagnostics) by the maleimide hinge method.[20] The TA2664-ALP conjugate was purified by gel filtration chromatography on Superdex 200HR.

Microtiter wells (FluoroNunc Maxisorp Black; Thermo Fisher Scientific, Waltham, MA, USA) were coated with TA2602 or TA2650 mAb and washed, blocked and dried as described above. Samples (50 μL) were added to each well together with 50 μL of assay buffer (pH 7.4). The wells were incubated for 1 h at 25°C and washed threefold. A 100-μL aliquot of ALP-conjugated TA2664 mAb was added to each well and incubated for 1 h at 25°C. After washing fivefold, 50 μL of substrate solution, CDP-Star with Sapphire II (Applied Biosystems, Bedford, MA, USA) was added. The relative luminescence intensity (RLI) was measured using a luminescence microplate reader (SpectraMax L; Molecular Devices, Sunnyvale, CA, USA). Serially diluted rIFN-λ3 was used as a standard in each assay. The standard log RLI was plotted against $\log_{10}$ concentration. IFN-λ3 concentrations were calculated from the calibration curve.

Statistical analysis

Statistical analyses were conducted using SPSS software (SPSS ver. 18J; SPSS, Chicago, IL, USA) and Microsoft Excel 2007 (Microsoft, Redmond, WA, USA). Discrete

variables were evaluated using Fisher's exact probability test. *P*-values were calculated using two-tailed Student *t*-tests or Wilcoxon rank sum test for continuous data and χ^2-test for categorical data. *P*-values less than 0.05 were considered statistically significant.

RESULTS

Development of gene-specific real-time PCR

A ROBUST QUANTIFICATION system specific for IFN-λ1, -λ2 and -λ3 is required to understand the specific roles of each of these cytokines in basic physiology and in human disease. To this end, we developed RTD–PCR specific for IFN-λ mRNA. The gene-specific PCR primers were designed to anneal directly to the cDNA sequences of each gene. To achieve specificity, a unique mRNA-specific fluorescent probe was required to discriminate the targets from non-specific and cross-reactive amplicons. The performance of the RTD–PCR assays was initially evaluated using cDNA clones of each IFN-λ that were diluted over seven orders of magnitude (from 10^7 to 10 copies per assay) and quantified by RTD–PCR (Fig. 1). The excellent linearity of the standard curve suggested that the gene-specific assay had a wide dynamic range of at least 7 log and was able to detect as few as 10 copies per reaction. As shown in Figure 1, cross-reaction did not occur in the IFN-λ1, -λ2 and -λ3 assays, suggesting that they achieved approximately 10^7-fold specificity for each gene.

Expression profile of IFN-λ

The performance of these assays was examined by analysis of total RNA samples from cultured cell lines using Raji, Jurkat, HT-60, HuH7 and HepG2. The cell lines were stimulated with LPS (3 ng/mL), poly I:C (30 ng/mL) or SeV (50 HA/mL) for 24 h following IFN-α treatment (100 U/mL) for 16 h. Figure 2 illustrates the expression profile of IFN-λ following stimulation with these PRR ligands; mRNA expression of the IFN-λ was detected in all cell lines under these conditions. SeV infection induced particularly high expression of IFN-λ mRNA compared with the other stimuli. Interestingly, the expression profiles varied between the cell lines (Fig. 2). Raji cells strongly expressed IFN-λ1, -λ2 and -λ3 in response to IFN-α only, but this cytokine had little effect, alone or in combination with PRR agonists, on IFN-λ expression in the other cell lines (Fig. 2a vs 2b–e). Hematopoietic cell lines expressed low levels of IFN-λ1 with or without PRR stimulation; however, cell lines derived from hepatocellular carcinomas expressed low

