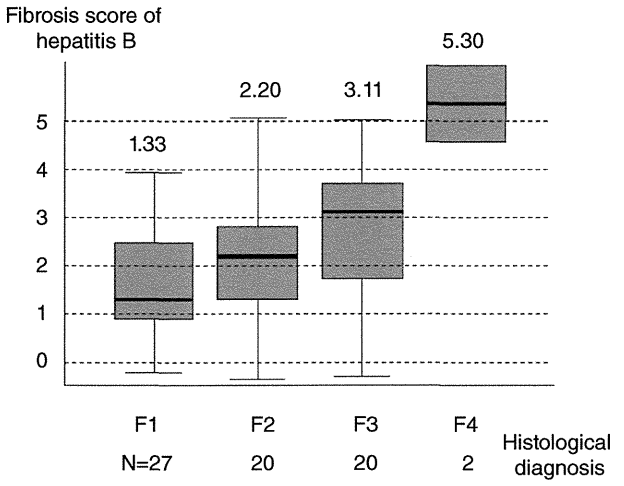

Figure 3 Box and whisker plots of fibrotic score of each group of histological fibrosis in the validation dataset. The fibrosis score of hepatitis B was generated by the function, $z = 1.40 \times ln$ (type IV collagen 7S) (ng/mL) $- 0.017 \times$ (platelet count) ($\times 1000^3/mm^3$) $+ 1.24 \times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

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

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

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

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

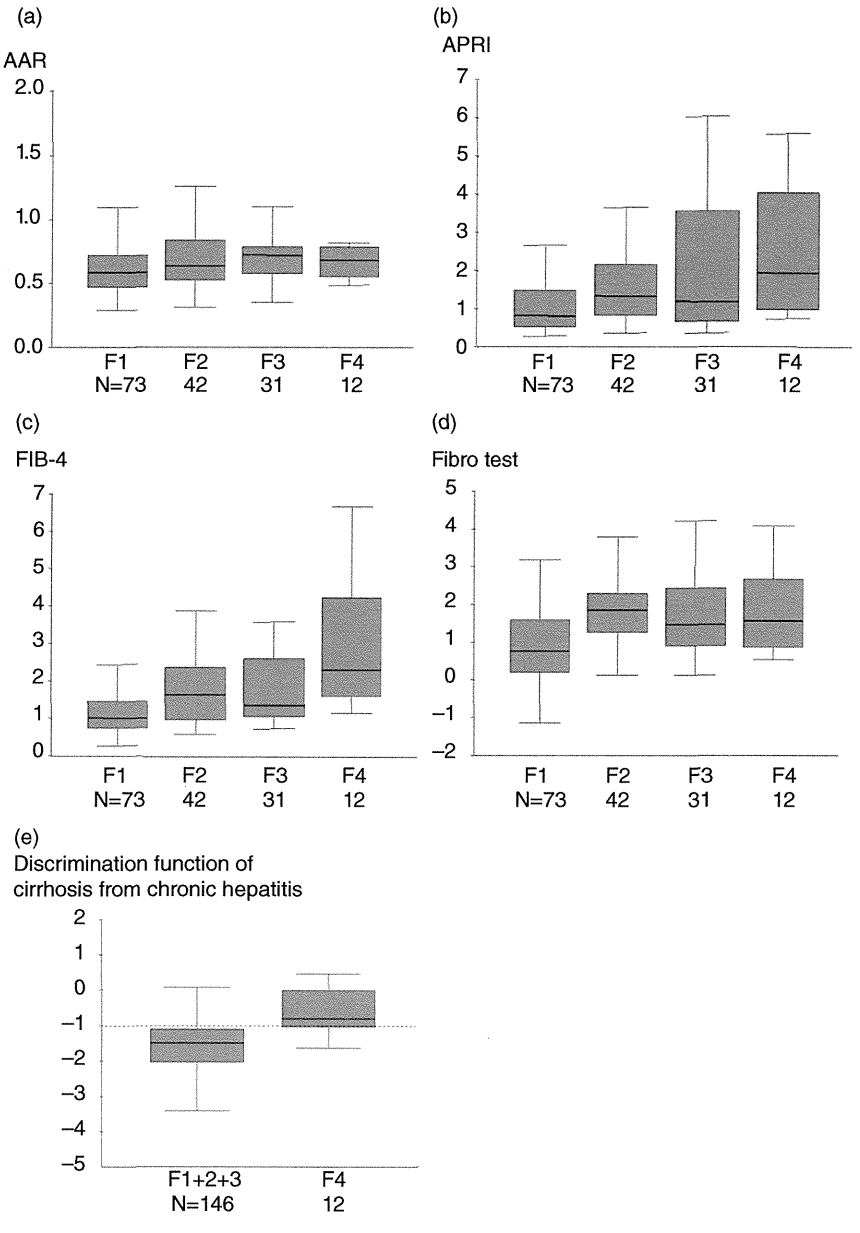

Figure 4 Previously published fibrosis scores. (a) Aspartate aminotransferase/alanine aminotransferase ratio (AAR),[19] (b) aspartate aminotransferase-to-platelet ratio index (APRI),[20] (c) FIB-4,[21] (d) FibroTest[22] and (e) discrimination function of cirrhosis from hepatitis in Japanese patients.[23]

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

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

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

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

ACKNOWLEDGMENTS

THIS STUDY WAS proposed and initiated by Dr Shiro Iino and the project was performed with a grant from the Viral Hepatitis Research Foundation of Japan.

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Hepatology Research

Hepatology Research 2013; **43**: 801–807 doi: 10.1111/hepr.12021

Case Report

Cutaneous sarcoidosis in a chronic hepatitis C patient receiving pegylated interferon and ribavirin therapy

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A 61-year-old Japanese woman suffered from a small, painful, subcutaneous nodule on the sole of her foot that was 10 mm across in diameter during pegylated interferon (PEG IFN) and ribavirin (RBV) combination therapy for chronic hepatitis C. Skin biopsy revealed multiple non-caseating granulomas composed of epithelioid histiocytes with multinucleate giant cells, which was consistent with sarcoidosis. Ophthalmologic examination revealed uveitis. Thoracic computed tomography (CT) showed multiple bilateral hilar lymphadenopathies and a diffuse micronodular interstitial pattern of the lungs. Genetic analysis indicated a probable homozygous haplotype of *A*02:01-C*15:02-B*51:01-DRB1*16:02-DQB1*05:02* in human leukocyte antigen regions. The patient was observed carefully without any additional medication because no significant systemic symptoms were noted. Combination therapy was continued for 2 months afterwards. She was asymptomatic for over 3 years of follow up, and repeated hematological and biological investigations and chest CT showed improvement. In conclusion, clinicians should bear sarcoidosis in mind as a complication during PEG IFN and RBV combination therapy. They should also be aware of the usually good prognosis of PEG IFN-induced cutaneous sarcoidosis in order not to prematurely discontinue a treatment necessary for liver disease; maintenance of PEG IFN treatment may be advised with careful follow up.

Key words: human leukocyte antigen, pegylated interferon, ribavirin, sarcoidosis

INTRODUCTION

PEGYLATED INTERFERON (PEG IFN) in combination with ribavirin (RBV) is the current standard of care for patients with chronic hepatitis C genotype 2, with the addition of a protease inhibitor for the treatment of genotype 1.[1,2] Side-effects are observed in almost 80% of patients treated with combination therapy[1] over the course of treatment, and the appearance of various skin conditions, such as eczematous and lichenoid eruptions and psoriasis, have been reported.[3] Cutaneous sarcoidosis in varying manifestations has also been reported to develop due to PEG IFN and RBV combination therapy.[4–11]

Sarcoidosis is a multisystem granulomatous disorder characterized by the presence of non-caseating granulomas in tissues. It typically appears in young to middle-aged women, but can affect both sexes and all age groups.[12] Although the etiology of sarcoidosis remains uncertain, it is known that highly-polarized T-helper type 1 (Th1) cells differentiated from activated $CD4^+$ cells produce excessive interleukin (IL)-2 and IFN-γ, thus activating macrophages and leading to the formation of granulomas.[13] The disease is currently thought to be triggered by various genetic and environmental factors, and evidence of familial and ethnic clustering

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Conflict of interest: none.
Received 22 August 2012; revision 21 October 2012; accepted 5 November 2012.

suggests the existence of a genetic predisposition to sarcoidosis.[14] Attempts to identify sarcoidosis susceptibility genes have focused on those residing in the major histocompatibility complex, and particularly the human leukocyte antigen (HLA) genes.[13]

We herein describe a patient with an extremely rare homozygous HLA genotype who became complicated with cutaneous sarcoidosis during PEG IFN and RBV therapy.

