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.

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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,

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.

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.

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

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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

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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

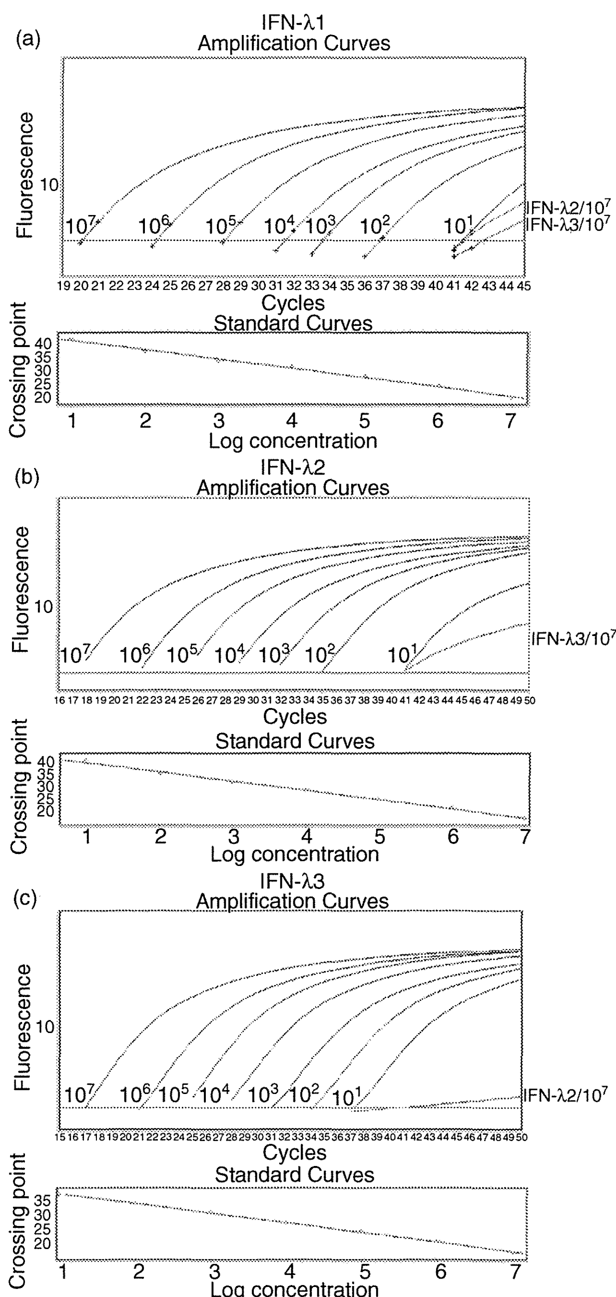

Figure 1 Standard curve and dynamic range of the gene-specific real-time detection polymerase chain reaction. Plasmids constructed with cDNA for each interferon (IFN)-λ gene were used to assess specificity among the IFN-λ family and to establish the standard curve and dynamic range. (a) IFN-λ1, (b) IFN-λ2 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.

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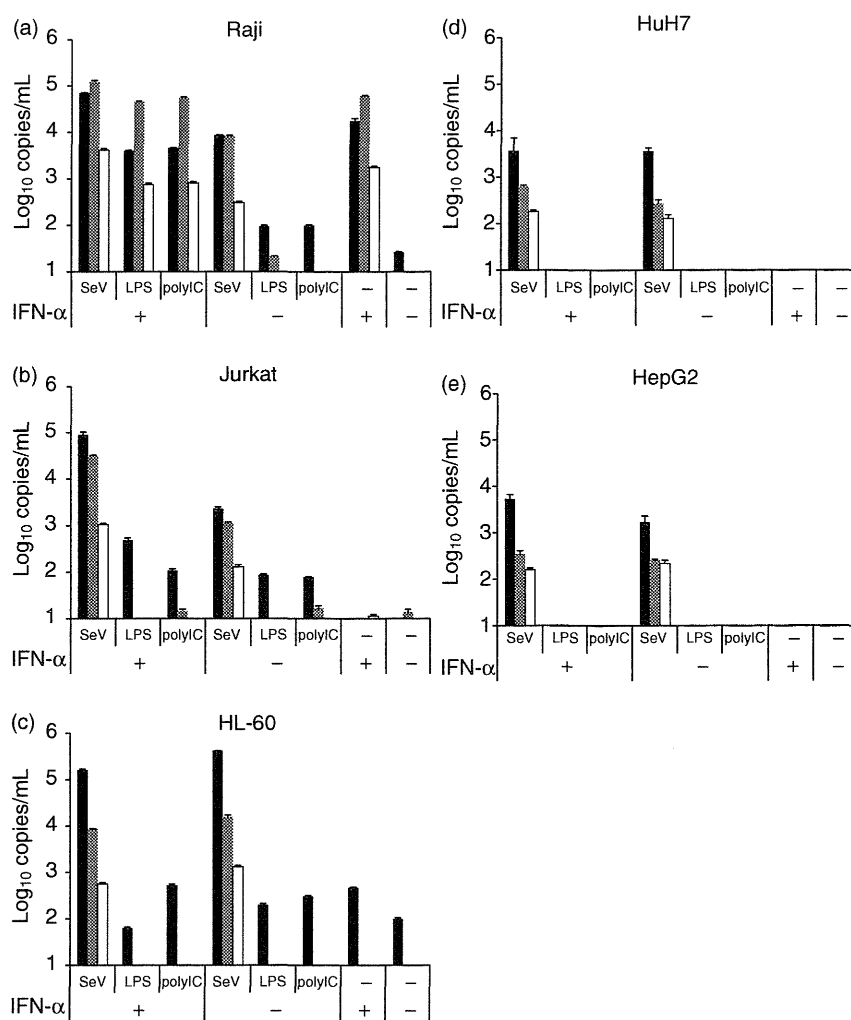