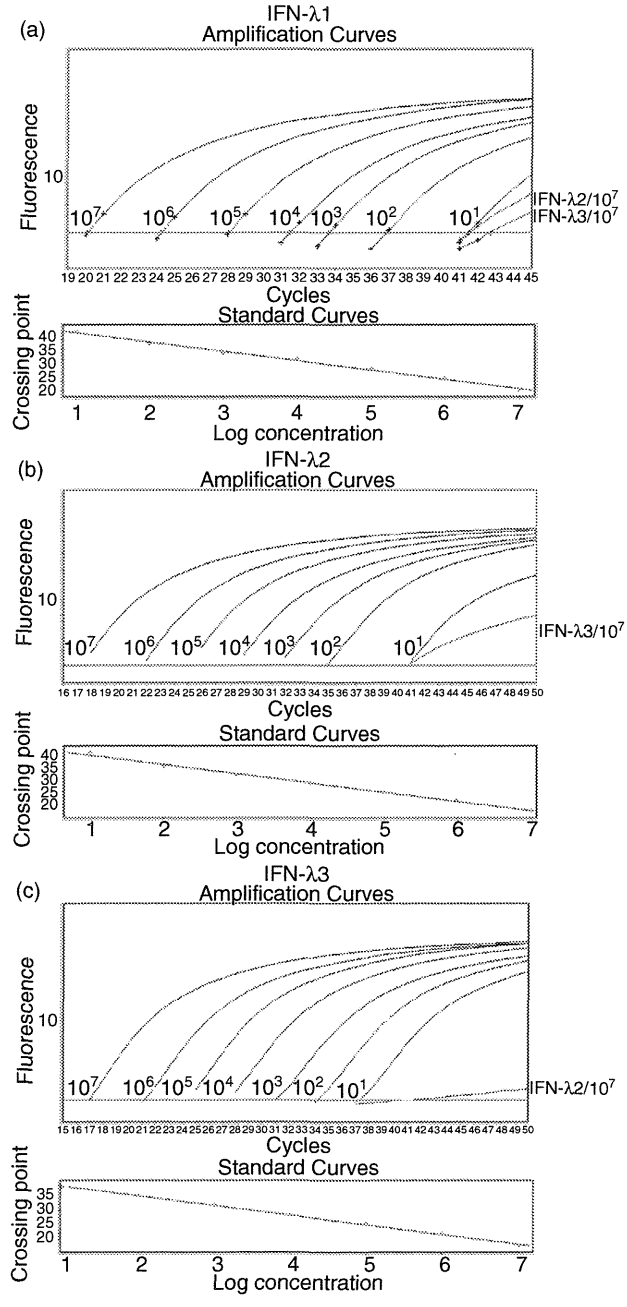

Figure 1 Standard curve and dynamic range of the gene-specific real-time detection polymerase chain reaction. Plasmids constructed with cDNA for each interferon (IFN)-λ gene were used to assess specificity among the IFN-λ family and to establish the standard curve and dynamic range. (a) IFN-λ1, (b) IFN-λ2 or (c) IFN-λ3. The amplification curve is shown in the upper panel. The standard curve is shown in the lower panel. Results of a representative experiment are shown in each figure based on three separate experiments.