CASE REPORT

A 61-YEAR-OLD JAPANESE woman was referred to our hospital by her primary care physician for treatment of hepatitis C virus (HCV) infection. She was suspected to have contracted HCV (genotype 1b) by a blood transfusion during childbirth 24 years prior. Serum HCV RNA was 5.2 log IU/mL, and serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) had both been consistently greater than 30 IU/L. Chronic hepatitis was histologically proven by liver tissue biopsy, which indicated scores of 3 for portal–portal bridging necrosis, portal inflammation and fibrosis, and scores of 1 for intralobular degeneration and focal necrosis, according to the Knodell Histological Activity Index classification.[15] Her single nucleotide polymorphism (SNP) of interleukin-28B at rs8099917[16] was T/T. She neither smoked nor habitually consumed alcohol. No history or findings of dermatological, pulmonary or autoimmune diseases were noted. A chest X-ray before treatment showed no abnormal findings (Fig. 1a).

Combination therapy was started with regular doses of PEG IFN-α-2b at 80 μg s.c. once weekly and RBV at 600 mg/day p.o. (MSD, Tokyo, Japan). Serum HCV RNA became undetectable in a TaqMan assay 12 weeks after the initiation of therapy. She showed occasional mild hematopenia without dose reductions of PEG IFN or RBV. Ten months after treatment commencement, the patient complained of a small, painful subcutaneous nodule on the sole of her foot that was 10 mm in diameter (Fig. 2). Combination therapy was continued for

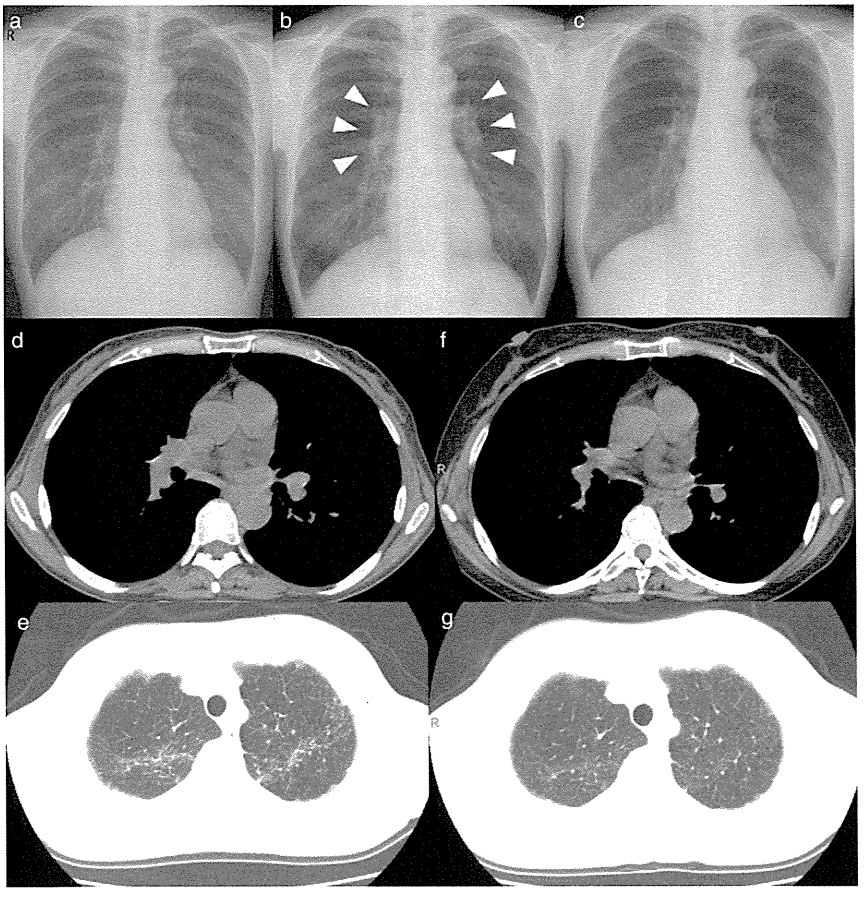

Figure 1 Chest X-ray before combination therapy showed no abnormal findings (a). Chest X-ray taken 2 months after combination therapy showed bilateral hilar lymphadenopathies (white arrowheads) (b). Thoracic computed tomography (CT) taken at the same time clearly showed multiple bilateral, paratracheal, subcarinal and hilar adenopathies (d) and a diffuse micronodular interstitial pattern of the lungs (e). Thoracic CT taken 20 months after combination therapy indicated improvement of bilateral hilar (f) and interstitial (g) lymphadenopathies. Chest X-ray taken 40 months after combination therapy depicted improvement of bilateral hilar lymphadenopathies (c).

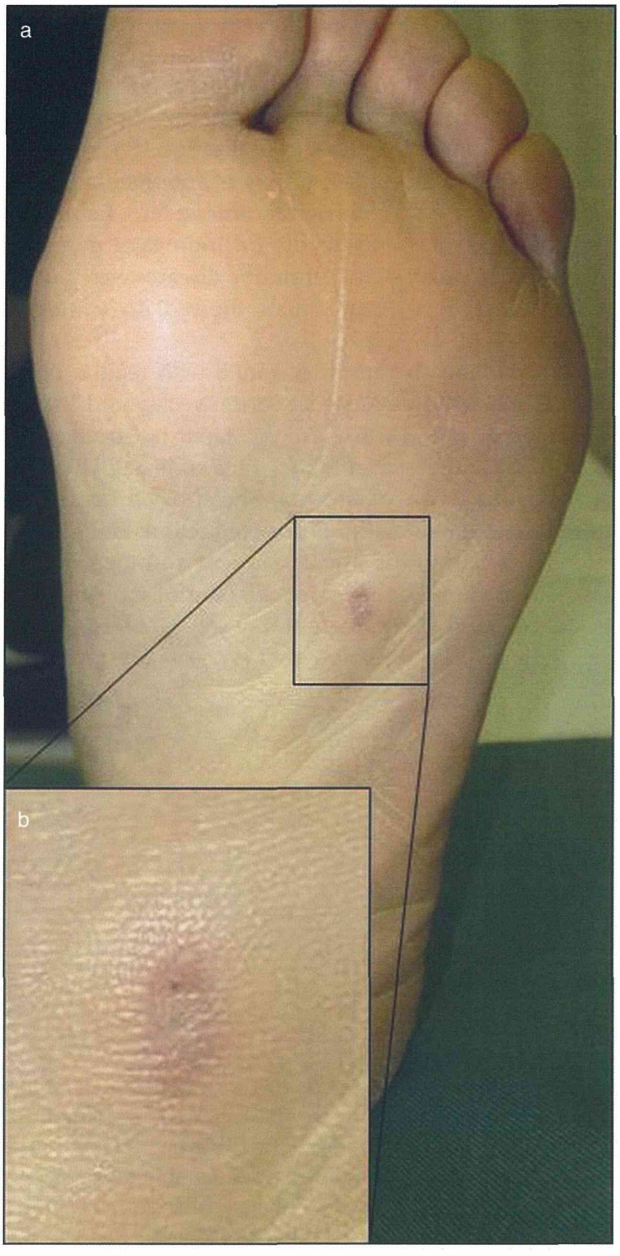

Figure 2 The patient developed a small, painful subcutaneous nodule (black circle) on the sole of her foot that was 10 mm in diameter 10 months after the initiation of therapy. The patient had no complaints about the sole of her foot before treatment.

2 months afterwards as no systemic symptoms were detected under close monitoring.

Skin biopsy of the nodule revealed multiple non-caseating granulomas composed of a compact aggregate of irregularly epithelioid histiocytes with multinucleated giant cells (Fig. 3). Specific stains showed no evidence of bacterial, fungal or mycobacterial organisms. Biopsy findings were interpreted as consistent with sarcoidosis, although the skin lesion disappeared around the time of biopsy. Further examination was performed to evaluate systemic involvement. Laboratory studies demonstrated moderate leukocytopenia (2800/μL), elevated lysozyme (10.5 μg/mL; normal, 5.0–10.2), and normal angiotensin-converting enzyme (16.9 U/mL; normal, 8.3–21.4) and serum calcium (9.1 mEq/L). Her tuberculin skin test was negative, but ophthalmologic examination revealed uveitis. A chest X-ray (Fig. 1b) and thoracic computed tomography (CT) taken 2 months after combination therapy showed multiple bilateral, paratracheal, subcarinal and hilar adenopathies (Fig. 1d), and a diffuse micronodular interstitial pattern of the lungs (Fig. 1e). Transbronchial lung biopsy revealed the presence of multiple non-caseating granulomas with multinucleated giant cells (Fig. 4). The bronchoalveolar lavage fluid level of lymphocytes was elevated at 38.7% compared with macrophages (56.3%) and neutrocytes (5.0%) in a total cell density of $1.67 \times 10^5/mm^3$. An increased ratio of CD4/CD8 cells of 2.33 was noted. Based on these findings, the patient was diagnosed as having sarcoidosis. She was observed carefully without any additional medication because no significant systemic symptoms were noted. A chest CT taken 20 months after combination therapy showed improvement (Fig. 1f,g). She was also asymptomatic for over 3 years of follow up, and repeated hematological