Figure 2 Gene-specific quantification of each interferon (IFN)-λ mRNA using real-time detection polymerase chain reaction. Cell lines were stimulated with Sendai virus (SeV) (50 HA/mL), lipopolysaccharide (LPS) (3 ng/mL), or polyinosinic–polycytidylic acid (poly I:C) (30 ng/mL) for 24 h following IFN-α 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

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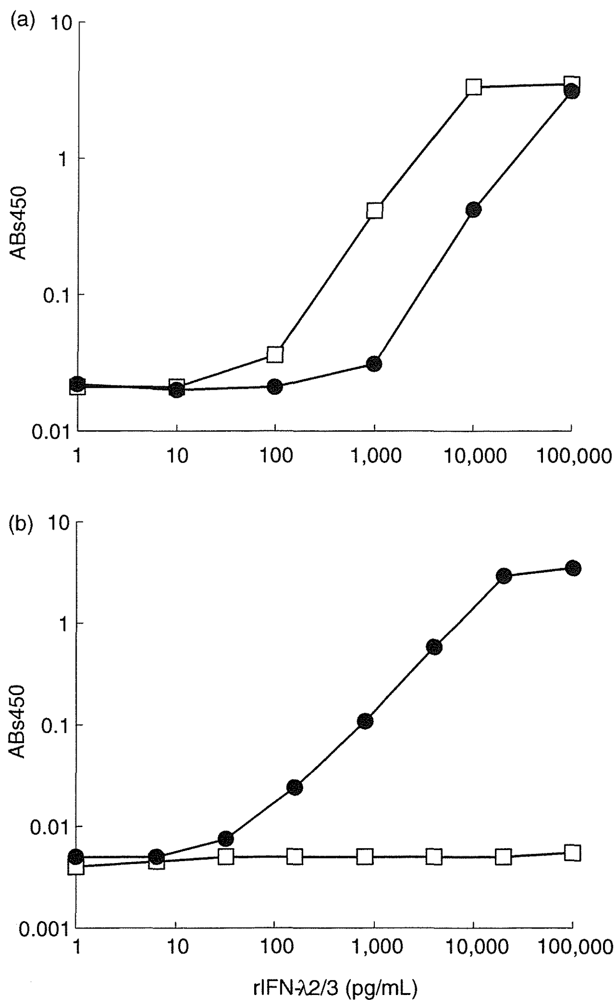

Figure 3 Specificity and the standard reactions of enzyme-linked immunoassays (ELISA) for interferon (IFN)-λ2 and -λ3. Serially diluted recombinant IFN (rIFN)-λ2 (□) or -λ3 (●) standards were measured by ELISA. (a) The results of ELISA using a set of commercially available anti-IFN-λ2 antibodies (R&D 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

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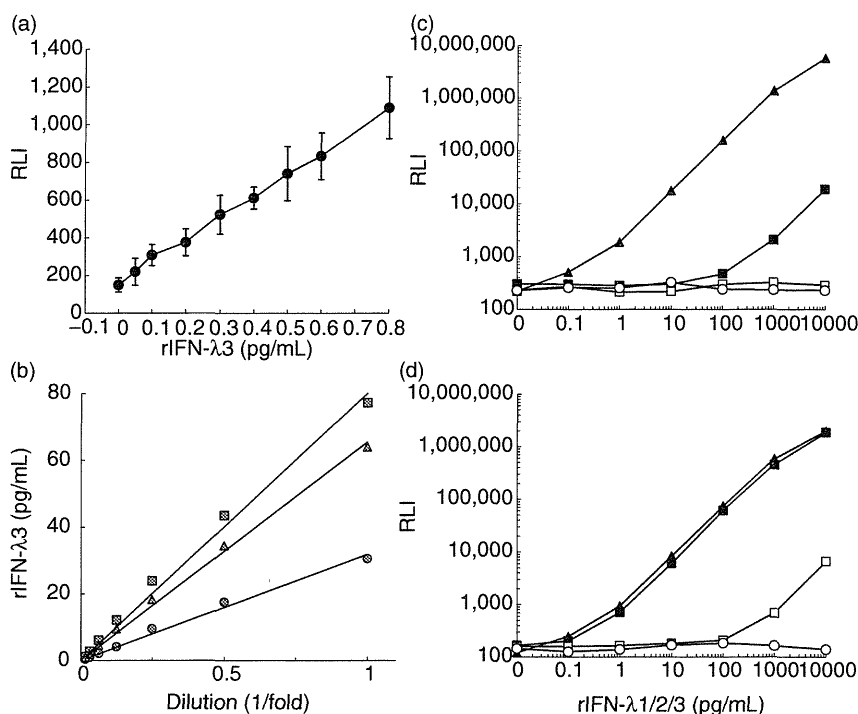

Figure 4 Detection limit, specificity and dilution linearity of interferon (IFN)-λ3 chemiluminescence enzyme immunoassay (CLEIA). The specificity and sensitivity of the newly developed CLEIA assay were assessed. (a) Detection limit was determined using serially diluted recombinant interferon (rIFN)-λ3. 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