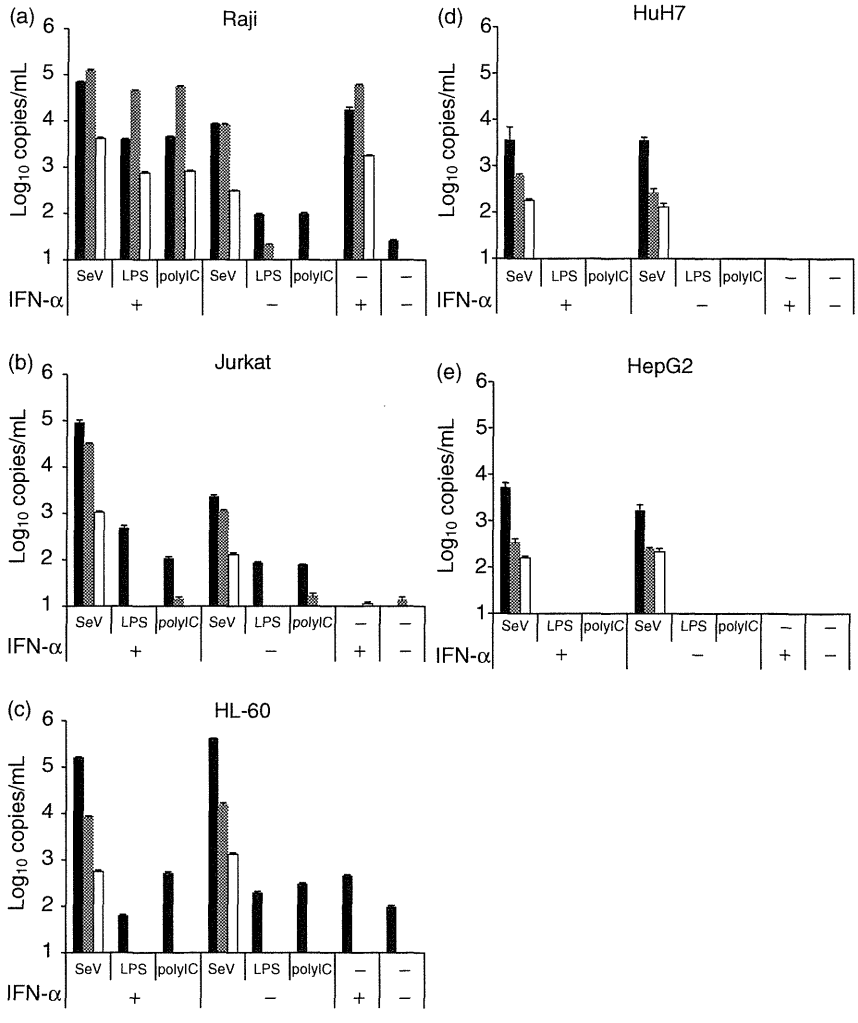

Figure 2 Gene-specific quantification of each interferon (IFN)-λ mRNA using real-time detection polymerase chain reaction. Cell lines were stimulated with Sendai virus (SeV) (50 HA/mL), lipopolysaccharide (LPS) (3 ng/mL), or polyinosinic–polycytidylic acid (poly I:C) (30 ng/mL) for 24 h following IFN-α treatment for 16 h. Total RNA was isolated, reverse-transcribed for cDNA synthesis, and used for gene-specific quantitative polymerase chain reaction, as described in Methods. (a) Raji cells, (b) Jurkat cells, (c) HL-60 cells, (d) HuH7 cells and (e) HepG2 cells. Results of a representative experiment, from a total of three separate experiments, are shown in each figure. ■, IFN-λ1; ▩, IFN-λ2; □, IFN-λ3.

levels of IFN-λ only following stimulation with SeV or SeV plus IFN-α (Fig. 2a–c vs 2d–e). These data suggested that the strong induction of IFN-λ was facilitated by hematopoietic cells in accord with a previous paper.[21,22] Overall, the level of mRNA expression was the highest for IFN-λ1, followed by IFN-λ2 and IFN-λ3 by a margin of approximately 2–10-fold, although this pattern was not observed in Raji cells.

Previous ELISA for IFN-λ and its specificity and sensitivity

In previous reports,[15–17] an ELISA was constructed to detect serum levels of IFN-λ using a set of commercial antibodies. We examined the specificity of the ELISA for the detection of IFN-λ2 or -λ3. The ELISA exhibited weak specificity for rIFN-λ2 or -λ3 because it reacted to both rIFN-λ2 and -λ3, but with approximately 10-fold stronger binding to IFN-λ2 (Fig. 3a). Linearity of detection for rIFN-λ2 and -λ3 was approximately 100–10 000 pg/mL and 1000–100 000 pg/mL, respectively. However, the accurate quantification of IFN-λ2 or -λ3 would be difficult in clinical samples because the secretion level is very low.