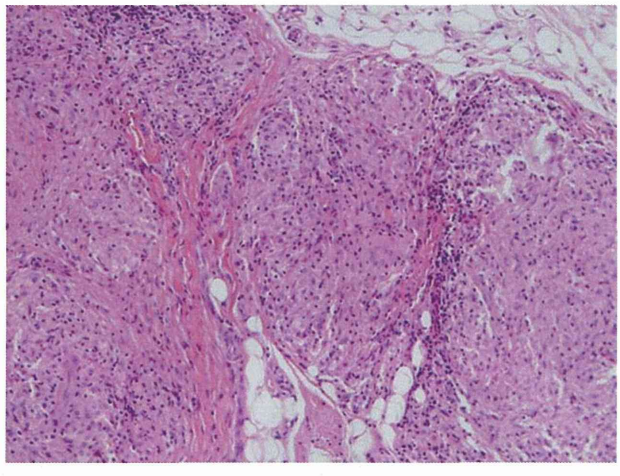

Figure 3 Skin biopsy revealed multiple non-caseating granulomas composed of a compact aggregate of irregularly arranged epithelioid histiocytes with multinucleated giant cells (hematoxylin–eosin, original magnification ×100).

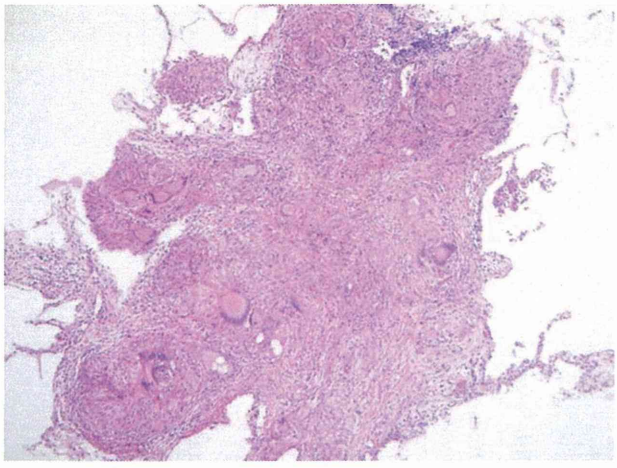

Figure 4 Transbronchial lung biopsy revealed the presence of multiple non-caseating granulomas with multinucleated giant cells (hematoxylin–eosin, original magnification ×200).

and biological investigations showed no exacerbation. A chest X-ray taken 40 months after combination therapy depicted no bilateral hilar lymphadenopathies (Fig. 1c). She also achieved a sustained virological response.

Genotyping of the HLA and microsatellite markers in the HLA region

After obtaining informed consent, genomic DNA was extracted from peripheral blood using either the QuickGene-800 kit (Fujifilm, Tokyo, Japan) or a standard phenol-chloroform method. HLA genotypes were determined by a Luminex multi-analyzer profiling system with a LAB type SSO One Lambda typing kit (One Lambda, Canoga Park, CA, USA). Additional polymerase chain reaction sequence-specific primers for sequence based typing reactions were employed to determine high-resolution alleles. Determination of the number of repeat units in the 23 microsatellites (*D6S210*, *D6S265*, *C5-2-7*, *C3-2-11*, *C4-2-7*, *C2-4-4*, *C2-2-2*, *C1-3-1*, *C1-2-5*, *C1-4-1*, *MIB*, *MICA*, *C1-2A*, *TNFA*, *TNFD*, *D6S273*, *D6S2924*, *D6S2919*, *D6S2888*, *D6S2886*, *DQCAR*, *D6S2445* and *M2-2-22*) in the HLA region was performed as described in a previous report.[17,18]

The patient's HLA genotype was *A*02:01/02:01*, *B*51:01/51:01*, *C*15:02/15:02*, *DRB1*16:02/16:02* and *DQB1*05:02/05:02*. As such, she was considered to be homozygous for the haplotype *A*02:01-C*15:02-B*51:01-DRB1*16:02-DQB1*05:02*. The frequency of this haplotype is 0.012% and very rare in a Japanese population. The alleles in all microsatellite loci were homozygous.

Genotyping of the IL-28B SNP (rs8099917) and BTNL2-SNP (rs2076530)

The patient was genotyped for *IL-28* (rs8099917) and *BTNL2* (rs2076530) polymorphisms using an SNP Genotyping Kit (Applied Biosystems, Tokyo, Japan), as previously reported.[19] The polymerase chain reaction was performed with a TaqMan Assay for Real-Time PCR (7500 Real Time PCR System; Applied Biosystems) following the manufacturer's instructions.

The patient's *IL-28* (rs8099917) allele was T/T and her *BTNL2* (rs2076530) allele was A/A.

DISCUSSION

PEGYLATED INTERFERON AND RBV therapy has numerous reported side-effects that include hematological disorders, influenza-like symptoms, neuropsychiatric disturbances, ophthalmologic disorders, glucose metabolism disruption, autoimmune disease exacerbation, dermatological complications, hair loss, thyroid dysfunction,[1] and even interstitial pneumonia in rare instances;[20] almost all of which can be managed with supportive care. Sarcoidosis induced by combination therapy is also considered to be a comparatively rare complication, for which the highest annual incidence has been observed in northern European countries (5–40 cases/100 000 people). The annual incidence in Japan ranges 1–2 cases/100 000 people.[21] The mechanism of PEG IFN/RBV-related sarcoidosis remains elusive, but is believed to be related to pathophysiological and immunomodulatory causes. One possible mechanism is that IFN promotes cytokine synthesis by macrophage activation and the development and enhancement of Th1-mediated responses. Persistent HCV infection induces chronic liver damage through a Th1 immune type of response, and the antigenicity and viral persistence seen in chronic HCV infection may serve as a trigger for the development of clinical sarcoidosis in susceptible individuals, which is exacerbated by the exogenous use of INF-α.[22,23] In addition, RBV has the ability to inhibit viral RNA replication, and may contribute to the pathogenesis of sarcoidosis by inhibiting the production of Th2 type cytokines by shifting the balance towards a Th1 response.[24] However, RBV may act solely as a facilitator in PEG IFN/RBV-related sarcoidosis induction because no such cases have been reported for RBV monotherapy.[9,25]

A total of eight known cases complicated with cutaneous sarcoidosis during PEG IFN and RBV combination therapy have been reported to date,[4–11] but with no definite associations made with regards to age, sex, onset

of sarcoidosis or type of PEG IFN (Table 1). Six cases[5–8,11] were described as cutaneous sarcoidosis without systemic lesions. Pulmonary disease accounts for the majority of the morbidity and mortality associated with primary sarcoidosis.[26] Three cases, including ours, were of cutaneous lesions with pulmonary disease.[4,9] Because cutaneous sarcoidosis leads to physical impairment in only a minority of patients and is not life-threatening, additional treatment is generally only indicated by considering the risks and benefits of treatment for patients with symptomatic, ulcerating or progressively worsening skin disease. All reported sarcoidosis cases, along with ours, showed an improvement in disease activity within several months of finishing or discontinuing PEG IFN and RBV therapy without immune-modulation therapy.

It is well-established that sarcoidosis is associated with HLA class II genes, especially HLA-DRB1 and HLA-DQB1, in several ethnic groups.[27–30] In Japanese sarcoidosis patients, a strong association with the HLA-DRB1*08 allele has been reported.[27] This is the first study to investigate HLA genotype in a patient with cutaneous sarcoidosis induced by PEG IFN and RBV combination therapy. Interestingly, our patient showed no previously reported susceptibility HLA genotypes or haplotypes.[28,31] However, she had an extremely rare HLA haplotype (*A*02:01-C*15:02-B*51:01-DRB1*16:02-DQB1*05:02*) present in only 0.012% of the Japanese population. Moreover, she appeared to have a homogeneous HLA haplotype from the results of microsatellite and HLA genotyping, which is estimated to exist at a frequency of 1.4×10^{-6}% in Japan.

Recently, an SNP within the butyrophilin-like 2 gene (*BTNL2*) at rs2076530 has been implicated as a risk factor for sarcoidosis.[32] Located only 170 kb from the *HLA-DRB1* gene telomerically on chromosome 6, *BTNL2* is a member of the immunoglobulin superfamily with suspected co-stimulatory activities in T-cell activation on the basis of its amino acid homology with B7 molecules.[33] The G/A transition of rs2076530 causes premature truncation of the protein, which disrupts membrane localization and downregulation of activated T cells (Th1).[34] As our analysis revealed the A/A homozygote of the *BTNL2* rs2076530 polymorphism, the truncated protein caused by the rs2076530A allele may have led to an increased risk of sarcoidosis in this patient.