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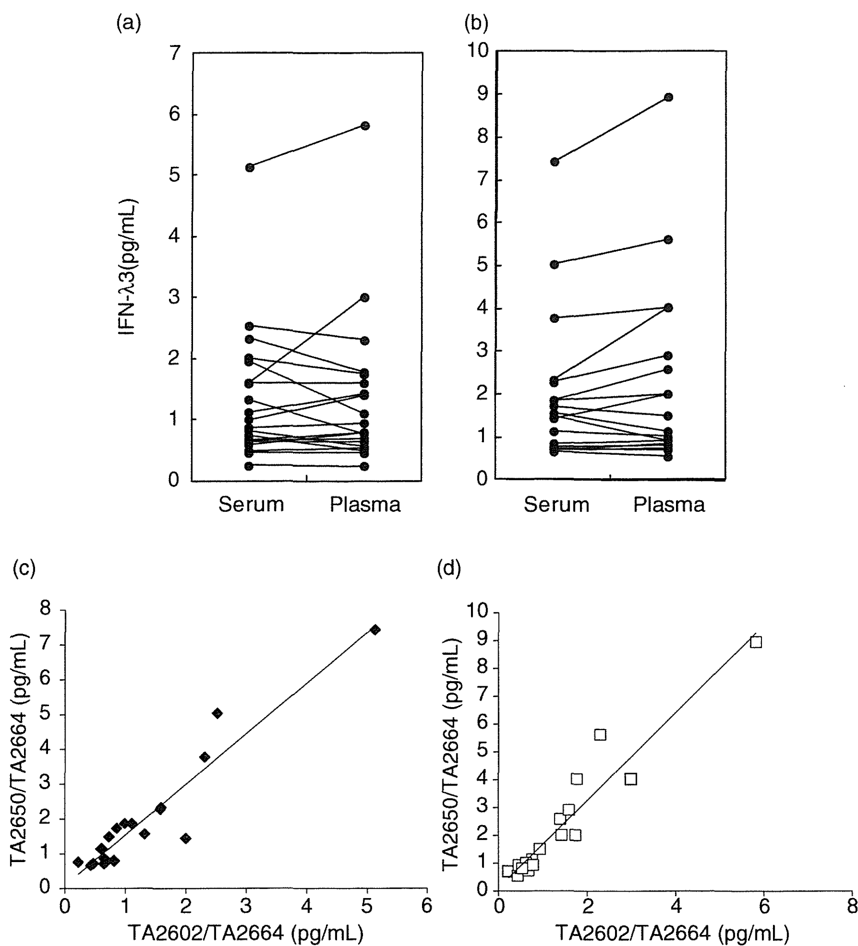

Figure 5 Interferon (IFN)-λ3 quantification in serum or plasma. IFN-λ3 concentration was measured by each chemiluminescence enzyme immunoassay (CLEIA) using serum/plasma pair specimens obtained from healthy volunteers ($n = 20$). (a) TA2602 coat/TA2664-alkaline phosphatase (ALP) CLEIA, (b) TA2650 coat/TA2664-ALP 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.

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IFN-α production in Raji cells is very low or undetectable under normal conditions.[27] Each Raji cell contains approximately 60 Epstein–Barr virus (EBV) genome equivalents,[28] and approximately 12 copies of the EBV genome can be integrated into the Raji cell genome.[29] EBV infection can regulate TLR expression and its signaling.[30–33] Therefore, IFN-α treatment alone was sufficient to induce high-level transcription of IFN-λ because PRR in the Raji cells were constitutively stimulated by endogenous EBV genome or protein. In Jurkat and HL-60 cells, high-level transcription of IFN-λ was only observed with SeV infection because these cells are otherwise free from virus infection.[22,34–36] IFN-λ production was strongly induced in viral infection for 24 h post-stimulation compared to external stimuli such as LPS or poly I:C. Intracellular PRR might be key regulators for IFN-λ induction. Viral infection could also be a trigger for extended IFN-λ expression. Further study is needed to characterize the regulation of IFN-λ gene expression.

Enzyme-linked immunoassays and CLEIA specific for IFN-λ3 were developed to detect low levels of IFN-λ3 in serum. These immunoassays did not suffer from cross-reactivity with IFN-λ2, even though there is 96.5% amino acid similarity (193/200) between IFN-λ3 and IFN-λ2. No immunoassays specific for IFN-λ3 have been previously developed that achieve sensitivity down to the pg/mL level. Unlike our newly developed assay, previous assays reacted to IFN-λ2 and -λ3.[15–17] Thus, this is the first report of an IFN-λ3-specific assay suitable for serum/plasma samples; previous assay systems were not sufficiently sensitive to detect the low levels of IFN-λ3 in serum.[16] The concentrations of IFN-λ3 in plasma or serum from healthy volunteers ranged 0.23–5.8 pg/mL, which could be detected by our high-sensitivity CLEIA, but not by the ELISA. Using an ELISA, Langhans *et al.* reported IFN-λ2/3 serum levels were above the detection limit (15.0 pg/mL) in only 27% of serum samples,[16] which is in accordance with our results. We detected IFN-λ3 in only 21.5% (6/28) of serum/plasma samples by ELISA (detection limit ~3 pg/mL). Although scatter plots between TA2602/TA2664 and TA2650/TA2664 showed high correlation on serum and plasma samples, TA2602/TA2664 assay revealed a slightly low value. These differences might be dependent on the difference of the capture antibody or IFN-λ3 genotype, which induces R74K substitution, and the expression levels of IFN-λ3 mRNA might be also different from each allele. The IFN-λ3-specific CLEIA will be a highly valuable tool to study the effects, functions and clinical uses of IFN-λ3.