Selection and characterization of mAb specific for IFN-λ3

To develop a detection system specific for IFN-λ3 protein, a total of 12 hybridoma cell lines producing anti-IFN-λ3 antibody were established following immunization of a mouse with rIFN-λ3. Of the 12 clones, one produced immunoglobulin (Ig)G2b-κ (clone TA2613), while the other clones produced IgG1-κ. Five mAb were specific for IFN-λ3 (TA2601, TA2602, TA2603, TA2613 and TA2664) but the remaining seven reacted with both

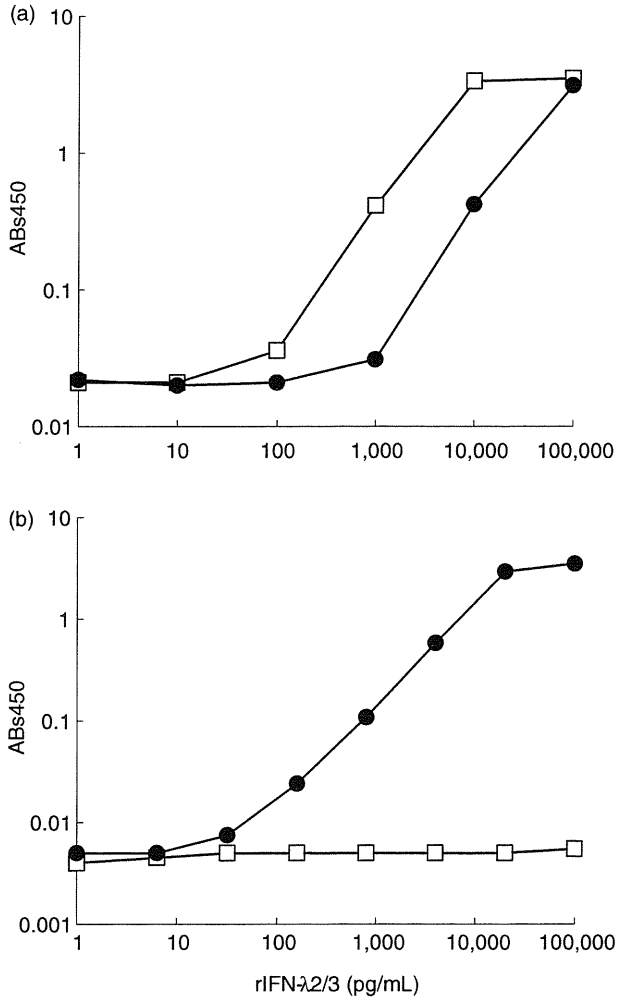

Figure 3 Specificity and the standard reactions of enzyme-linked immunoassays (ELISA) for interferon (IFN)-λ2 and -λ3. Serially diluted recombinant IFN (rIFN)-λ2 (□) or -λ3 (●) standards were measured by ELISA. (a) The results of ELISA using a set of commercially available anti-IFN-λ2 antibodies (R&D Systems).[15–17] (b) Standard curve using the IFN-λ3 ELISA developed in this study (using TA2602-coated/TA2664-biotin monoclonal antibodies). Results of a representative experiment are shown in each figure, based on three separate experiments. □, rIFN-λ2; ●, rIFN-λ3.

IFN-λ3 and -λ2 (TA2607, TA2608, TA2611, TA2622, TA2650, TA2651 and TA2670).

For the development of an IFN-λ3-specific assay, we selected TA2602 or TA2650 as the immobilized mAb for capturing IFN-λ3 and TA2664 as a detector mAb by the screening of all mAb. We sought to identify the specific epitope recognized by the mAb using 20-mer synthetic peptides from IFN-λ3 with 10 overlapping amino acids. Although all 12 mAb reacted with rIFN-λ3, none of them reacted with the synthetic peptides, suggesting that the mAb all recognized conformational epitopes.

Development of ELISA for IFN-λ3

Two new ELISA specific for IFN-λ3 were established based on the mAb described above. The calibration curve was determined using rIFN-λ3 as a standard (Fig. 3b). The lower and upper detection limits of each ELISA were 10 and 10 000 pg/mL, respectively. These ELISA were used to measure IFN-λ3 concentration in culture medium and serum/plasma. Although IFN-λ3 was successfully detected in the culture medium from hematopoietic cells, it could not be detected in 22 of the 28 serum/plasma samples (78.5%) (data not shown), suggesting that the detection range of these ELISA was not sufficient for assaying clinical samples.