In conclusion, clinicians should bear sarcoidosis in mind as a complication during PEG IFN and RBV combination therapy. They should also be aware of the usually good prognosis of PEG IFN-induced cutaneous

Table 1 Published cases of complicating cutaneous sarcoidosis in PEG IFN and RBV combination therapy in the English-language published work

Case (reference)	Age (years)	Sex	Genotype	Type of PEG IFN	PEG IFN (μg/week)	RBV (mg/day)	Duration (weeks)	Onset (weeks)	Other sites of involvement	Therapy	Result
1[4]	63	Male	2	α-2a	135	800	48	45	Lung	Observation	Resolved
2[5]	55	Male	1b	α-2a	150	1200	48	40	–	Observation	Resolved
3[6]	44	Male	1a	α-2b	1.5 μg/kg	1,000	48	40	–	Observation	Resolved
4[7]	59	Female	2 and 4	α-2a	180	800	48	52†	–	Topical steroids	Resolved
5[8]	60	Female	1	α-2a	180	800	24	24	–	Discontinuation	Resolved
6[9]	47	Female	N.D.	α	N.D.	N.D.	48	40	–	Observation	Resolved
7[10]	53	Female	N.D.	α	N.D.	N.D.	N.D.	N.D.	–	Observation	Resolved
8[11]	54	Female	N.D.	α	1.4 μg/kg	1000	N.D.	16	Lung	Observation	Resolved
Our case	61	Female	1b	α-2b	80	600	48	40	Lung, uveitis	Observation	Resolved

†Onset after PEG IFN and RBV combination therapy.
N.D., not described; PEG IFN, pegylated interferon; RBV, ribavirin.

sarcoidosis in order not to prematurely discontinue a necessary treatment for liver disease. Maintenance of PEG IFN treatment in such patients may be advised with careful follow up.

ACKNOWLEDGMENTS

WE THANK TREVOR Ralph for his English editorial assistance.

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KIR, *HLA*, and *IL28B* Variant Predict Response to Antiviral Therapy in Genotype 1 Chronic Hepatitis C Patients in Japan

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Abstract

Natural killer cell responses play a crucial role in virus clearance by the innate immune system. Although the killer immunoglobulin-like receptor (KIR) in combination with its cognate human leukocyte antigen (HLA) ligand, especially *KIR2DL3-HLA-C1*, is associated with both treatment-induced and spontaneous clearance of hepatitis C virus (HCV) infection in Caucasians, these innate immunity genes have not been fully clarified in Japanese patients. We therefore investigated 16 KIR genotypes along with *HLA-B* and *-C* ligands and a genetic variant of interleukin (IL) 28B (rs8099917) in 115 chronic hepatitis C genotype 1 patients who underwent pegylated-interferon-α2b (PEG-IFN) and ribavirin therapy. *HLA-Bw4* was significantly associated with a sustained virological response (SVR) to treatment (P = 0.017; odds ratio [OR] = 2.50,), as was the centromeric A/A haplotype of *KIR* (P = 0.015; OR 3.37). In contrast, SVR rates were significantly decreased in patients with *KIR2DL2* or *KIR2DS2* (P = 0.015; OR = 0.30, and P = 0.025; OR = 0.32, respectively). Multivariate logistic regression analysis subsequently identified the *IL28B* TT genotype (P = 0.00009; OR = 6.87, 95% confidence interval [CI] = 2.62 - 18.01), *KIR2DL2/HLA-C1* (P = 0.014; OR = 0.24, 95% CI = 0.08 - 0.75), *KIR3DL1/HLA-Bw4* (P = 0.008, OR = 3.32, 95% CI = 1.37 - 8.05), and white blood cell count at baseline (P = 0.009; OR = 3.32, 95% CI = 1.35 - 8.16) as independent predictive factors of an SVR. We observed a significant association between the combination of *IL28B* TT genotype and *KIR3DL1-HLA-Bw4* in responders (P = 0.0019), whereas *IL28B* TT along with *KIR2DL2-HLA-C1* was related to a non-response (P = 0.0067). In conclusion, combinations of *KIR3DL1/HLA-Bw4*, *KIR2DL2/HLA-C1*, and a genetic variant of the *IL28B* gene are predictive of the response to PEG-IFN and ribavirin therapy in Japanese patients infected with genotype 1b HCV.

Citation: Nozawa Y, Umemura T, Joshita S, Katsuyama Y, Shibata S, et al. (2013) *KIR*, *HLA*, and *IL28B* Variant Predict Response to Antiviral Therapy in Genotype 1 Chronic Hepatitis C Patients in Japan. PLoS ONE 8(12): e83381. doi:10.1371/journal.pone.0083381

Editor: Golo Ahlenstiel, University of Sydney, Australia

Received September 3, 2013; **Accepted** November 2, 2013; **Published** December 12, 2013

Funding: This work was supported by a grant from the Ministry of Health, Labor, and Welfare of Japan. Takeji Umemura and Eiji Tanaka report receiving grant support from MSD. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: MSD partly funded this study. There are no patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease worldwide. Chronic HCV infection often develops into chronic hepatitis, which may progress to liver cirrhosis and/or hepatocellular carcinoma (HCC)[1]. HCC is a leading cause of death from malignant neoplasms in Japan[2]. Since approximately 70% of Japanese HCC patients are infected with HCV, the successful eradication of this virus, defined as a sustained virological response (SVR), is considered important to decrease the incidence of HCC.

Natural killer (NK) cells are key components of the innate antiviral immune response that are controlled by a balance of activation and inhibitory receptors. NK cell activation receptors include C-type lectin-like receptors (NKG2C, NKG2D, and NKG2E), natural cytotoxicity receptors (NKp30, NKp44, and NKp46), and CD16, while known inhibitory receptors include killer cell immunoglobulin-like receptors (KIRs) and the CD94/NKG2 family, which also contains a C-type lectin-like receptor (NKG2A) [3,4]. Sixteen *KIR* genes and pseudogenes have been identified that are encoded by a family of genes located on human chromosome 19q13.4. One particular feature of *KIRs* is their substantial genetic diversity. Some inhibitory *KIRs*

recognize human leukocyte antigen (HLA) class I molecules as their ligands; *KIR2DL1* recognizes *HLA*-C group 2 (*HLA-C2*) allotypes having lysine at amino acid position 80, whereas *KIR2DL2* and KIR2DL3 recognize *HLA-C* group 1 (*HLA-C1*) allotypes having asparagine at amino acid position 80 [5]. *KIR2DL2* and *KIR2DL3* also recognize HLA-B*4601 acquiring the-C1 epitope by gene conversion [6]. Furthermore, *KIR3DL1* recognizes subsets of *HLA*-A and *HLA*-B allotypes having the -Bw4 epitope determined by amino acid positions 77-83 [7].

It has been well documented that certain KIR-HLA receptor-ligand combinations are associated with susceptibility to infectious diseases, such as HCV, as well as with disease progression and treatment response [8-15]. Recent reports have also identified a relationship between interleukin (IL) 28B gene polymorphisms and treatment and spontaneous resolution of HCV infection[16-19]. Dring et al. observed that the presence of *IL28B* gene polymorphisms and *KIR* genotypes synergized to increase the risk of chronic HCV infection[20], although this finding is under debate[21]. Suppiah et al. [22] recently reported that genotyping for *IL28B*, *HLA*-C, and *KIR* genes was useful for predicting HCV treatment response in patients of European descent. As these gene associations have not yet been studied in the Japanese population, we evaluated whether HLA-KIR interactions, in addition to an *IL28B* polymorphism, would influence the outcome of pegylated-interferon-α (PEG-IFN) and ribavirin therapy in Japanese patients with chronic hepatitis C.

Table 1. Clinical features of sustained and non-sustained virological response patients with chronic hepatitis C.