In recent genome-wide association studies, it was found that genetic polymorphisms near the IFN-λ3 gene were strongly associated with sustained viral response of pegylated IFN therapy and spontaneous viral clearance in HCV patients.[10–14] However, it is unknown how these polymorphisms affect antiviral host responses. Among those SNP near the IFN-λ3 gene that are associated with spontaneous resolution and successful treatment of HCV infection is a non-synonymous SNP (rs8103142) located in the third exon of IFN-λ3 that causes a change from Lys to Arg (K74R). Interestingly, the amino acid at position 74 in wild-type IFN-λ2 is arginine. We and others previously reported that the substitution itself at position 74 did not affect the capacity of IFN-λ3 to bind its receptor and induce activity at the IFN-stimulated response element (ISRE).[18,21] Based on the present study, we predict that the K74R substitution induces conformational change in IFN-λ3. The IFN-λ3-specific ELISA (TA2602/TA2664) detected wild-type rIFN-λ3 (74 K) but not rIFN-λ3 with the 74R substitution. mAb TA2602 and TA2664 recognize conformational epitopes because they did not react to any of the IFN-λ3 peptides.

The crystal structure of mature IFN-λ3 protein identifies three intramolecular disulfide bridges between Cys^{41} and Cys^{140}, Cys^{75} and Cys^{173}, and Cys^{192} and Cys^{199} (amino acids are numbered as a 200-residue protein). In addition, Cys^{73} forms an intramolecular disulfide bond that connects two molecules of IFN-λ3.[4] The K74R substitution could influence the disulfide bridge between Cys^{75} and Cys^{173} due to its proximity to Cys^{75}. IFN-λ3 with the K74R substitution could potentially adopt one of two conformations with disulfide bridges between Cys^{73} and Cys^{173} or between Cys^{75} and Cys^{173} because the substitution generates a tandem sequence of Cys^{73}-Arg^{74}-Cys^{75}-Arg^{76} in the region.

In conclusion, previous assays have not been able to adequately distinguish IFN-λ3 from IFN-λ1 and -λ2, particularly because these latter two IFN appear to be expressed at much higher levels than IFN-λ3. The highly specific and sensitive assay for IFN-λ3 that we have developed will be particularly valuable for understanding the role of this cytokine in human disease, particularly HCV infection.

ACKNOWLEDGMENTS

THIS WORK WAS supported by a Grant-in-Aid from the Ministry of Health Labor and Welfare of Japan. Laboratory work by Ms Ayano Ito, Ms Mami Ohashi, Ms Sachiko Sato, Ms Miki Yoshida, Ms Chieko Haga, Ms Dinara Kurbanova and Ms Ikue Kirikae is highly appreciated. We also thank Ms Masako Awano, Ms Miwa Noda and Ms Mikako Kajio for secretarial support. We

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are grateful to Hisahiko Yamashita and Rie Aoi for the establishment of mAb and for their technical assistance.

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SUPPORTING INFORMATION

ADDITIONAL SUPPORTING INFORMATION may be found in the online version of this article:

Table S1 Recovery of interferon (IFN)-λ3 chemiluminescence enzyme immunoassay (CLEIA).

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Accepted Article

Human BDCA3$^+$ dendritic cells are a potent producer of IFN-λ in response to hepatitis C virus

Sachiyo Yoshio[1], Tatsuya Kanto[1]*, Shoko Kuroda[1], Tokuhiro Matsubara[1], Koyo Higashitani[1], Naruyasu Kakita[1], Hisashi Ishida[1], Naoki Hiramatsu[1], Hiroaki Nagano[2], Masaya Sugiyama[3], Kazumoto Murata[3], Takasuke Fukuhara[4], Yoshiharu Matsuura[4] , Norio Hayashi[5], Masashi Mizokami[3], and Tetsuo Takehara[1]

1 Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka, Japan

2 Department of Surgery, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka, Japan

3 The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, 1-7-1, Kohnodai, Ichikawa 272-8516, Japan.

4 Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1002/hep.26182

Accepted Article

5 Kansai Rosai Hospital, 3-1-69 Amagasaki, Hyogo, Japan

*Corresponding author: Tatsuya Kanto, M.D., Ph.D.

Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, 565-0871 Japan

E-mail: kantot@gh.med.osaka-u.ac.jp

Telephone: +81-6-6879-3621, Fax: +81-6-6879-3629

Keywords

IL-28B, single nucleotide polymorphism; CD81; JFH-1; TLR3

Funding: Supported in part by a Grant-In-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan and a Grant-In-Aid from the Ministry of Health, Labor, and Welfare of Japan.

Conflicts of interest: None of the authors do have commercial or other associations that might

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pose a conflict of interest.

Abbreviations : Ab, antibody; HCV, hepatitis C virus; HCVcc, cell-cultured hepatitis C virus; HSV, herpes simplex virus; IHL, intrahepatic lymphocyte; IRF, interferon regulatory factor; ISGs, interferon-stimulated genes; JEV, Japanese encephalitis virus; Lin, lineage; mDC, myeloid DC; MOI, multiplicity of infection; PBMC, peripheral blood mononuclear cell; pDC, plasmacytoid DC; Poly IC, polyinosine-polycytidylic acid; RIG-I, retinoic acid-inducible gene-I; SNPs, single nucleotide polymorphisms; TLR, Toll-like receptor; TRIF, TIR-domain-containing adapter-inducing interferon-β

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ABSTRACT

The polymorphisms in *IL-28B* (IFN-λ3) gene are strongly associated with the efficacy of HCV clearance. Dendritic cells (DCs) sense HCV and produce IFNs, thereby playing some cooperative roles with HCV-infected hepatocytes in the induction of interferon-stimulated genes (ISGs). BDCA3^{+} DCs are discovered as a producer of IFN-λ upon toll-like receptor 3 (TLR3) agonist. We thus aimed to clarify the roles of BDCA3^{+}DCs in anti-HCV innate immunity. Seventy healthy subjects and 20 patients with liver tumors were enrolled. BDCA3^{+}DCs, in comparison with plasmacytoid DCs and myeloid DCs, were stimulated with TLR agonists, cell-cultured HCV (HCVcc) or Huh7.5.1 cells transfected with HCV/JFH-1. BDCA3^{+}DCs were treated with anti-CD81 antibody, inhibitors for endosome acidification, TRIF-specific inhibitor or ultraviolet-irradiated HCVcc. The amounts of IL-29/IFN-λ1, IL-28A/IFN-λ2 and IL-28B were quantified by subtype-specific ELISA. The frequency of BDCA3^{+}DCs in PBMC was extremely low but higher in the liver. BDCA3^{+}DCs recovered from PBMC or the liver released large amounts of IFN-λs, when stimulated with HCVcc or HCV-transfected Huh7.5.1.