Development of CLEIA for IFN-λ3

The ELISA described above was adapted to detect low level IFN-λ3 by using ALP-labeled antibody and its chemiluminescent substrate to develop a CLEIA. The Fab' fragment of TA2664 was directly labeled with ALP for use as a detection antibody. The analytical lower detection limit was 0.1 pg/mL, determined as the lowest concentration at which the mean −2 standard deviations (SD) of the RLI did not overlap with the mean +2 SD of the zero calibrator ($n = 8$) (Fig. 4a). Therefore, the CLEIA assays had high sensitivity and displayed a broad dynamic range, from 0.1–10 000 pg/mL.

The linearity of these assays was examined using three serum samples containing high levels of IFN-λ3, which were serially diluted in assay buffer (Fig. 4b). The IFN-λ3 concentration decreased linearly with serial dilution through to the zero point. Interassay reproducibility was assessed from five measurements of the three serum specimens. The mean IFN-λ3 values of the specimens were 11.4, 7.9 and 5.0 pg/mL, and the coefficients of variation (CV) were 8.6%, 8.6% and 16.2%, respectively. For assessment of the recovery rate, 10-μL aliquots of rIFN-λ3 were added to 90 μL of reference serum and the IFN-λ3 concentration in these samples was measured. Recovery rates ranged 81.0–97.8%, indicating that blood components did not interfere with the assay (Table S1). TA2650/TA2664 CLEIA showed similar results to that of TA2602/2664 CLEIA.

Specificity of the IFN-λ3 CLEIA

The specificity of the IFN-λ3 CLEIA (TA2602/TA2664) was investigated using rIFN-λ1, -λ2 and -λ3. The standard