Characteristic	All	SVR	Non-SVR	*P*
	(n = 115)	(n = 56)	(n = 59)	
Age (yr)	60 (24 - 80)	59 (25 - 80)	60 (24 - 75)	0.43
Male	66 (57)	34 (61)	32 (54)	0.48
Alanine aminotransferase (IU/L)	46 (17 - 389)	48 (17 - 389)	45 (17 - 309)	0.81
Aspartate aminotransferase (IU/L)	43 (17 - 246)	42 (17 - 231)	43 (17 - 246)	0.49
White blood cells (/μL)	4410 (2280 - 8240)	4740 (2700 - 8170)	4070 (2280 - 8240)	0.011
Hemoglobin (g/dL)	14.4 (9.2 - 18.2)	15.1 (11.0 - 18.2)	13.9 (9.2 - 17.4)	0.002
Platelet count (10^4/μL)	15.9 (6.7 - 33.6)	16.6 (8.3 - 26.2)	15.6 (6.7 - 33.6)	0.30
APRI	0.89 (0.21 - 5.40)	0.59 (0.22 - 5.40)	0.66 (0.21 - 5.06)	0.41
HCV RNA (log_{10} IU/mL)	6.4 (5.0 - 7.3)	6.1 (5.0 - 6.8)	6.5 (5.0 - 7.3)	< 0.001

Data are expressed as median (range) or n (%) as appropriate. SVR, sustained virological response; HCV, hepatitis C virus

doi: 10.1371/journal.pone.0083381.t001

Materials and Methods

Ethics statement

This study was approved by the ethical committee of Shinshu University School of Medicine, Matsumoto, Japan, and written informed consent was obtained from all participants. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Subjects

One hundred and fifteen consecutive IFN-treatment-naïve patients with chronic hepatitis C were enrolled in this study. All subjects were seen at Shinshu University Hospital or one of its affiliated hospitals. The clinical and demographic characteristics of our cohort are shown in Table 1. Diagnosis of chronic hepatitis C was based on previously reported criteria [23]: 1) presence of serum HCV antibodies and detectable viral RNA; 2) absence of detectable hepatitis B surface antigen and antibody to the human immunodeficiency virus; and 3) exclusion of other causes of chronic liver disease or a history of decompensated cirrhosis or HCC. Serum levels of HCV RNA were determined using Cobas Amplicor assays (sensitivity: 50 IU/mL; Roche Diagnostic Systems, Tokyo, Japan). HCV genotypes were determined using INNO-LiPA HCV II kits (Innogenetics, Gent, Belgium). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and other relevant biochemical tests were performed using standard methods[24]. Liver fibrosis was assessed using the AST to platelet ratio index (APRI) in this study. APRI has been recognized as a noninvasive test to estimate the degree of liver fibrosis in chronic liver disease with HCV infection[25]. APRI was calculated for all study subjects as follows: AST/upper limit of normal (45 IU/L) × 100/platelet count (10^9/L). Patients received PEG-IFN-α2b (Pegintron; MSD KK, Tokyo, Japan; 1.5 μg/kg of body weight by subcutaneous injection once per week) and ribavirin (Rebetol; MSD KK; 600-1000 grams daily, according to body weight) for 48 weeks, as described previously[26]. Patients achieving a sustained HCV response were defined as those whose serum HCV RNA was undetectable 24 weeks after completing therapy. Patients who did not meet this criterion, who included non-responders and relapsers, were regarded as treatment failures.

HLA, KIR, and IL28B (rs8099917) Genotyping

Genomic DNA was isolated from whole blood samples using QuickGene-800 assays (Fujifilm, Tokyo, Japan). We genotyped *HLA*-B, *HLA*-C, and *KIR* using a Luminex multi-analyzer profiling system with a LAB type® HD and KIR SSO genotyping kit (One Lambda, Inc., Canoga Park, CA), which is based on PCR sequence-specific oligonucleotide probes[27]. Subjects were identified as having the B/x or A/A genotype as defined previously[28]. Genotypes for the centromeric (*Cen*) and telomeric (*Tel*) parts of the *KIR* locus were determined according to the presence or absence of one or more B haplotype-defining *KIR* genes. Thus, *Cen-A1* and *Tel-A1* were the centromeric and telomeric motifs, respectively, of the canonical *A KIR* haplotype in the present study, *Cen-B1* and *Cen-B2* were alternative centromeric motifs of common *B KIR* haplotypes, and *Tel-B1* was the common telomeric motif of *B* haplotypes[29]. For much of this analysis, *Cen-B1* and *-B2* were grouped together as *Cen-B*, whereas *Cen-A1* was shortened to *Cen-A* and *Tel-A1* to *Tel-A*, as reported

previously[30,31]. Genotyping of an *IL28B* SNP (rs8099917) was performed using a TaqMan 5' exonuclease assay with primers supplied by Applied Biosystems[32]. Probe fluorescence signals were detected using a TaqMan assay for Real-Time PCR (7500 Real Time PCR System, Applied Biosystems) according to the manufacturer's instructions.

Statistical Analysis

The Mann-Whitney *U* test was employed to analyze continuous variables. Pearson's chi-squared test was used for the analysis of categorical data. We adopted Fisher's exact test when the number of subjects was less than 5. The Bonferroni correction for multiple testing was applied to our data of KIR-HLA combinations using the number of comparisons performed by our primary factors of interest in Table 2 (i.e., 8 tests = 4 combinations × 2 comparisons between two groups). A P value of < 0.05 was considered to be statistically significant. Association strength was estimated by calculating the odds ratio (OR) and 95% confidence interval (CI). Our model was checked by regression diagnostic plots to verify normality, linearity of data, and constant variance. Stepwise logistic regression analysis with a forward approach was performed to identify independent factors associated with an SVR after continuous variables were separated into 2 categorical variables by each median value. Statistical analyses were performed using SPSS software version 21.0J (IBM, Tokyo, Japan). Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated to determine the reliability of the predictors of therapy response.

Results

Patient Characteristics and Treatment Outcome

All patients in our test cohort were infected with HCV genotype 1b. Of the 115 patients receiving PEG-IFN-α2b and ribavirin therapy, 56 (49%) achieved an SVR. The remaining 59 patients were non-responders, 28 of whom experienced a relapse and 31 who were null responders. The median white blood cell count (P = 0.011) and hemoglobin value (P = 0.002) in the SVR group were significantly higher than those in the non-SVR group prior to treatment. HCV viral load at baseline was significantly associated with treatment outcome (P < 0.001).

Association of HLA and KIR with a Sustained Virological Response

We first determined the frequency of *HLA-Bw* and *HLA-C* alleles in SVR and non-SVR patients (Figure 1). The frequency of *HLA-Bw4Bw6* in responders was significantly higher than that in non-responders (55% [31/56] vs. 36% [21/59]; P = 0.033; OR = 2.24, 95% CI = 1.06 - 4.75). Conversely, patients with the *HLA-Bw6* homozygote had a higher non-SVR rate (32% [18/56] vs. 54% [32/59]; P = 0.017; OR = 0.40, 95% CI = 0.19 - 0.85). Overall, *HLA-Bw4* was associated with an SVR among patients (68% [38/56] vs. 46% [27/59]; P = 0.017; OR = 2.50, 95% CI = 1.17 - 5.35). The frequencies of HLA-C were not statistically significant. We further checked whether particular HLA-Bw or HLA-C alleles were beneficial to treatment outcome. The HLA-B*35:01 allele was more frequently found in patients with an SVR than in those without (13% [15/102] vs. 4% [5/118]; P = 0.014 [Pc = 0.36]; OR = 3.49, 95% CI = 1.23 - 9.97).

Table 2. Frequency of *IL28B* genotype, *KIR3DL1/HLA-Bw4*, and *KIR2DL2/HLA-C1* combinations in 56 patients with a sustained virological response (SVR) and 59 patients with a non-SVR to pegylated interferon and ribavirin therapy of chronic hepatitis C.

KIR3DL1/ HLA-Bw4	*KIR2DL2/HLA-C1*	SVR	Non-SVR	P (Pc)	OR (95% CI)
		(n = 56)	(n = 59)		
+/+	+/+	5 (9%)	7 (12%)	0.61	
+/+	Other	31 (55%)	19 (32%)	0.012 (0.1)	2.61 (1.22 - 5.58)
Other	+/+	1 (2%)	10 (17%)	0.014 (0.12)	0.09 (0.01 - 0.72)
Other	Other	19 (34%)	23 (39%)	0.57	
IL28B	*KIR3DL1/ HLA-Bw4*	SVR	Non-SVR	P (Pc)	OR (95% CI)
		(n = 56)	(n = 59)		
TT	+/+	27 (48%)	13 (22%)	0.003 (0.024)	3.29 (1.47 - 7.39)
TT	Other	17 (30%)	14 (24%)	0.42	
TG/GG	+/+	9 (16%)	13 (22%)	0.42	
TG/GG	Other	3 (5%)	19 (32%)	0.00062 (0.0005)	0.12 (0.03 - 0.43)
IL28B	*KIR2DL2/ HLA-C1*	SVR	Non-SVR	P (Pc)	OR (95% CI)
		(n = 56)	(n = 59)		
TT	Other	38 (68%)	18 (31%)	0.000062 (0.0005)	4.81 (2.19 - 10.58)
TT	+/+	6 (11%)	9 (15%)	0.47	
TG/GG	Other	12 (21%)	24 (41%)	0.026 (0.21)	0.40 (0.17 - 0.91)
TG/GG	+/+	0 (0%)	8 (14%)	0.013 (0.1)	-

Data are expressed as n (%).

doi: 10.1371/journal.pone.0083381.t002

The distribution of *KIR* genes and their association with treatment outcome are shown in Figure 2. No statistically significant differences were found for any allele combination apart from *KIR2DL2* and *KIR2DS2*; patients with these genes had significantly decreased SVR frequencies compared with those without (P = 0.015 [Pc = 0.48]; OR = 0.30, 95% CI = 0.11 - 0.82 and P = 0.025 [Pc = 0.8]; OR = 0.32, 95% CI = 0.12 - 0.90, respectively).