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BDCA3^{+}DCs were able to induce ISGs in the co-existing JFH-1-positive Huh7.5.1 cells. The treatments of BDCA3^{+}DCs with anti-CD81 antibody, cloroquine or bafilomycin A1 reduced HCVcc-induced IL-28B release, whereas BDCA3^{+}DCs comparably produced IL-28B upon replication-defective HCVcc. The TRIF-specific inhibitor reduced IL-28B release from HCVcc-stimulated BDCA3^{+}DCs. In response to HCVcc or JFH-1-Huh7.5.1, BDCA3^{+}DCs in healthy subjects with IL-28B major (rs8099917, TT) released more IL-28B than those with IL-28B minor genotype (TG). ***Conclusion:*** Human BDCA3^{+}DCs, having tendency of being accumulated in the liver, recognize HCV by a CD81-, endosome- and TRIF-dependent manner and produce substantial amounts of IL-28B/IFN-λ3, the ability of which is superior in subjects with IL-28B major genotype.

Journal of Medical Virology 85:449–458 (2013)

Model Incorporating the *ITPA* Genotype Identifies Patients at High Risk of Anemia and Treatment Failure With Pegylated-Interferon Plus Ribavirin Therapy for Chronic Hepatitis C

Masayuki Kurosaki,[1] Yasuhito Tanaka,[2] Nao Nishida,[3] Naoya Sakamoto,[4] Nobuyuki Enomoto,[5] Kentaro Matsuura,[2] Yasuhiro Asahina,[6] Mina Nakagawa,[6] Mamoru Watanabe,[6] Minoru Sakamoto,[5] Shinya Maekawa,[5] Katsushi Tokunaga,[3] Masashi Mizokami,[7] and Namiki Izumi[1]*

[1]*Department of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan*
[2]*Department of Virology, Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan*
[3]*Department of Human Genetics, Graduate School of Medicine, University of Tokyo, Tokyo, Japan*
[4]*Department of Gastroenterology and Hematology, Hokkaido University, Sapporo, Japan*
[5]*First Department of Internal Medicine, University of Yamanashi, Yamanashi, Japan*
[6]*Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan*
[7]*Research Center for Hepatitis and Immunology, International Medical Center of Japan Konodai Hospital, Ichikawa, Japan*

This study aimed to develop a model for predicting anemia using the inosine triphosphatase (*ITPA*) genotype and to evaluate its relationship with treatment outcome. Patients with genotype 1b chronic hepatitis C (n = 446) treated with peg-interferon alpha and ribavirin (RBV) for 48 weeks were genotyped for the *ITPA* (rs1127354) and *IL28B* (rs8099917) genes. Data mining analysis generated a predictive model for anemia (hemoglobin (Hb) concentration <10 g/dl); the CC genotype of *ITPA*, baseline Hb <14.0 g/dl, and low creatinine clearance (CLcr) were predictors of anemia. The incidence of anemia was highest in patients with Hb <14.0 g/dl and CLcr <90 ml/min (76%), followed by Hb <14.0 g/dl and *ITPA* CC (57%). Patients with Hb ≥14.0 g/dl and *ITPA* AA/CA had the lowest incidence of anemia (17%). Patients with two predictors (high-risk) had a higher incidence of anemia than the others (64% vs. 28%, $P < 0.0001$). At baseline, the *IL28B* genotype was a predictor of a sustained virological response [adjusted odds ratio 9.88 (95% confidence interval 5.01–19.48), $P < 0.0001$]. In patients who achieved an early virological response, the *IL28B* genotype was not associated with a sustained virological response, while a high risk of anemia was a significant negative predictor of a sustained virological response [0.47 (0.24–0.91), $P = 0.026$]. For high-risk patients with an early virological response, giving >80% of the planned RBV dose increased sustained virological responses by 24%. In conclusion, a predictive model incorporating the *ITPA* genotype could identify patients with a high risk of anemia and reduced probability of sustained virological response. ***J. Med. Virol. 85:449–458, 2013.***

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KEY WORDS: hemolytic anemia; ribavirin; creatinine clearance; antiviral therapy

INTRODUCTION

Hepatitis C virus (HCV) infection is a leading cause of cirrhosis and hepatocellular carcinoma worldwide [Kim, 2002]. The rate of eradication of HCV by pegylated interferon (PEG-IFN) plus ribavirin (RBV), defined as a sustained virological response, is around 50% in patients with HCV genotype 1 [Manns et al., 2001; Fried et al., 2002]. Failure of treatment is attributable to the lack of a virological response or relapse after completion of therapy. Genome-wide association studies and subsequent cohort studies

Grant sponsor: Ministry of Health, Labor and Welfare, Japan.

Conflicts of interest and financial disclosures: None reported.

*Correspondence to: Namiki Izumi, MD, PhD, Department of Gastroenterology and Hepatology, Musashino Red Cross Hospital, 1-26-1 Kyonan-cho, Musashino-shi, Tokyo 180-8610, Japan. E-mail: nizumi@musashino.jrc.or.jp

Accepted 19 November 2012

DOI 10.1002/jmv.23497

Published online 7 January 2013 in Wiley Online Library (wileyonlinelibrary.com).