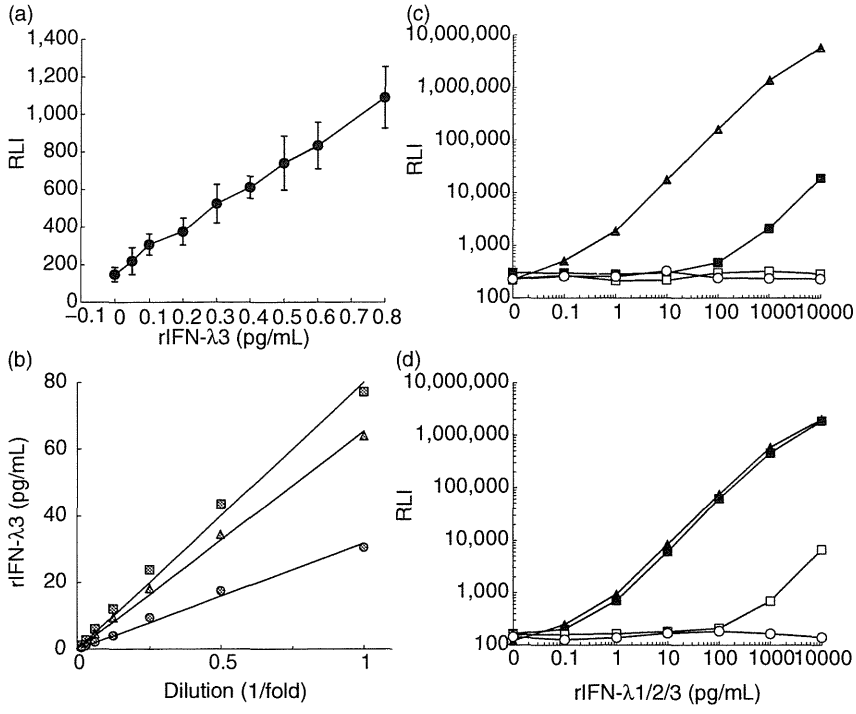

Figure 4 Detection limit, specificity and dilution linearity of interferon (IFN)-λ3 chemiluminescence enzyme immunoassay (CLEIA). The specificity and sensitivity of the newly developed CLEIA assay were assessed. (a) Detection limit was determined using serially diluted recombinant interferon (rIFN)-λ3. Results are means of eight assays and the error bars show 2 standard deviations (SD). The dotted line indicates the mean +2 SD of zero calibrator. (b) Dilution linearity was examined by serial dilution of three serum samples from donors with high levels of IFN-λ3. Data are from the enzyme-linked immunoassay (ELISA) using TA2602-coated/TA2664-biotin monoclonal antibodies. Results of a representative experiment are shown in each figure, based on three separate experiments. (c) TA2602 coat/TA2664-alkaline phosphatase (ALP) CLEIA or (d) TA2650 coat/TA2664-ALP CLEIA. Serially diluted rIFN-λ1 (R&D; ○), -λ2 (R&D; □), -λ3 wild-type (HeLa; ▲) or -λ3 with K74R substitution (HeLa; ■) were measured by IFN-λ3-specific CLEIA. The assay reactivity is shown as relative luminescence intensity (RLI). Results of a representative experiment are shown in each figure, based on three separate experiments. ▩, serum 1; ▲, serum 2; ◍, serum 3. ▲, rIFN-λ3 wild; ■, rIFN-λ3 K74R; ○, rIFN-λ1; □, rIFN-λ2.

curve of each IFN-λ3 CLEIA indicated that the upper detection limit was at least 10 000 pg/mL (Fig. 4c,d). The assay had no cross-reactivity with IFN-λ1 or -λ2 (Fig. 4c). Reactivity with the K74R substitution of IFN-λ3 was also examined because the SNP responsible for this substitution, rs8103142, was one of the SNP significantly associated with the response to pegylated IFN-α administration in chronic hepatitis C patients.[12,13] The reactivity of the TA2602/TA2664 CLEIA with the K74R substitution was reduced by a factor of 1000 compared to wild-type IFN-λ3 (Fig. 4c), indicating that this assay was specific to wild-type IFN-λ3 with no cross-reactivity to rIFN-λ1 and -λ2. The specificity of the TA2650/TA2664 CLEIA was also tested. The TA2650-coated/TA2664-ALP sandwich assay reacted with K74R rIFN-λ3 to the same extent as it did with wild-type rIFN-λ3 (Fig. 4d). This assay slightly cross-reacted with rIFN-λ2, with 1/500th the strength of its reaction with IFN-λ3.

IFN-λ3 detection in serum/plasma pair samples

Levels of IFN-λ3 were examined in serum/plasma pair samples from healthy volunteers ($n = 20$). IFN-λ3 was detectable in all samples and the median concentrations of IFN-λ3 in serum or plasma were 0.92 (range, 0.23–5.12) and 0.86 (0.23–5.81) pg/mL, respectively, using the TA2602/TA2664-ALP CLEIA (Fig. 5a), and 1.52 (range 0.66–7.43) and 1.32 (0.55–8.95) pg/mL, respectively, using the TA2650/TA2664-ALP CLEIA (Fig. 5b). The IFN-λ3 levels in plasma were not significantly different from serum ($P = 0.88$, =0.10). TA2602/TA2664 assay represented lower value compared with that of