KIR genotype profiles were determined by the presence or absence of each *KIR* locus in patients (Figure 3). Since strong linkage disequilibrium is a prominent feature in the *KIR* region, *KIR* gene profiles were classified based on *Cen* and *Tel* motifs. When we evaluated SVR according to genotype and *Cen* and *Tel* frequencies, we observed that virologic clearance with *Cen-A/A* was significantly higher than that without (54% [50/92] vs.

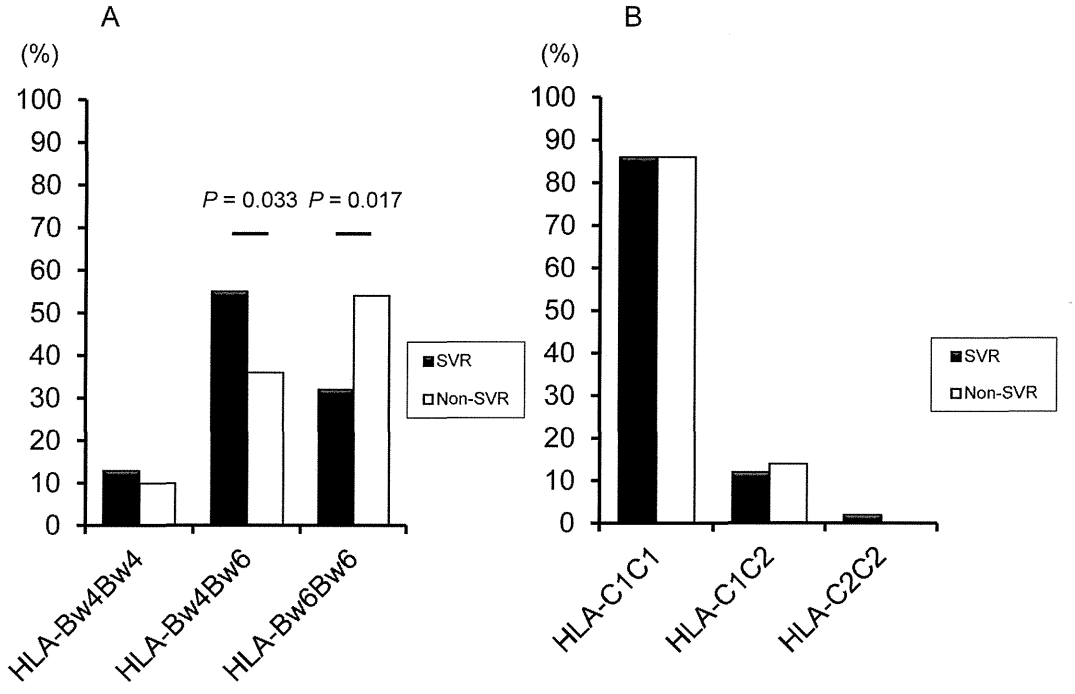

Figure 1. Frequency of *HLA-Bw* and -C alleles in 56 patients with a sustained virological response (SVR) and 59 patients with a non-SVR to pegylated interferon and ribavirin therapy of chronic hepatitis C.

doi: 10.1371/journal.pone.0083381.g001

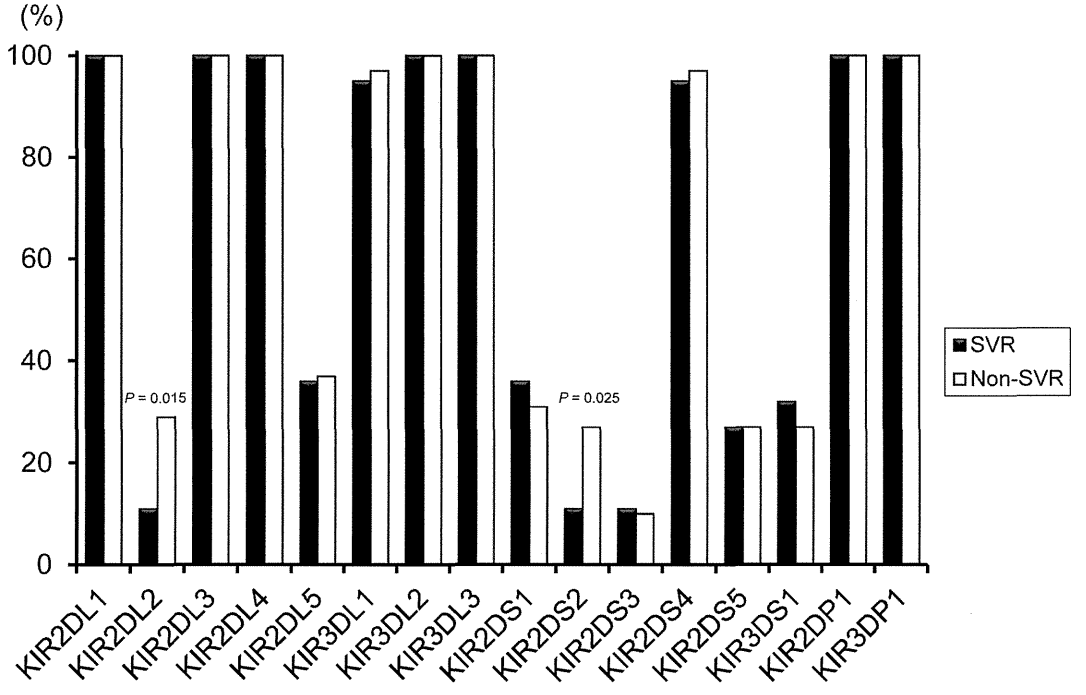

Figure 2. Frequency of each *KIR* gene in 56 patients with a sustained virological response (SVR) and 59 patients with a non-SVR to pegylated interferon and ribavirin therapy of chronic hepatitis C.

doi: 10.1371/journal.pone.0083381.g002

26% [6/23], $P = 0.015$; OR = 3.37, 95% CI = 1.22 - 9.33). There were no significant differences regarding *AA* genotype and *Tel*.

We next analyzed combinations of activation/inhibitory *KIR*s and their *HLA* ligands for possible associations with an SVR. Among the combinations of *KIR3DL1-HLA-Bw4*, *KIR2DL2-HLA-C1*, and KIR2DL1-*HLA*-C2, patients who carried the inhibitory *KIR3DL1* receptor and its ligand *HLA-Bw4* had a significantly higher response rate than those without *KIR3DL1* or *HLA-Bw4* (58% [36/62] vs. 38% [20/53]; $P = 0.030$ [*Pc* = 0.12]; OR = 2.29, 95% CI = 1.08 - 4.84). In contrast, the *KIR2DL2-HLA-C1* combination resulted in a significantly lower SVR rate (26% [6/23] vs. 54% [50/92]; $P = 0.015$ [*Pc* = 0.06]; OR = 0.30, 95% CI = 0.11 - 0.82). Although several studies have found that *KIR2DL3-HLA-C1* carriers are associated with treatment-induced and spontaneous clearance of HCV in Caucasians, no such association was found in our cohort (data not shown).

Patients with *KIR3DL1-HLA-Bw4* but without *KIR2DL2-HLA-C1* had a higher SVR rate (55% [31/56] vs. 32% [19/59]; $P = 0.012$ [*Pc* = 0.1]; OR = 2.61, 95% CI = 1.22 - 5.58) (Table 2). Conversely, the frequency of the *KIR2DL2-HLA-C1* positive, but *KIR3DL1-HLA-Bw4* negative condition was significantly higher in non-responders (17% [10/59] vs. 2% [1/56]; P = 0.014 [*Pc* = 0.12]; OR = 0.09, 95% CI = 0.01 - 0.72).

Prediction of a Sustained Virological Response by KIR-HLA and IL28B

Examination of the *IL28B* rs8099917 SNP in our cohort revealed significant differences in SVR frequencies. The SVR rate in patients with the *IL28B* TT genotype was significantly higher in those with TG or GG genotypes (62% [44/71] vs. 27% [12/44), $P = 0.0003$; OR = 4.35, 95% CI = 1.92 - 9.85). In subjects with *IL28B* TT and *KIR3DL1-HLABw4*, virologic clearance was significantly increased over other combinations (68% [27/40] vs. 39% [29/75]; $P = 0.003$ [*Pc* = 0.024]; OR 3.29, 95% CI = 1.47 - 7.39).

We next evaluated several factors found in association with an SVR to PEG-IFN and ribavirin therapy for independence by logistic regression analysis. Fifty-six responders were compared with 59 non-responders by means of a forward stepwise likelihood ratio logistic regression method; estimated OR coefficients, 95% CI, and *P* values are summarized in Table 3 for the variables that remained in equation at the last step. *IL28B* TT genotype ($P = 0.00009$; OR = 6.87, 95% CI = 2.62 - 18.01), *KIR2DL2-HLA*-C1 ($P = 0.014$; OR = 0.24, 95% CI = 0.08 - 0.75), white blood cell count ≥ 4410/μL ($P = 0.009$; OR = 3.32, 95% CI= 1.35 - 8.16), and *KIR3DL1-HLA-Bw4* ($P = 0.008$; OR = 3.32, 95% CI = 1.37 - 8.05) were all identified as independent parameters that significantly influenced an SVR.