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have shown that single nucleotide polymorphisms (SNPs) located near the *IL28B* gene are the most important determinant of virological response to PEG-IFN/RBV therapy [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Rauch et al., 2010]. On the other hand, among patients with a virological response, the probability of a sustained virological response decreases when the patients become intolerant to therapy because of RBV-induced hemolytic anemia and receive a reduced dose of RBV [McHutchison et al., 2002; Kurosaki et al., 2012]. Genome-wide association studies have shown that variants of the inosine triphosphatase (*ITPA*) gene protect against hemolytic anemia [Fellay et al., 2010; Tanaka et al., 2011]. These variants are associated with a reduced requirement for an anemia-related dose reduction of RBV [Sakamoto et al., 2010; Thompson et al., 2010a; Kurosaki et al., 2011d; Seto et al., 2011]. However, factors other than the *ITPA* gene also contribute to the risk of severe anemia or RBV dose reduction [Ochi et al., 2010; Kurosaki et al., 2011d] and the results of studies on the impact of the *ITPA* genotype on treatment outcome are inconsistent [Ochi et al., 2010; Sakamoto et al., 2010; Thompson et al., 2010a, 2011; Kurosaki et al., 2011d].

Data mining is a novel statistical method used to extract relevant factors from a plethora of factors and combine them to predict the incidence of the outcome of interest [Breiman et al., 1980]. Decision tree analysis, a primary component of data mining analysis, has found medical applications recently [Averbook et al., 2002; Miyaki et al., 2002; Baquerizo et al., 2003; Leiter et al., 2004; Garzotto et al., 2005; Zlobec et al., 2005; Valera et al., 2007] and has proven to be a useful tool for predicting therapeutic efficacy [Kurosaki et al., 2010, 2011a,b,c, 2012] and adverse events [Hiramatsu et al., 2011] in patients with chronic hepatitis C treated with PEG-IFN/RBV therapy. Because the results of data mining analysis are presented as a flowchart [LeBlanc and Crowley, 1995], they are easily understandable and usable by clinicians lacking a detailed knowledge of statistics.

For the general application of this genetic information in clinical practice, this study aimed to construct a predictive model of severe anemia using the *ITPA* genotype, together with other relevant factors. This study also aimed to analyze the impact of the risk of anemia on treatment outcome, after adjustment for the *IL28B* genotype. These analyses were carried out at baseline and during therapy, when the early virological response became evident.

MATERIALS AND METHODS

Patients

Data were collected from a total of 446 genotype 1b chronic hepatitis C patients who were treated with PEG-IFN alpha and RBV at five hospitals and universities throughout Japan. The inclusion criteria were: (1) infection by hepatitis C genotype 1b; (2) no co-infection with hepatitis B virus or human immunodeficiency virus; (3) no other causes of liver disease such as autoimmune hepatitis and primary biliary cirrhosis; and (4) availability of DNA for the analysis of the genetic polymorphisms of *IL28B* and *ITPA*. Patients received PEG-IFN alpha-2a (180 μg) and 2b (1.5 μg/kg) subcutaneously every week and a daily weight-adjusted dose of RBV (600 mg for patients weighing <60 kg, 800 mg for patients weighing 60–80 kg, and 1,000 mg for patients weighing >80 kg) for 48 weeks. Dose reduction or discontinuation of PEG-IFN and RBV was primarily based on the recommendations on the package inserts and the discretion of the physicians at each university and hospital. The standard duration of therapy was set at 48 weeks. No patient received erythropoietin or other growth factors for the treatment of anemia. Written informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the institutional ethics review committees.

Laboratory Tests

Blood samples obtained before therapy were analyzed for hematologic data, blood chemistry, and HCV RNA. Genetic polymorphisms in SNPs of the *ITPA* gene (rs1127354) and the *IL28B* gene (rs8099917) were determined using ABI TaqMan Probes (Applied Biosystems, Carlsbad, CA) and the DigiTag2 assay, respectively. Baseline creatinine clearance (CLcr) levels were calculated using the formula of Cockcroft and Gault [1976]: for males, CLcr = [(140 − age in years) × body weight in kg] ÷ (72 × serum creatinine in mg/dl) and for females, CLcr = 0.85 × [(140 − age in years) × body weight in kg] ÷ (72 × serum creatinine in mg/dl). The stage of liver fibrosis was scored according to the METAVIR scoring system: F0 (no fibrosis), F1 (mild fibrosis: portal fibrosis without septa), F2 (moderate fibrosis: few septa), F3 (severe fibrosis: numerous septa without cirrhosis), and F4 (cirrhosis). A rapid virological response was defined as undetectable HCV RNA by qualitative PCR with a lower detection limit of 50 IU/ml (Amplicor, Roche Diagnostic Systems, Pleasanton, CA) at week 4 of therapy and a complete early virological response was defined as undetectable HCV RNA at week 12. A sustained virological response was defined as undetectable HCV RNA at 24 weeks after completion of therapy. Severe anemia was defined as hemoglobin (Hb) <10 g/dl.