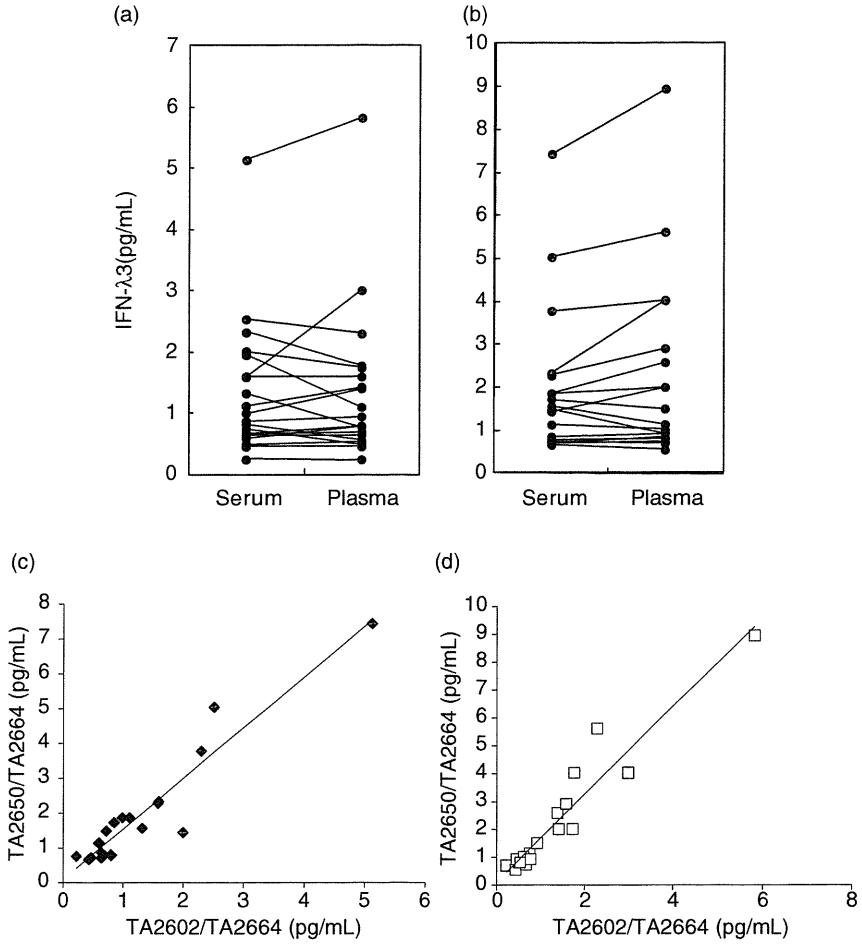

Figure 5 Interferon (IFN)-λ3 quantification in serum or plasma. IFN-λ3 concentration was measured by each chemiluminescence enzyme immunoassay (CLEIA) using serum/plasma pair specimens obtained from healthy volunteers ($n = 20$). (a) TA2602 coat/TA2664-alkaline phosphatase (ALP) CLEIA, (b) TA2650 coat/TA2664-ALP CLEIA. Scatter plot between TA2602/TA2664 and TA2650/TA2664 was shown on (c) serum and (d) plasma. IFN-λ3 concentrations are indicated as pg/mL. The samples were processed in duplicate. ◆, serum; □, plasma.

TA2650/TA2664 assay because of low reactivity to K74R (Fig. 4c,d). Scatter plots between TA2602/TA2664 and TA2650/TA2664 assay showed high correlation on serum and plasma ($R^2 = 0.90$ and $= 0.91$, respectively) (Fig. 5c,d), suggesting that these assays will be useful for both serum and plasma specimens.

DISCUSSION

THE IFN-λ3 GENE attracted much attention following genome-wide association studies that identified SNP surrounding this gene to be associated with the response to pegylated IFN-α therapy in chronic hepatitis C patients.[10–13] To further understand the relationship between IFN-λ3 and the response to IFN-α therapy, IFN-λ3-specific quantification methods are required. In the present study, we developed IFN-λ1-, -λ2- and -λ3-specific RTD–PCR and quantitative ELISA and CLEIA immunoassays specific for IFN-λ3. The RTD–PCR was able to detect IFN-λ3 over a wide range (10–10^7 copies/assay) with high specificity (~10^7-fold specificity), which is superior to a previously reported assay (100-fold sensitivity).[21] Cell lines derived from hepatocytes (HuH7 and HepG2) showed relatively low expression levels of IFN-λ whereas hematopoietic cell lines induced approximately 10-fold greater expression of IFN-λ and responded to several types of PRR ligands. These data support previous reports that the main source of IFN-λ are cells of the hematopoietic lineage.[3,22–24]

RIG-I recognizes 5′-triphosphate RNA with base-paired structures,[25] while melanoma differentiation-associated gene 5 (MDA5) senses long dsRNA.[26] The engagement of these receptors by their cognate ligands activates signaling cascades that lead to the expression of IFN-α, -β and -λ. The expression pattern of IFN-λ in Raji cells was different from that in Jurkat and HL-60 cell lines. Raji cells induced high levels of IFN-λ following IFN-α stimulation with or without PRR stimulation.