The frequency of the *IL28B* TT genotype with *KIR3DL1-HLA-Bw4* in responders was significantly higher than in non-responders (48% [27/56] vs. 22% [13/59]; $P = 0.003$ [*Pc* = 0.024]; OR = 3.29, 95% CI = 1.47 - 7.39) (Table 2). Patients with the *IL28B* TT genotype without *KIR2DL2-HLA-C1* had a significantly higher SVR rate (68% [38/56] vs. 31% [18/59]; $P = 0.000062$ [*Pc* = 0.0005]; OR = 4.81, 95% CI = 2.19 - 10.58). The frequency of a non-SVR was significantly higher in patients with the *IL28B* non-TT genotype both with and without

KIR profile	Geno type	Cen motif	Tel motif	3DL3	2DS2	2DL2	2DL3	2DP1	2DL1	3DP1	2DL4	3DL1	3DS1	2DL5	2DS3	2DS5	2DS1	2DS4	3DL2	SVR (n = 56)	Non-SVR (n = 59)
1	AA	A/A	A/A	+	-	-	+	+	+	+	+	+	-	-	-	-	-	+	+	34 (60.7)	28 (47.5)
2	Bx	A/A	A/B	+	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	9 (16.1)	10 (16.9)
3	Bx	A/B	A/A	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	2 (3.6)	8 (13.6)
4	Bx	A/A	A/B	+	-	-	+	+	+	+	+	+	+	+	+	-	+	+	+	4 (7.1)	2 (3.4)
5	Bx	A/A	B/B	+	-	-	+	+	+	+	+	-	+	+	-	+	+	-	+	3 (5.4)	1 (1.7)
6	Bx	A/B	A/B	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	1 (1.8)	2 (3.4)
7	Bx	A/B	A/B	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	1 (1.8)	2 (3.4)
8	Bx	A/B	A/A	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+	+	0 (0.0)	3 (5.1)
9	Bx	A/B	A/B	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	1 (1.8)	0 (0.0)
10	Bx	A/B	A/B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1 (1.8)	0 (0.0)
11	Bx	A/B	B/B	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	+	1 (1.8)	0 (0.0)
12	Bx	A/B	A/A	+	-	+	+	+	+	+	+	+	-	-	-	-	-	+	+	0 (0.0)	1 (1.7)
13	Bx	A/A	A/A	+	-	-	+	+	+	+	+	+	-	+	+	-	-	+	+	0 (0.0)	1 (1.7)

Figure 3. *KIR* gene profile frequencies in 56 patients with a sustained virological response (SVR) and 59 patients with a non-SVR to pegylated interferon and ribavirin therapy of chronic hepatitis C. Numerical data represent the number of individuals (%). The presence of *KIR* genes is indicated by gray shading. Cen, centromeric; Tel, telomeric.

doi: 10.1371/journal.pone.0083381.g003

Table 3. Logistic regression analysis of variables contributing to a sustained virological response to pegylated interferon and ribavirin.

Factor	Odds ratio	95% confidence interval	*P*
IL28B TT genotype	6.87	2.62 - 18.01	0.00009
KIR2DL2/HLA-C1	0.24	0.08 - 0.75	0.014
White blood cells ≥ 4410/μL	3.32	1.35 - 8.16	0.009
KIR3DL1/HLA-Bw4	3.32	1.37 - 8.05	0.008

Only variables achieving statistical significance ($P < 0.05$) in multivariate logistic regression analysis are shown.

doi: 10.1371/journal.pone.0083381.t003

KIR2DL2-HLA-C1 (14% [8/59] vs. 0% [0/8]; $P = 0.013$ [*Pc* = 0.1] and 41% [24/59] vs. 21% [12/56]; $P = 0.026$ [*Pc* = 0.21]; OR = 0.40, 95% CI = 0.17 - 0.91, respectively). The ability to predict an SVR by *IL28B* genotype and *KIR3DL1-HLA-Bw4* and *KIR2DL2-HLA-C1* was next evaluated. Corresponding values for sensitivity, specificity, PPV, and NPV are listed in Table S1 in File S1. A combination of the *IL28B* TT genotype and *KIR3DL1-HLA-Bw4* demonstrated high predictive specificity (78%), as did the combination of *IL28B* TT genotype and *KIR2DL2-HLA-C1* (86%).

Lastly, we analyzed combinations of the three factors of *IL28B* genotype, *KIR3DL1-HLA-Bw4*, and *KIR2DL2-HLA-C1* for prediction of treatment outcome (Table S2 in File S1). The frequencies of *IL28B* TT, *KIR2DL2-HLA-C1*-negative, with and without *KIR3DL1-HLA-Bw4* were significantly higher among responders (38% [21/56] vs. 19% [11/59]; P = 0.024 [Pc = 0.29]; OR = 2.62, 95% CI = 1.12 - 6.12 and 30% [17/56] vs. 12% [7/59]; P = 0.015 [Pc = 0.18]; OR = 3.24, 95% CI = 1.22 - 8.57, respectively).

Discussion

The present study examined *HLA*, *KIR*, and *IL28B* gene variant associations with an SVR following PEG-IFN and ribavirin therapy in Japanese patients with chronic hepatitis C. We found a significant association of *HLA-Bw* alleles with treatment outcome, although the frequency of *HLA-C* alleles did not differ significantly between responders and non-responders. Functional analyses have demonstrated that NK cells in *HLA-C1C1* subjects exhibit a more rapid and stronger antiviral response that those in *HLA-C2C2* subjects due to differing responses of *HLA-C*-inhibited NK subsets[33]. *HLA-C2C2* homozygousity is strongly associated with treatment failure in HCV patients of European ancestry [11,22], but we could not assess its role in our study because this genotype was found in only 1 of 115 patients.

We uncovered a significant association between the presence of *KIR2DL2* or *KIR2DS2* and lower SVR rates. Several reports have shown that *KIR2DL3-HLAC1* in Caucasians [11,22] and *KIR2DL5* in Brazilians [34] are associated with treatment outcome of antiviral therapy. Since our results showed no such statistical significances, these conflicting interpretations may reflect differences in patient selection, genetic background, sample size, and/or treatment regimen. Further studies are required to clarify this discrepancy in the Japanese population.

A study by Dring et al. examined *KIR* haplotypes in patients with HCV infection and showed that a centromeric *KIR* haplotype was increased in chronic HCV infection as compared with resolved cases [20]. We therefore determined *KIR* haplotypes and *Cen-A/B* and *Tel-A/B* in our patients as well, and found an interesting association between *Cen-A/A* and an SVR to antiviral therapy (P = 0.015; OR 3.37). Since *Cen-A/B* is determined by *KIR2DL3* and *KIR2DS2* and/or *KIR2DL2*, this finding is consistent with our results demonstrating a relationship between *KIR2DS2* and *KIR2DL2* genotypes and treatment failure.

The most significant finding in this study was the association between KIR-HLA receptor-ligand pairings and treatment outcome in chronic hepatitis C. Among the inhibitory KIR-HLA receptor-ligand pairs, patients with *KIR3DL1-HLA-Bw4* exhibited a significantly higher SVR rate when compared to those without this pair (P = 0.03; OR 2.29). Conversely, virologic clearance in patients with *KIR2DL2-HLA-C1* was significantly lower than in those without (P = 0.015; OR = 0.30). Stratification analysis of the 4 groups of *KIR3DL1-HLA-Bw4* (presence or absence) and *KIR2DL2-HLA-C1* (presence or absence) revealed a higher frequency of responders with *KIR3DL1-HLA-Bw4* presence, *KIR2DL2-HLA-C1* absence compared with those possessing *KIR2DL2-HLA-C1* presence, *KIR3DL1-HLA-Bw4* absence (62% vs. 9%; P = 0.0044; OR =16.32). When these KIR-HLA pairs were both either positive or negative, SVR rates were similar at 42% and 45%, respectively. Together with the results of logistic regression analysis, we clearly showed that *KIR3DL1-HLA-Bw4* was positively associated with an SVR (OR = 3.32) and that *KIR2DL2-HLA-C1* had a negative association (OR = 0.24) with treatment outcome. As almost one half of the Japanese population have the functional *KIR3DL1-HLA-Bw4* combination, this inhibitory receptor-ligand interaction is potentially important in understanding NK cell diversification. The NK-cell surface expression of KIR3DL1 is higher in individuals having Bw4 than in those lacking it [35]. Therefore, these cells might be more weakly controlled by inhibitory signals than other NK cells, more easily activated by viral infection, and more readily promoted for cytolysis and IFN-gamma production.