Statistical Analysis

Database for analysis included the following variables: age, sex, body mass index, serum aspartate aminotransferase (AST) levels, alanine aminotransferase (ALT) levels, gamma-glutamyltransferase (GGT) levels, creatinine levels, CLcr, Hb, platelet count, serum levels of HCV RNA, and the stage of liver fibrosis

TABLE I. Patients' Baseline Characteristics

Age (years)	58.6	(9.6)
Gender: male (n, %)	185	(42%)
Body mass index (kg/m^2)	23.1	(3.7)
AST (IU/L)	59.9	(53.8)
ALT (IU/L)	69.8	(53.8)
GGT (IU/L)	48.5	(41.6)
Creatinine (mg/dl)	0.7	(0.2)
Creatinine clearance (ml/min)	89.5	(23.0)
Hemoglobin (g/dl)	14	(1.4)
Platelet count (10^9/L)	154.5	(52.1)
HCV RNA > 600,000 IU/ml (n, %)	354	(79%)
Liver fibrosis: F3-4 (n, %)	108	(24%)
Initial ribavirin dose (n, %)		
600 mg/day	300	(67%)
800 mg/day	138	(31%)
1,000 mg/day	9	(2%)
Pegylated interferon (n, %)		
alpha2a 180 mcg	58	(13%)
alpha2b 1.5 mcg/kg	388	(87%)
ITPA rs1127354: CC (n, %)	317	(71%)
IL28B rs809917: TT (n, %)	311	(70%)

AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase.
Data expressed as mean (standard deviation) unless otherwise mentioned.

(Table I). Based on these data set, a model for predicting the risk of developing severe anemia was constructed by data mining analysis using the IBM-SPSS Modeler 13 as described previously [Kurosaki et al., 2010, 2011a,b,c; Hiramatsu et al., 2011]. Briefly, the software was used to explore the database automatically to search for optimal predictors that discriminated most efficiently patients with severe anemia from those without. The software also determined the optimal cutoff values of each predictor. Patients were divided into two groups according to the predictor and each of the two groups was repeatedly divided in the same way until no significant factor remained or 20 or fewer patients were in a group.

The incidence of severe anemia, the total dose of RBV, and treatment outcome were compared between groups with high and low risks of anemia. On univariate analysis, Student's *t*-test was used for continuous variables, and Fisher's exact test was used for categorical data. Logistic regression was used for multivariate analysis. *P* values of <0.05 were considered significant. SPSS Statistics 18 was used for these analyses.

RESULTS

Predictive Model of Severe Anemia

The incidence of severe anemia in the whole cohort was 49% (Fig. 1). The best predictor of severe anemia was the baseline Hb concentration. Patients with a low baseline Hb concentration (<14 g/dl) were more likely to develop severe anemia (67%) than those with a higher Hb (>14 g/dl) (34%). The second best predictor for those patients with a baseline Hb <14.0 g/dl was CLcr. Patients with a CLcr below 90 ml/min had the highest incidence of severe anemia (76%). In those with a CLcr above >90 ml/min the incidence of severe anemia was 57% in patients with the CC allele of the *ITPA* gene while it was 37% in patients with the CA or AA allele. On the other hand, the second best predictor for those patients with a baseline Hb concentration above 14 g/dl was the *ITPA* genotype. Patients with the AA or AC allele had the lowest incidence of anemia (17%). For those with the *ITPA* CC allele, CLcr was the third best predictor; the optimal cutoff value was 85 ml/min for this group. The incidence of severe anemia was 49% in patients with a CLcr below 85 ml/min while it was 32% in those with a CLcr above 85 ml/min.

Following this analysis, the patients were divided into six groups, with the incidence of severe anemia ranging from 17% to 76%. Three groups with two predictors, having an incidence of anemia >40%, were defined as the high-risk group and the remainder were defined as the low-risk group. The incidence of severe anemia was higher in the high-risk group than the low-risk group (65% vs. 28%, $P = 0.029$) (Fig. 2). Comparison of the *ITPA* genotype and the predictive model showed that the sensitivity for the prediction of severe anemia was similar (75.9% vs. 76.4%) but the specificity of the predictive model was greater (33.6% vs. 59.3%).

The Risk of Anemia Impacts on Sustained Virological Responses by Patients Who Achieved an Early Virological Response

The impact of *IL28B* genotype, *ITPA* genotype, and risk group of anemia on the rate of sustained virological response was studied at baseline and week 12. At baseline, patients with the TT allele of the *IL28B* gene had a significantly higher rate of sustained virological response than those with the TG or GG allele (43% vs. 10%, $P < 0.0001$), the high-risk group for anemia had a significantly lower rate of sustained virological response than the low-risk group (28% vs. 40%, $P = 0.011$), and the *ITPA* genotype was not associated with a sustained virological response (Fig. 3A–C). At week 4, patients with rapid virological response had a high rate of sustained virological response, irrespective of the *IL28B* genotype (TT vs. TG/GG; 97% vs. 100%, $P = 1.000$), the *ITPA* genotype (CC vs. CA/AA; 95% vs. 100%, $P = 1.000$), and the risk of anemia (high vs. low; 95% vs. 100%, $P = 1.000$). Among the patients who did not achieve a rapid virological response, those with the *IL28B* TT allele had a significantly higher rate of sustained virological response than those with the TG or GG allele (38% vs. 8%, $P < 0.0001$), and the high-risk group for anemia had a significantly lower rate of sustained virological response than the low-risk group (24% vs. 35%, $P = 0.015$). At week 12, in patients who achieved a complete early virological response, the *IL28B* genotype was not associated with a sustained virological response, while the high-risk group for anemia had a