This study confirmed that the *IL28B* TT genotype is a strong predictor of an SVR in Japanese patients[18,32]. Furthermore, SVR frequencies were positively correlated with a combination of the *IL28B* TT genotype and *KIR3DL1-HLA-Bw4* (P = 0.0019) and negatively associated with the *IL28B* TT genotype and *KIR2DL2-HLA-C1* (P = 0.0067). These combinations were also highly specific for virologic response prediction. In light of these findings, patients with poor expected treatment outcome may be advised to wait for the use of combinations of direct acting antiviral agents[36]. Akuta et al. reported that a combination of amino acid substitutions in the core region of HCV and *IL28B* genotype was a useful predictor of PEG-IFN, ribavirin, and telaprevir therapy results in Japan[37]. Since we could not collect sera before treatment for all patients, we were not able to assess the effect of amino acid substitutions in the HCV core region. Furthermore, interferon-free combinations of direct-acting antiviral agents have become an area of considerable clinical interest. Chu et al. have reported that IL28B genotype appears to affect early viral kinetics in patients with chronic hepatitis C receiving interferon-free treatment [38]. Recently, two groups have discovered *IFN* lambda *4* (*IFNL4*), a new gene that may account for associations of spontaneous and IFN-based treatment clearance of HCV [39,40]. The IFN-λ 4 protein is generated by individuals who carry the ΔG allele of the ss469415590 variant, and the presence of this protein is strongly associated with impaired clearance of HCV. Linkage disequilibrium is strong between the *IFNL4-ΔG* allele and the unfavorable rs12979860-T allele (*IL28B*) in subjects of European or Asian ancestry, whereas this linkage disequilibrium is moderate in individuals of African ancestry [39]. We have confirmed that the linkage disequilibrium between the *IFNL4-ΔG* allele and *IL28B* SNP (rs8099917) is high and that the *IFNL4-ΔG* allele is strongly associated with treatment failure of PEG-IFN and ribavirin therapy in patients with Japanese chronic hepatitis C [41]. Hence, the clinical impacts of HLA-KIR genetic variants, IL28B genotype, and the IFNL4 allele should be explored.

In conclusion, the present study showed significant associations of *KIR3DL1-HLA-Bw4, KIR2DL2-HLA-C1*, and *IL28B* combinations with an SVR to PEG-IFN and ribavirin therapy in Japanese patients with genotype 1 HCV. The clinical significance of *IL28B* genotyping combined with HLA/KIR pairs to predict treatment outcome warrants further validation for triple therapy.

Supporting Information

File S1. Table S1, Sensitivity, specificity, and predictive values of IL28B TT genotype and KIR3DL1/HLA-Bw4 or

KIR2DL2/HLA-C1 for a sustained virological response in 115 patients with chronic hepatitis C. Data are expressed as % (n). PPV, positive predictive value; NPV, negative predictive value. Table **S2**, Frequency of ***IL28B*** genotype and ***KIR3DL1/HLA-Bw4*** and ***KIR2DL2/HLA-C1*** combinations in 56 patients with a sustained virological response (SVR) and 59 patients with a non-SVR to pegylated interferon and ribavirin therapy of chronic hepatitis C. Data are expressed as n (%).
(DOC)

Acknowledgements

The authors thank Yuki Akahane, Asami Yamazaki, and Toyo Amaki for their technical assistance, and Trevor Ralph for his editorial assistance.

Author Contributions

Conceived and designed the experiments: YN TU ET MO. Performed the experiments: YN TU YK MO. Analyzed the data: YN TU YK MO. Contributed reagents/materials/analysis tools: YN TU SJ YK SS TK SM MK AM ET. Wrote the manuscript: TU MO.

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Journal of Clinical Microbiology

Application of a Newly Developed High-Sensitivity HBsAg Chemiluminescent Enzyme Immunoassay for Hepatitis B Patients with HBsAg Seroclearance

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J. Clin. Microbiol. **2013, 51(11):3484. DOI: 10.1128/JCM.00726-13.**
Published Ahead of Print 14 August 2013.

JCM
Journals.ASM.org

Application of a Newly Developed High-Sensitivity HBsAg Chemiluminescent Enzyme Immunoassay for Hepatitis B Patients with HBsAg Seroclearance

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We modified and automated a highly sensitive chemiluminescent enzyme immunoassay (CLEIA) for surface antigen (HBsAg) detection using a combination of monoclonal antibodies, each for a specific epitope of HBsAg, and by improving an earlier conjugation technique. Of 471 hepatitis B virus (HBV) carriers seen in our hospital between 2009 and 2012, 26 were HBsAg seronegative as determined by the Abbott Architect assay. The Lumipulse HBsAg-HQ assay was used to recheck those 26 patients who demonstrated seroclearance by the Abbott Architect assay. The performance of the Lumipulse HBsAg-HQ assay was compared with that of a quantitative HBsAg detection system (Abbott Architect) and the Roche Cobas TaqMan HBV DNA assay (CTM) (lower limit of detection, 2.1 log copies/ml) using blood serum samples from patients who were determined to be HBsAg seronegative by the Abbott Architect assay. Ten patients had spontaneous HBsAg loss. Of 8 patients treated with nucleotide analogues (NAs), two were HBsAg seronegative after stopping lamivudine therapy and 6 were HBsAg seronegative during entecavir therapy. Eight acute hepatitis B (AH) patients became HBsAg seronegative. Of the 26 patients, 16 were HBsAg positive by the Lumipulse HBsAg-HQ assay but negative by the Abbott Architect assay. The differences between the two assays in terms of detectable HBsAg persisted over the long term in the spontaneous loss group (median, 10 months), the NA-treated group (2.5 months), and the AH group (0.5 months). In 9 patients, the Lumipulse HBsAg-HQ assay detected HBsAg when HBV DNA was negative by the CTM assay. HBsAg was also detected by the Lumipulse HBsAg-HQ assay in 4 patients with an anti-HBs concentration of >10 mIU/ml, 3 of whom had no HBsAg escape mutations. The automatic, highly sensitive HBsAg CLEIA Lumipulse HBsAg-HQ is a convenient and precise assay for HBV monitoring.

Today, >400 million people worldwide are hepatitis B virus (HBV) carriers (1). We have monitored HBV markers, such as HBV DNA, hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), and HB core-related antigen (HBcrAg), in chronic hepatitis B patients. The measurement of HBV DNA levels by a PCR-based method is the state-of-the-art technique for monitoring HBV replication in clinical practice (2). However, it is suboptimal for chronic hepatitis B patients who are medicated with nucleotide analogues (NAs), as those, in many cases, can decrease HBV DNA to below the limit of detection.

HBsAg is a secreted envelope protein that is continuously shed into the blood as long as HBV infection persists, irrespective of viral replication. Recent advances in HBsAg quantification (qHBsAg) have opened up new perspectives in the study of HBV; qHBsAg levels are correlated with intrahepatic covalently closed circular (ccc) DNA, which is used as a template for viral transcription and maintains the chronic HBV infection state (3–5). Additionally, a correlation between qHBsAg and HBV DNA has been suggested, with the possibility of a role for qHBsAg as a surrogate marker for viral replication put forward, which might identify chronic hepatitis B patients who are likely to be cured with pegylated alpha interferon (6–9).

In Japan, two HBsAg quantification assays are available: the Architect HBsAg-QT (Abbott Japan) (detection range, 50 to 250,000 mIU/ml) and the HISCL HBsAg (Sysmex) (detection range, 30 to 2,500,000 mIU/ml). These two methods have a good correlation and are sensitive over a wide detection range. Recently, Matsubara et al. (10) reported a novel highly sensitive chemiluminescent enzyme immunoassay (CLEIA) that was developed for quantitative HBsAg detection by combining monoclonal antibodies, each specific for a different epitope of the antigen, and employing an improved conjugation technique. It is as sensitive as nucleic acid testing for detecting early HBV infection. We further modified and improved the high-sensitivity assay reagent described above for adaptation to both ferrite microparticles as the solid phase and the automated analyzer system by modification of the optimum combination of monoclonal antibodies. As was recently reported (11), this assay (Lumipulse HBsAg-HQ) had good accuracy, reproducibility, specificity, and sensitivity, and the results correlate well with those of the Abbott Architect. The coefficient of variation in the Lumipulse HBsAg-HQ is <5.9% for samples with a low concentration of HBsAg (11), and the assay was approved by the Japanese government in 2013.

The sensitivity of this assay (5 mIU/ml) was approximately 10-fold higher than that of the Abbott Architect assay (50 mIU/ml). Here, we adapted this assay to monitor chronic hepatitis B

Received 18 March 2013 **Returned for modification** 29 April 2013
Accepted 6 August 2013
Published ahead of print 14 August 2013
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doi:10.1128/JCM.00726-13