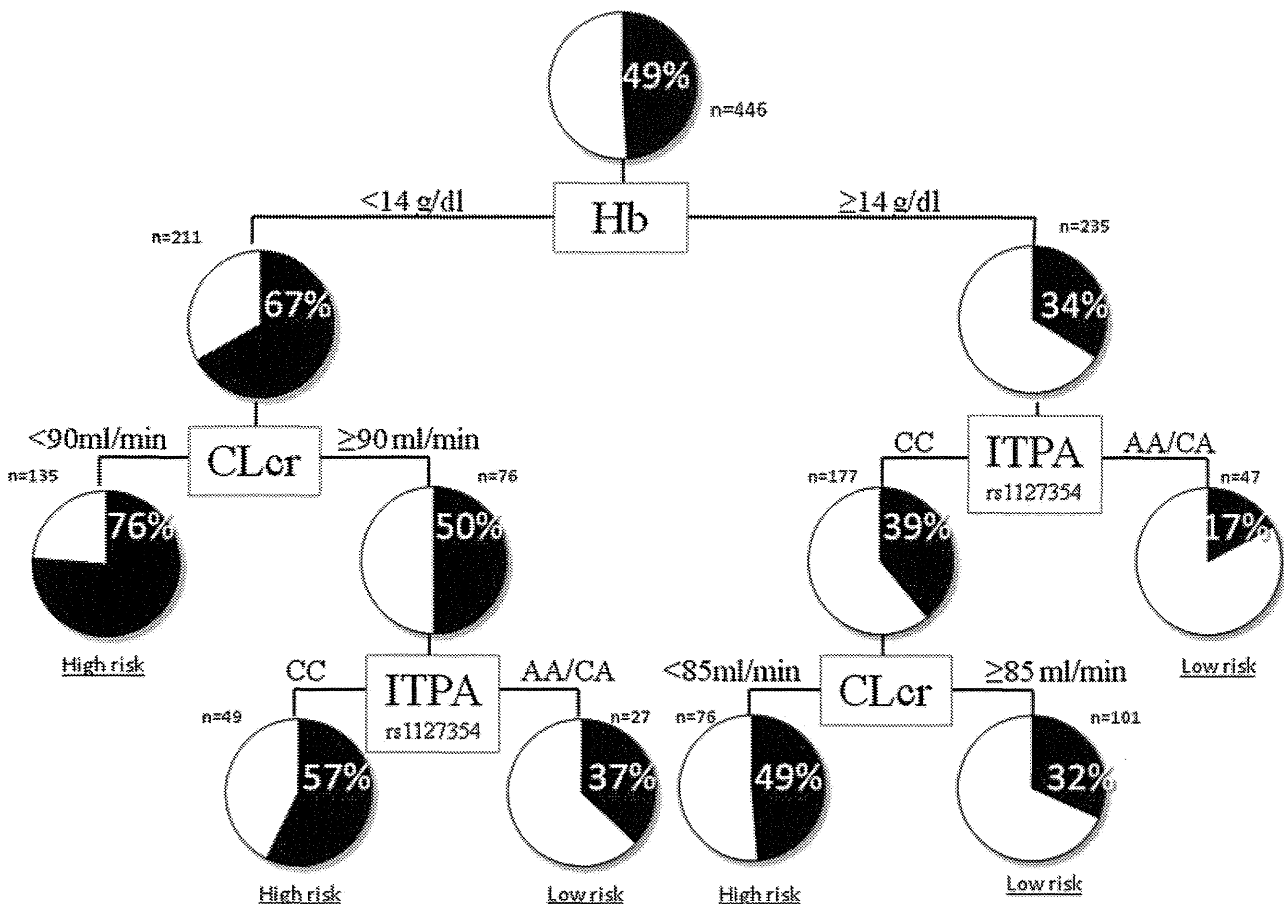

Fig. 1. The predictive model for severe anemia. The boxes indicate the factors used to differentiate patients and the cutoff values for the different groups. The pie charts indicate the rate of severe anemia (Hb <10.0 g/dl) for each group of patients, after differentiation. Terminal groups of patients differentiated by analysis are classified as at high risk if the rate is >40% and low risk if the rate is <40%. *ITPA*, inosine triphosphatase; CLcr, creatinine clearance; Hb, hemoglobin.

significantly lower rate of sustained virological response than the low-risk group (59% vs. 76%, $P = 0.013$) (Fig. 3D–F). In patients who did not achieve a complete early virological response, the *IL28B* genotype was a significant predictor of a sustained virological response (TT vs. TG/GG; 14% vs. 2%, $P < 0.0001$) but a high risk for anemia was not (high vs. low; 10% vs. 6%, $P = 0.361$).

From multivariate analysis (Table II), the *IL28B* genotype was the most important predictor of a sustained virological response at baseline [adjusted odds ratio 9.88 (95% confidence interval 5.01–19.48), $P < 0.0001$], along with female sex [0.42 (0.26–0.68), $P < 0.0001$], platelet count [1.09 (1.04–1.15), $P < 0.0001$], advanced fibrosis [0.49 (0.27–0.91), $P = 0.024$], and baseline HCV RNA load [4.14 (2.27–7.55), $P < 0.0001$]. At week 4, in patients without a rapid virological response, the *IL28B* genotype remained the most important predictor of a sustained virological response [7.16 (3.60–14.25), $P < 0.0001$], along with female sex and platelet count. At week 12, in patients with a complete early virological response, the risk of anemia was an independent and significant predictor of a sustained virological response [0.47 (0.24–0.91), $P = 0.026$], together with the platelet count and HCV RNA load, but the *IL28B* genotype was not associated with a sustained virological response. In patients without a complete early virological response, the *IL28B* genotype was a predictor of a sustained virological response [9.13 (2.02–41.3), $P = 0.004$] along with the platelet count. Thus, *IL28B* was a significant predictor of a sustained virological response at baseline and among virological non-responders at weeks 4 and 12. On the other hand, once a complete early virological response was achieved, the *IL28B* genotype was no longer associated with a sustained virological response but the risk of anemia was an independent predictor of a sustained virological response.

The Risk of Anemia, RBV Dose, and Treatment Outcome in Patients With a Complete Early Virological Response

Patients who achieved a complete early virological response were stratified according to adherence to